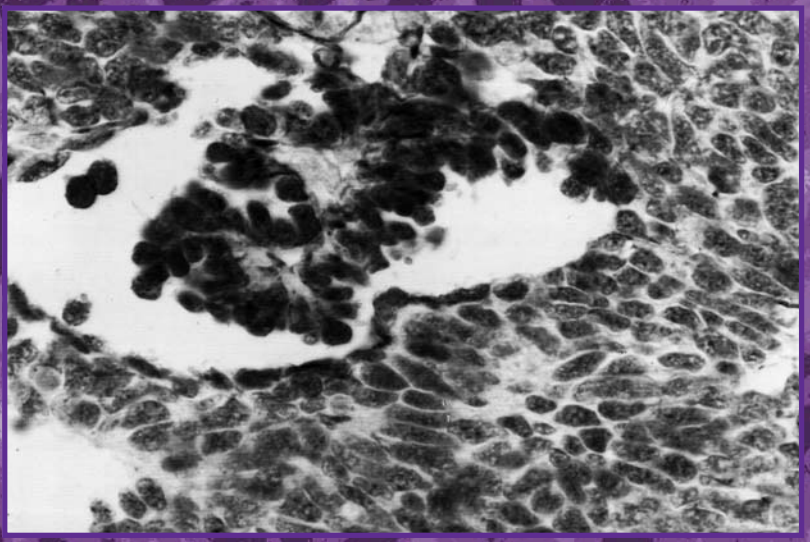
CANCER DRUG DISCOVERY AND DEVELOPMENT

# Tumor Suppressor Genes in Human Cancer

Edited by

David E. Fisher, MD, PhD



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# Tumor Suppressor Genes in Human Cancer

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# TUMOR SUPPRESSOR GENES IN HUMAN CANCER

Edited by

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Cover illustration: The cover shows the histologic appearance of a Wilm's tumor stained with hematoxylin and eosin (purple) or immunostained for the Wilm's tumor suppressor protein (black and white inset). Cover photos were generously provided by Scott Granter, MD, Department of Pathology, Brigham and Women's Hospital, Boston, MA.

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# PREFACE

The war against cancer has seen enormous successes, but also painful frustration. While major conceptual breakthroughs have been made in our understanding of how cell proliferation is regulated, the translation of this information into effective treatment discoveries has lagged terribly behind. Modern molecular oncology has begun to inch closer to treatment-related questions because the pathways under study are now known to regulate events such as cell death, the precise goal of cancer treatment. Because tumor suppressor biology has not yet translated into a therapy-oriented discipline, the purpose of *Tumor Suppressor Genes in Human Cancer* is to present a view of the current field which simultaneously highlights the clinically relevant directions which have already emerged while stimulating the discovery of new ones.

Through the detailed presentation of tumor suppressor genes with a molecular biological and genetic perspective, two paradigms emerge: 1) a finite number of discrete pathways exist into which tumor suppressors and dominant oncogenes reside and 2) cancer biology rests heavily on both regulators of cell proliferation and cell death. In the current climate of informatics, genomics, and molecularly driven drug discovery, cancer research holds greater promise than ever. *Tumor Suppressor Genes in Human Cancer* first sets the stage by presenting the background of systems for the study of tumor suppressor genes as well as the fields of apoptotic cell death and cancer drug discovery. The second section of *Tumor Suppressor Genes in Human Cancer* proceeds to present detailed analyses of major tumor suppressors and, most important, the pathways into which they fit. The intended audience is the student of cancer biology, from those engaged in graduate or medical education to clinicians or drug development professionals seeking to understand the context of cancer cell biology and its promise for therapeutic gains in the coming years.

The concept that individual genes underlie the biology of malignant transformation hearkens back to the early 1900s with the discovery by Peyton Rous of avian sarcomas that were caused by infectious viruses. Many decades later, the identification of the *Src* oncogene placed into focus the notion of the dominant oncogene, a factor whose inappropriate activation confers cellular changes associated with malignant transformation. Alfred Knudsen predicted the existence of a second class of oncogenes whose contribution to cancer is recessively inherited. His hypothesis was based upon clinical observations of cancer risk in familial cancer inheritance patterns and the notion that disease predisposition may represent a multi-hit phenomenon with loss-of-function mutations contributing to the malignant phenotype. Thus the concept of tumor suppressor gene was born and has been abundantly validated by observations that span bench to bedside.

The most striking validation of the tumor suppressor concept comes from the discovery of inactivating mutations or deletions of candidate genes in cancer

prone families. Originally discovered for retinoblastoma, the list has been dramatically extended to include p53, p16/Ink4a/ARF, the NF family, DNA mismatch repair genes, Wilms, von Hippel Lindau, Fanconi Anemia, and other genes. In these cases heterozygous germline disruption of a single allele is associated with cancer predisposition in affected family members. Loss of heterozygosity is frequently observed at the genetic locus within tumors that develop in affected individuals. Mechanisms for tumor suppressor inactivation are diverse and are still being discovered today. For example, in addition to traditional loss of function mutations or deletions, the more recently appreciated inactivating mechanisms include transcriptional silencing (e.g., p16/Ink4a), targeted protein degradation (e.g., p53), and functional disruption of tumor suppressing gene activities (e.g., bcl-2 or Mdm2). These diverse mechanisms of tumor suppressor inactivation highlight one of the most striking breakthroughs in cancer biology, the discovery of discrete pathways in which dominantly acting and tumor suppressing genes converge.

The functional convergence of dominant oncogenes and tumor suppressor genes in cellular growth or survival pathways represents such a powerful clue in the puzzle of carcinogenesis that the ability to fit into a known growth regulatory pathway has become a virtual requirement for a gene's acceptance as a true cancer modifier. Moreover much of the data defining these interactions stemmed from the convergence of clinically derived questions with more basic laboratory science. For example, the retinoblastoma tumor suppressor was found to be targeted by multiple dominant oncogenes discovered through the analysis of animal DNA tumor viruses. The existence of additional interactions between tumor suppressors and dominant oncogenes has cemented the notion that key cellular pathways produce the phenotypes associated with cancer, and the homeostatic regulators of these pathways are potent and common targets of carcinogenic disruption.

The pathways that tumor suppressor genes modulate in cancer have been found to cluster around regulation of the cell cycle, cell death, growth factor signaling, DNA damage responses, and other stress responses. Nearly all tumor suppressors are thought to act through modulation of one (or several) of these pathways. Cell cycle regulation has been the traditional pathway thought to be targeted in the etiology of cancer. More recent observations have added a dramatic new dimension to this view in suggesting that cell survival pathways exist as distinct, genetically selected entities and may profoundly influence behaviors we associate with malignancy. Either dysregulated growth or inefficient death (or both) are strongly associated with tumorigenesis. The current revolution in molecular oncology has been fueled largely by the ability to place individual cancer genes within such functional pathways of known importance. Perhaps more important, these functional classifications have in some cases led to investigations that relate more than ever before to cancer treatment.

The study of cancer cell death carries with it the hope of intervening in the very same process for therapeutic benefit. Rarely has a field of fundamental basic

science become so mainstream in biologic inquiry while simultaneously focusing on questions of direct therapeutic importance. The interface between research on cell death and clinical ramifications of that work is well illustrated by the actions of tumor suppressor genes, many of which are now recognized to regulate cell survival.

*Tumor Suppressor Genes in Human Cancer* is not an attempt to fully synthesize cancer biology and treatment, since the field has not arrived at a stage where such a synthesis is yet possible. However the convergence of new technologies suggests that the coming years will begin to see treatment discoveries more directly interface with basic research. Genomics and systematic gene expression technologies will provide thorough catalogs of information whose discovery currently occupies substantial research effort. Linkage of these catalogs to clinical data including treatment responses (pharmacogenomics) promises to dramatically alter drug discovery and treatment design. The pillar of this revolution is the basic biology of disease, and tumor suppressor genes lie at the core of that pillar.

***David E. Fisher***

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# I Analysis and Clinical Implications of Tumor Suppressor Genes

# 1 Animal Models for Tumor Suppressor Genes

*Jordan A. Kreidberg, MD, PhD and Thomas A. Natoli, PhD*

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## 1. INTRODUCTION

The observation that bilateral retinoblastoma (RB) occurred with earlier onset than unilateral disease led Knudson to formulate the two-hit theory for tumorigenesis *(1)*. This theory provided the underpinning for the search for tumor suppressor genes (TSGs). In 1986–1987, the *Rb* gene was identified, appearing to fulfill the criteria of a TSG for RB, and therefore was among the early candidates for gene-targeting experiments in mice *(2–4)*. Since the initial development of gene-targeting technology, several hundred genes have been mutated, including many of the currently defined TSGs.

This chapter summarizes results obtained from animal models for TSGs, and discusses how these models have improved understanding of tumor development. Most of the results dealt with in this chapter are derived from mice carrying targeted deletions, and therefore a brief summary of this technology is appropriate. Readers desiring a

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more comprehensive summary should consult one of several recent reviews of this area *(5–9)*.

The goal of gene targeting is to introduce specific mutations into the germ line of mice, so that heterozygous and/or homozygous mice can be derived. These experiments make use of embryonic stem (ES) cells, which are carried as cell lines that can be introduced into preimplantation mouse blastocysts, and contribute to embryonic tissue. ES cell lines were originally derived from the inner cell mass of the preimplantation mouse blastocyst; this is the group of cells that are totipotent and give rise to the entire embryo after implantation. As described below, it is possible to target mutations to specific genes in ES cells, then use mutated ES cells to derive mutant mice. The general approach to homologous recombination is shown and described in Fig. 1. Once the ES cells carrying the desired mutation are obtained, they are microinjected into mouse blastocysts, where they recombine with the inner cell mass, and contribute to the resultant mouse (termed a chimera, because of its dual origin). Chimeric mice whose germ cells are derived from ES cells are able to transmit the mutation to their offspring, which are then true heterozygotes for the mutation. Male and female heterozygotes can be mated, and homozygous mutants, if viable, can be obtained. Among the several hundred reported experiences with gene targeting, the full range of expected results has been observed, from phenotypes that are apparent in heterozygotes, to animals that are apparently normal as homozygous mutants. Mutations that result in observable phenotypes in homozygous animals range from those that cause lethality very early in embryogenesis, to those that result in no morphological impairment, but cause functional impairment after birth.

In this chapter, the emphasis is placed on those TSGs that have thus far received the most intense study. A comprehensive table (Table 1) of TSGs studied in mice or rats includes those not more fully discussed in the text. New TSGs are continually identified, and many of these have not been studied in animal models beyond their initial identification. Therefore, these genes will be given less attention, or are not discussed. Included are some emerging technologies aimed at identifying novel TSGs.

## 2. P53

*p53* is the most commonly mutated TSG in human tumors, and therefore was an obvious candidate for which to obtain an animal model. As discussed in greater depth in Chapter of this volume, the p53 protein appears to function mostly as a transcription factor involved in the regulation of the progression through the cell cycle and entry into cell death pathways, particularly in response to DNA damage. One might expect that a protein involved in such basic activities important to all cell types would be essential for normal development, and that embryos unable to produce it might arrest early in development. In the event, the opposite was observed upon targeting the *p53* gene *(10–12)*. At least some portion of *p53–/–* mice are developmentally normal, although defects are observed, as is discussed below. Despite developing normally, all *p53* homozygous mutant mice succumb to tumors by about 9 mo of age *(10,12)*. It is generally assumed that most, if not all, genes that are found within the genome are present because of evolutionary selection against their loss. Therefore, when a mouse is found to survive and to reproduce without the function of a particular gene, it is usually suggested that the function of the gene product is redundant or overlapping with another

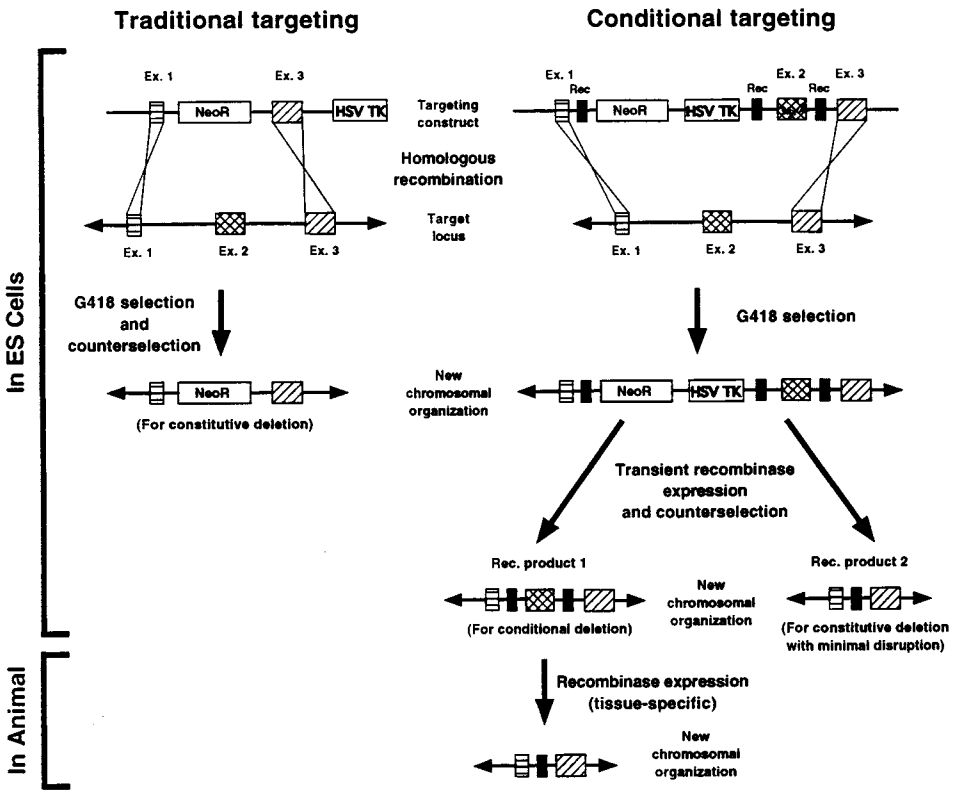


**Fig. 1.** Schematic representation of traditional and conditional gene-targeting approaches. Traditional targeting: Vectors for traditional gene targeting are designed with an antibiotic resistance marker (NeoR), introducing an interruption or deletion at the site to be mutated (in this figure, exon 2 is to be deleted). The *NeoR* gene is flanked upstream and downstream by DNA sequences homologous to those bordering to be mutated. The herpes virus thymidine kinase gene *(HSVTK)* is placed outside this region of homology. Upon transfection of ES cells with this vector, homologous recombination between the vector and genomic DNA inserts the *NeoR* gene, but not the *HSVTK* gene; random integration is more likely to insert both genes. ES cells are electroporated with the targeting construct, and selected with G418. G418 is used to select ES cells that have incorporated the *NeoR* gene, and gancyclovir is used to counterselect against those that have incorporated the *HSVTK* gene. Conditional targeting: Vectors for conditional targeting are designed with site-specific recombinase recognition sites (*lox* sites or Frt sites, indicated by black boxes labeled "Rec") flanking both the region to be deleted and the selective markers. After transfection of ES cells, G418-resistant colonies are screened for homologous recombination (generating the indicated chr organization). Cells from properly targeted colonies are transiently transfected with the recombinase gene *(cre* or *flp),* and gancyclovir is used to select cells that have lost the *neo* resistance and *HSVTK* genes, because site-specific recombination. There are two potential products of this reaction. Site-specific recombination between the left and center sites removes only the selective markers, leaving the exon to be deleted flanked by recombinase sites. Resultant mice from these ES cells are used for conditional mutations, in which expression of the recombinase under the control of a tissue-specific promoter allows cell-type-specific deletion of the exon. Recombination between the left and right sites deletes both the selectable markers and the exon. This type of deletion can be useful when it is desirable to eliminate a single exon, while otherwise maintaining expression of the gene. Because recombination sites are much smaller than the *Neo* gene, (e.g., 34 bp vs 2.5 kb), it is less likely that a single recombination site will interfere with gene expression.

**Table 1**
**Phenotypes of Animals with Mutations in TSGs**

| *Gene* | *Heterozygote phenotype* | *Homozygote phenotype* | *Ref(s).* |
|---|---|---|---|
| APC | Intestinal adenomas | Embryonic lethal before E11 | *(74,76,139)* |
| ATM | Normal | Normal development; lymphoma, radiation sensitivity | *(67,68)* |
| *E-Cadherin* | Normal | Embryonic lethal at E4.5 | *(140,141)* |
| *BRCA1* | Normal | Embryonic lethal at E7.7–E13 | *(111,112)* |
| *BRCA2* | Normal | Embryonic lethal at E8.5–9.5; lymphomas (truncation mutation) | *(111,113,115,116)* |
| *E2F-1* | Normal | Reproductive tract sarcomas lung adenocarcinomas, lymphomas | *(49)* |
| $G\alpha_{i2}$ | Normal | Normal development; ulcerative colitis, colon adenocarcinoma | *(142)* |
| *α-inhibin* | Normal | Normal development; gonadal tumors | *(143)* |
| *Mgmt* | Normal | Normal development; lymphomas, adenomas induced with MNU | *(107,144)* |
| *Mlh1* | Normal | Defective spermatogenesis; DNA repair defects, intestinal adenomas, adenocarcinoma, lymphoma | *(96,145)* |
| *Msh2* | Normal | Lymphoma intestinal neoplasia in older mice | *(93,95)* |
| *Msh6* | Normal | Normal development; GI tumors, lymphoma | *(100)* |
| *Nf1* | Adrenal tumors in older mice | Embryonic lethal around E13.5 with heart defects | *(146)* |
| *Nf2* | Range of metastatic tumors in older mice | Embryonic lethal at E6.5–7 | *(147,148)* |
| $p16^{Ink4a}$–$p19^{ARF}$ | Normal | Normal development; sarcomas and lymphomas | *(66)* |
| $p21^{Cip1/Waf1}$ | Normal | Normal development Cell cycle defects in vitro | *(57)* |
| $p27^{Kip1}$ | Tumors after irradiation | Organ hyperplasia tumors after irradiation | *(61–63)* |
| *p53* | Sarcomas and lymphomas in older mice | Normal development or neural tube defects; lymphomas and sarcomas | *(10,12,13)* |
| $p57^{Kip}$ | Normal or same as –/– (imprinted gene) | Neonatal lethal with areas of hyperplasia, renal dyspasia | *(64)* |
| *p107* | Normal | Normal; dysplastic retinas in p107–/–Rb+/– | *(52)* |
| *p130* | Normal | Normal; skeletal defects in p130/p107–/– | *(51)* |
| *Pms2* | Normal | Normal development; DNA repair defects, lymphomas and sarcomas | *(96,97)* |
| *Rb1* | Pituitary tumors | Embryonic lethal at E14–15 with defective hematopoeis and neurogenesis | *(41,42)* |
| *Smad3* | Normal | Small size; metastatic colorectal tumors | *(92)* |
| *Smad4* | Intestinal tumors in Apc+/– compound heterozygotes | Embryonic lethal at E7 | *(91)* |
| *Tsc2* | (Eker rat) renal cell carcinoma | Embryonic lethal around E13 | *(149)* |
| *Vhl* | Normal | Embryonic lethal at E9.5–10.5 | *(128)* |
| *WT1* | Normal | Embryonic lethal E13–14 No kidneys or gonads | *(65)* |
| *Xpa* | Normal | Normal development; UV-induced skin tumors | *(101,103)* |
| *Xpc* | Normal | Normal development; UV-induced skin tumors | *(102)* |

gene, or that some other gene is able to functionally compensate in its absence. However, in the case of the *p53* gene, there is probably not the need to invoke either of these explanations. Even though *p53–/–* mice do progress through embryogenesis, and in some cases are able to reproduce, the uniform development of tumors by several months of age implies that the TSG function of *p53* offered sufficient evolutionary advantage to maintain this gene.

Though a portion of *p53*-mutant mice were born without developmental defects, studies of large numbers of these homozygous mutants revealed that 23% of female *p53–/–* embryos failed to achieve normal closure of their neural tube, and developed anencephaly, leading to embryonic or neonatal death *(13)*. This phenotype was to some degree influenced by the genetic background of the mice. The reason for the female preponderance in the anencephaly phenotype is not yet understood, although a similar female predisposition to neural tube defects is seen in humans. Other defects observed in homozygous mutant embryos included dental and ocular anomalies.

A spectrum of tumor types is observed in *p53–/–* mice, although lymphomas account for nearly 75% of observed tumors *(10,12)*. Other tumors observed include hemangiosarcomas, rhabdomyosarcomas, fibrosarcomas, anaplastic sarcomas, and teratomas. Consistent with the original Knudson two-hit hypothesis for the development of tumors, mice heterozygous for the *p53* mutation develop tumors at later points, and, additionally, the spectrum is somewhat different *(10,12)*. Instead of the great predominance of lymphomas, the most frequently observed tumors are osteosarcomas, lymphomas, hemangiosarcomas, brain tumors, and rhabdomyosarcomas, as well as numerous other tumors at lower frequencies. Also consistent with the two-hit hypothesis, tumor cells from heterozygous mice have in many cases lost the remaining wild-type (wt) allele.

It is of obvious interest to compare *p53*-mutant mice to human individuals with the Li-Fraumeni syndrome, who are heterozygous for constitutional mutations of *p53 (14)*. These individuals predominantly have sarcomas and tumors of the brain and breast. Therefore, there are some similarities, but not complete overlap, between *p53* mice and the Li-Fraumeni syndrome. It is not unexpected that humans and mice will differ in the spectrum of tumors that develop, even in response to similar mutations in identical oncogenes. Mice, in general, tend to develop a different spectrum of tumors than humans, with sarcomas being more frequent than carcinomas: The opposite is true in humans. This variability presumably results from differences in genetic background that exist between species, or even within noninbred individuals of a particular species. Examples of such differences may be slight but significant polymorphisms in protein structure, or differences in gene expression patterns.

An analysis of the *p53* mutation in different strains of mice reveals the effect of genetic background on tumor development *(15,16)*. It is well known that different strains of inbred mice are predisposed to distinct types of tumors or leukemias/lymphomas. This is probably related to their complements of endogenous retroviruses and oncogenes, and other genes that interact with them. Donehower et al. *(15)* compared survival rates and the spectrum of tumors in strains 129/Sv inbred and C57B1/6 X 129/Sv mixed-inbred mice, and observed some differences in these two groups. Survival was slightly poorer in 129-inbred mice, both in homozygous and heterozygous mutant mice. For example, they noted prominent difference in the frequency of malignant teratomas in the testicles of male mice. These tumors were frequent in 129-inbred

males, infrequent in the mixed genetic background, and were not observed in wt 129 males with intact *p53* genes. This demonstrates that 129 males are predisposed to testicular tumors when *p53* mutations are present, and it will be of great interest to eventually determine the responsible gene(s) that are presumably polymorphic between strains 129 and C57, and responsible for this difference. Genetic background also affected the types of lymphomas that arose in *p53*-mutant mice. Lymphomas in *p53–/–* mice on a mixed 129Sv/C57BL/6 background are predominantly of $CD4^+CD8^+$ T-cell origin; pre-B-lymphomas made up about 13% of lymphomas observed in *p53–/–* C.B.17-C57BL/6 mice. The recent accomplishments of the mouse genome project *(17),* in providing a detailed genetic map of the mouse, now make the identification of these modifier genes a reasonable goal.

*p53*-mutant mice have become a valuable tool for experimentally approaching the role of this gene in tumorigenesis, as evidenced by numerous, recently published studies *(18–21).* For example, *p53* homozygous mutant mice succumbed more quickly to chronic treatment with a carcinogen, dimethylnitrosamine, presumably because of a decreased ability of cells in treated animals to respond appropriately to carcinogen-induced DNA damage *(19).* However, the absence of *p53* did not result in an increased accumulation of point mutations in a transgene target, either in untreated or mutagen-treated mice, suggesting that increased sensitivity to carcinogen in the abovementioned study may reflect DNA damage other than point mutations *(22). p53*-mutant mice were also used to study the effect of the *p53* gene dosage in a model for chemically induced skin tumors. Topical treatment with dimenthylbenzanthracene (DMBA) resulted in initiation of similar numbers of papillomas in wt, heterozygous, or homozygous mutant mice *(23).* In contrast, progression of these lesions to malignant carcinomas upon treatment with the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), occurred more rapidly in homozygous mutant mice than in the heterozygotes *(23).* As in other studies, loss of heterozygosity occurred during the malignant transition, so that the heterozygotes lost the wt allele. Carcinomas that arose in *p53*-mutant mice appeared less differentiated than those from wt mice, which is again consistent with the general observation that absence of *p53* may accelerate the accumulation of genetic alterations in cells.

Several studies have also examined susceptibility to radiation-induced mutation and tumors in *p53* transgenic and mutant mice, and cells derived from mutant mice *(24–27).* These studies have demonstrated a decreased apoptotic response to irradiation, both in tissues and cells in culture, and an increase in the accumulation of mutations. In one study, transgenic mice expressing a dominant-negative mutant *p53* gene developed tumors, mostly sarcomas, in response to γ-irradiation *(27).* Chromosomal damage induced by γ-irradiation in wt, *p53*-transgenic, or *p53*-null mice, was evaluated by examining the presence of micronuclei in red blood cells. Micronuclei were increased in both *p53*-null and transgenic mice in comparison with wt mice *(27).* Thus, these results are consistent with the role of *p53* in surveillance and destruction of genetically damaged cells that are the likely precursors to tumors.

The effect of *p53* mutations in mice has been studied in some detail in relation to mammary carcinomas, a tumor for which mouse models have existed for some time *(28–31).* Mammary tumors in mice are often caused by the integration of mouse mammary tumor virus (MMTV), which carries a promoter/enhancer that confers mammary-specific expression. Mouse models for mammary carcinoma have included mice

transgenic for the c-*myc,* v-Ha-*ras, neu, Wnt1,* or *int2* proto-oncogenes, under control of the MMTV promoter enhancer. Li et al. *(30)* expressed a mutant *p53* gene in transgenic mice, under control of the whey acidic protein promoter, to confer mammary-specific expression, and demonstrated that treatment with DMBA-reduced tumor latency. Nuclear morphology was more irregular in tumors in transgenic mice than in wt, again indicating a role for *p53* in the maintenance of genome integrity. Using mice carrying the targeted deletion of the *p53* gene, Donehower et al. *(28)* showed that a combination of a *Wnt1* transgene and the absence of *p53* resulted in acceleration of mammary tumor formation. Chromosomal aneuploidy, amplification, and deletion were more commonly observed in tumor cells arising in *p53*-nullizygous mice. Additionally, tumor cell proliferation was enhanced in the absence of *p53 (31).*

In contrast, another study examined mice carrying both an MMTV–c-*myc* transgene and homozygous *p53*-null alleles for the onset of tumors. These mice developed more aggressive lymphomas than mice carrying either transgene/mutation alone, but the presence of the *p53*-null alleles did not appear to accelerate the appearance of mammary carcinomas *(29).* Although these latter results correlate with earlier observations that mammary carcinomas are not frequently observed in *p53*-mutant mice, the formerly mentioned studies demonstrate that mutation or absence of *p53* can contribute to mammary carcinogenesis, in collaboration with a subset of oncogenes, or when *p53* mutant genes are deliberately expressed in mammary tissue.

The wt *p53* gene has also been used as an antitumor agent in a mammary tumor model. Adenovirus vectors, containing either *p53* wt or interleukin-2 cDNAs, were injected in combination into mammary tumors induced in transgenic mice expressing the polyoma middle T-antigen under control of the MMTV promoter/enhancer *(32).* Tumor regression was greatly enhanced by the combination therapy, compared to therapy with interleukin-2 alone, and only with the combination therapy were antitumor cytotoxic T-lymphocytes produced *(32).*

*p53* interacts with many other proteins, and also serves as a transcription factor (TF) involved in the regulation of expression of target genes such as $p21^{WAF1/CIP1}$ (33). Therefore, it was of interest to study the effect of the *p53*-targeted-mutation in combination with other mutations, to determine the effect on viability and/or tumor formation (*p53/Rb* double mutants will be discussed in subheading 3 on the *Rb* mutant mouse). A striking effect was observed when mice carrying mutations in *p53* and *mdm2* were intercrossed *(34,35).* Amplification of *mdm2* is observed in human tumors, particularly frequently in sarcomas, defining it as an important human oncogene *(36).* Physical interaction of the *mdm2* gene product with p53 inactivates the transcriptional activation function of the p53 protein *(37).* Mouse embryos homozygous for a targeted deletion of the *mdm2* gene arrest around the time of implantation *(34,35),* which suggests that negatively regulating p53 function may be crucial during early embryogenesis, when cell division is rampant. This hypothesis was supported by the interesting observation that the *mdm2* mutant phenotype is rescued in the absence of *p53:* embryos carring homozygous mutations in both the *mdm2* and *p53* genes survive and grow to adults, demonstrating that a major, if not sole, function of *mdm2* is indeed to regulate p53 activity *(34,35).* A study of double-mutant mice demonstrated that the spectrum of tumors in *p53–/–mdm2–/–* double-mutant mice did not differ from that observed in *p53–/–* mice, further supporting the hypothesis that the only function of *mdm2* is to regulate p53 activity *(38).*

*p53*-mutant mice were also intercrossed with *scid* mice that carry a mutation in the gene encoding DNA protein kinase, which is involved both in the response to DNA damage, and in joining DNA ends that break during immunoglobulin gene rearrangement *(39).* T-cell development was enhanced in double-mutant mice, and the development of lymphomas occurred earlier in double-mutant mice than in *p53–/–scid+/–* mice. Nacht et al. *(39)* suggested that p53 may be involved in eliminating cells with faulty immunoglobulin gene rearrangements that resulted in activation of oncogenes. Alternatively, the combined absence of the *scid* gene-encoded DNA protein kinase and *p53* may lead to a more severe defect in DNA repair than would result from the absence of either gene by itself.

The high frequency of tumor formation in *p53*-mutant mice allows them to be used as a model for chemoprevention and chemotherapy of tumors. In one such study, it was demonstrated that dyhydroepiandrosterone delayed the onset of tumors; several other compounds had no effect *(20).*

## 3. RB AND ASSOCIATED GENES

The details of molecular pathways that involve the *Rb* gene are discussed in greater detail in Chapter 6 of this volume, and it suffices for this chapter to mention that it is involved in regulating transcription in a cell-cycle-dependent manner. The two salient features of this regulatory system are that the Rb protein is hyperphosphorylated by cyclin-dependent kinases during late G1 phase, and this hyperphosphorylated form is found mostly in the cytoplasm. The hypophosphorylated form is found in the nucleus during G0/early G1, where it complexes with TFs such as E2F-1, preventing E2F-1 from activating transcription of genes whose activity normally increases during S phase *(40).* In this fashion, the *Rb* gene product functions as part of a molecular switch to control the proliferative state of cells. It is apparent that such a switch may figure prominently in determining entry of a cell into a tumorigenic state, but it is less obvious why cells of the retina may be most affected by loss of this ubiquitously expressed gene. Therefore, the *Rb* gene was mutated in mice, with the expectation of clarifying this issue, and contributing to the general understanding of tumor development.

Unlike viable *p53* homozygous mutant mice, *Rb–/–* mice do not survive past embryonic day 14 (E14), apparently because of a major defect in hepatic hematopoesis *(41,42). Rb–/–* mice were also observed to have abnormal cell division and cell death in the nervous system *(41,42).* No developmental abnormalities were observed in the eye itself, and the retina, did not display the cell death otherwise widely present in the nervous system. Although the embryonic demise of mutant mice did not allow observation of homozygous mutants for tumor development, this was possible with *Rb+/–* mice, and indeed these mice developed tumors between 2 and 11 mo of age *(42,43).* Unlike humans, however, tumors in *Rb+/–* mice developed from melanotrophic cells in the intermediate lobe of the pituitary (PIT) gland *(42,43).* As observed in tumors developing in *p53+/–* mice, these PIT tumors lost the remaining wt allele of the *Rb* gene, again confirming Knudson's original two-hit hypothesis for tumor development.

The *Rb* gene also demonstrated the use of conditional gene targeting to study TSGs. This emerging technology is employed to target mutations at specific points during or

after embryonic development, in specific locations, so that the effect of that mutation can be observed in a specific tissue at a specific time, without affecting the entire embryo or animal *(5,6,44,45)*. This technology involves homologous recombination in ES cells to place target sites for site-specific recombination on either side of the portion of the chromosome (chr) to be deleted. A site-specific recombinase-encoding transgene is then expressed at a desired point, usually by placing it under the control of a tissue-specific promoter (*see* Fig. 1). In the case of the conditional targeting of the *Rb* gene, *Frt* sites, which are target sites for site-specific recombination catalyzed by the yeast enzyme, Flp, were placed flanking exon 19 of the *Rb* gene *(46)*. *Flp* recombinase was expressed under control of the rat pro-opiomelanocortin promoter, which primarily expresses in melanotrophic cells of the intermediate lobe. In mice carrying the *Flp* transgene and *Frt* sites in the *Rb* gene, PIT tumors were observed *(46)*. As observed with the original mutation, if only one *Rb* allele carried the *Frt* sites, tumors that developed subsequently lost the wt *Rb* allele. *Rb* loss appeared to be more widespread in the intermediate lobe than actual tumor development, and pretumor histological stages were observed, suggesting that additional genetic events, subsequent to losing *Rb* gene, may be involved in tumorigenesis *(46)*.

Human tumors have been identified in which both the *p53* and *Rb* genes have been inactivated (*see* refs. in ref. *47*). In addition, both genes are involved in controlling progression through the cell cycle. These two important characteristics provided the motivation for intercrossing mice carrying *Rb* and *p53* mutations, to determine the phenotype resulting from the loss of these two prominent TSGs. Because *Rb*–/– mice are not viable, *p53*–/–*Rb*+/– mice were studied, as well as double heterozygotes. Similar to *Rb*+/– mice, PIT tumors were most frequently found in *p53*–/–*Rb*+/– mice, although earlier onset was found *(47,48)*. Medullary thyroid carcinoma was also frequently observed, as were lymphomas, sarcomas, and pancreatic islet cell carcinomas. Retinal dysplasia (but not hyperplasia) was also detected, but animals died of other tumors before retinal tumors might have arisen. Williams et al. *(47)* discuss three possible models for the cooperation observed between *p53* and *Rb* mutations: In the first, *p53* and *Rb* may each negatively regulate growth, so that double-mutant cells undergo relatively unrestricted proliferation; second, the absence of *p53* function leads to an increased rate of loss of the *Rb* gene, which relates to the general observation that accumulation of mutations is increased in the absence of *p53*; third, the absence of *p53* allows for the survival of *Rb*–/– cells, which takes into account the extreme cell death observed in *Rb*–/– embryos, and indicates that *p53* loss may allow these cells to transform instead *(47)*.

Because the *Rb* gene product exerts at least a portion of its regulatory effect by complexing with the TF E2F-1, it became of interest to target a mutation to the *E2F-1* gene, and observe potential similarities with *Rb*-mutant mice. In contrast to *Rb*–/– mice, mice homozygous for a mutation in the *E2F-1* gene survive and are fertile, although they demonstrate hypertrophy of exocrine glands, and males experience testicular atrophy *(49)*. This indicates that some vital functions of Rb do not rely on its interaction with E2F-1. *E2F-1* also appears to function as a tumor suppressor, and homozygous- and heterozygous-mutant mice develop reproductive tract sarcomas, lung adenocarcinomas, lymphomas, and other tumors, at lower frequencies *(49)*. Upon derivation of *E2F-1*–/– *Rb*+/– mice, the frequency of PIT and thyroid tumors was found to be reduced, and the life-span of animals lengthened, indicating that the tumorigenic phenotype of

*Rb*+/– mice is at least in part a consequence of the failure of *Rb* to adequately downregulate the expression of E2F-1-activated genes *(50).*

Two proteins with structural and functional similarities to Rb are known: p107 and p130. This raises the question of whether there is either overlap or the potential for compensation in their respective functions. As with *p53,* this issue is raised because *Rb* fulfills a basic role in the control of cell proliferation, and so it is not completely apparent why embryos progress normally more than halfway through embryogenesis in the absence of *Rb* function. The *p107* and *p130* genes were separately targeted, and in both cases homozygous mutant mice were viable, fertile, and without apparent major defects *(51,52).* In neither case did mutant mice develop tumors at increased frequency, even after long observation. *p107–/–p130–/–* double mutants were then obtained, and, although cartilage and skeletal abnormalities were observed, tumors were still not evident *(51).* However, when *Rb*+/–*p107–/–* mice were derived, pronounced growth retardation and early mortality were evident *(52).* The spectrum of tumors observed, however, did not differ from *Rb*+/– mice among those mice that survived to adulthood, nor did tumors occur with earlier onset. *Rb*+/–*p107–/–* mice developed retinal dysplasia, providing an indication of the importance of *Rb* function in this tissue. When PIT tumors from *Rb*+/–*p107*+/– were examined, it was found that the wt *Rb* allele was lost, but not the wt *p107,* indicating that *Rb* is the more important determinant of tumor formation, although both genes are important in growth control *(52).* Demonstrating that there was indeed functional overlap between *Rb* and *p107,* embryonic death occurred earlier in *Rb–/–p107–/–* embryos than in absence of *Rb* alone, with widespread apoptosis in the liver and nervous system *(52).*

The retinal dysplasia observed in *Rb*+/–*p107–/–* mice suggests that these two genes may serve as coordinately acting tumor suppressors in the retina, and raises the question of whether retinal tumors would be observed in double-homozygous mutants, if they were otherwise viable. This issue represents a general problem with gene-targeting technology: It is only possible to observe the earliest phenotype that results in arrest of development, and later potential phenotypes remain unseen. One way of approaching this problem is to use the previously discussed technology for conditional gene targeting.

An alternate approach that is perhaps technically less challenging, but has seen some important success, is to derive ES cells that have undergone targeting of both alleles of a gene, then use these ES cells to derive chimeric mice. Two approaches are generally available to target second alleles: a second targeting vector, carrying a different antibiotic-resistance gene than *neo* may be used, or it has been observed that in some instances, raising the G418 concentration in the ES cell culture media results in homozygosing of the original targeted allele. Once homozygously targeted ES cells are obtained and injected into wt blastocysts, they often contribute to various tissues in chimeric embryos or animals, so that cells missing both alleles are rescued beyond the point at which an entirely homozygous mutant tissue would be viable. When homozygous mutant cells are retained in particular tissues, they often represent a significant portion of the cells, so that an abnormal phenotype is observed, even though the whole animal remains viable.

This type of experiment was performed using ES cells that carried homozygous mutations in both the *Rb* and *p107* genes. Chimeric mice derived with these doubly targeted ES cells developed RBs *(53).* Retinal cells in chimeras, derived using ES cells

carrying only a double targeting of the *Rb* gene alone, underwent apoptosis, indicating that loss of *p107* was also required for retinal tumor development in the mouse. Furthermore, not all chimeras made with double-mutant ES cells developed RB, suggesting that additional mutation events may be required for development of the malignant phenotype *(53).* These results demonstrate that *Rb* and *p107* are TSGs in the mouse, and further indicate that murine and human tissues are differentially sensitive to the loss of specific TSGs.

Adenovirus-mediated gene therapy has also been attempted using the *Rb* gene. Adenoviral vectors carrying the *Rb* gene were injected into PIT tumors arising in *Rb*+/– mice. Cells of injected tumors showed lower proliferative rates, and animals with injected tumors survived longer than controls, indicating a potential use for the *Rb* gene as a therapeutic agent *(54).*

## 4. CELL CYCLE GENES

Mice carrying mutations in *p53* and *Rb,* two genes involved in the control of cell proliferation, have already been discussed. Several general observations suggested that targeting mutations to other genes, whose products are involved in control of the cell cycle, could also be informative about tumorigenesis. First, cancer is a disease of abnormal cell proliferation. Therefore, genes involved in the control of cell proliferation are obvious candidates to study in an animal model. Second, well-described TSGs, such as *p53, p16$^{Ink4a}$*, and *Rb,* were shown to be involved in pathways that control passage through the cell cycle. Finally, cell cycle regulatory proteins, such as cyclins, were found to be mutated in human cancers *(55).* Two set of genes, both functioning as CDK inhibitors, exert negative regulatory effects on passage through the cell cycle. One set, the Kips, includes *p21$^{Cip1/Waf1}$*, *p27$^{Kip1}$*, and *p57$^{Kip2}$*. The other, the Inks, includes *p16$^{Ink4a}$*, *p15, p18,* and *p19$^{Arf}$*. Overall, the study of additional members of these sets of genes in mutant mice has been less yielding of actual models of tumorigenesis, but, nonetheless, has been informative about the importance of the cell proliferation regulation at the level of the whole organism. Readers wishing a more comprehensive review of these mutant mice should consult the recent review by Kiyokawa and Koff *(56).*

As discussed in subheading 2, *p21$^{Cip1/Waf1}$* is a regulatory target of *p53,* which raises the question of whether the tumor suppressor activity of *p53* is mediated by *p21$^{Cip1/Waf1}$* and downstream targets. However, very few *p21$^{Cip1/Waf1}$* mutations have been found thus far in human tumors. When *p21$^{Cip1/Waf1}$* knockout mice were derived, homozygous mutants experienced normal development *(57).* Unlike *p53* mutant mice, no tumors developed in *p21$^{Cip1/Waf1}$*–/– mice. In addition, the absence of *p21$^{Cip1/Waf1}$* did not rescue the lethality of the *mdm2* mutation in mice *(58),* further indicating that *p53* must act more broadly than simply regulating *p21$^{Cip1/Waf1}$* expression. Although *p21$^{Cip1/Waf1}$* mice appeared normal, study of *p21$^{Cip1/Waf1}$*-deficient cells in vitro did reveal abnormalities. When *p21$^{Cip1/Waf1}$*–/– cells were observed in tissue culture, defects in G1 checkpoint control were found, and cells entered G0 at higher densities than normal, a phenomenon also displayed by *p53*–/– cells *(57,59).* In contrast to *p53*–/– thymocytes, which are refractory to apoptosis, apoptosis could be induced in *p21$^{Cip1/Waf1}$*–/– thymocytes identically to wt cells *(57).* Another study examining the potential involvement of *p21$^{Cip1/Waf1}$* in tumorigenesis demonstrated that ras-transformed primary keratinocytes

from $p21^{Cip1/Waf1}$–/– mice developed more aggressive tumors than did transformed keratinocytes from wt mice *(60)*.

Disruption of the $p27^{Kip1}$ gene in mice resulted in large mice with multiorgan hyperplasia, indicating a failure to properly regulate cell division *(61,62)*. Nodular hyperplasia and adenomas of the PIT gland intermediate lobe were found in $p27^{Kip1}$–/– mice, reminiscent of the phenotype of *Rb*+/– mice, suggesting a particular sensitivity of this tissue to defects in control of the cell cycle *(61,62)*. Although low levels of $p27^{Kip1}$ are found in human tumor cells, $p27^{Kip1}$ mutations have generally not been found in human tumors. Likewise, with the exception of the PIT adenomas, tumors do not develop in $p27^{Kip1}$–/– mice, despite increased cell proliferation. However, when $p27^{Kip1}$–/– or +/– mice are challenged with γ-irradiation or carcinogens, tumors in multiple tissues are obtained, with homozygous mutants developing tumors at approximately twice the frequency of heterozygous mice *(63)*. In contrast to observations with *p53*- or *Rb*-mutant mice, tumors in heterozygotes appear not to have undergone loss of heterozygosity (LOH) at the $p27^{Kip1}$ locus *(63)*. Thus, $p27^{Kip1}$ appears to be a TSG in mice, but, in contrast to most TSGs, a single copy of this gene appears to be insufficient to prevent tumors.

Mice carrying a targeted deletion of the $p57^{Kip2}$ gene also have a phenotype characteristic of a defect in the regulation of cellular proliferation, including omphalocele, cleft palate, incomplete differentiation of chrondrocytes, renal medullary dysplasia, lens cell hyperplasia, and adrenal cortical hyperplasia *(64)*. $p57^{Kip2}$ is an imprinted gene, meaning that it is only expressed from the chromosome inherited from one parent; in the case of $p57^{Kip2}$, expression of the maternally inherited chromosome is observed. Therefore, the full mutant phenotype can be found in heterozygotes that have inherited the mutant allele from their female parent. The $p57^{Kip2}$ gene maps to the chromosome locus that harbors deletions in individuals with the Beckwith-Wiedeman syndrome (BWS). Several aspects of the murine $p57^{Kip2}$ phenotype are also characteristic of the BWS in humans, including omphalocele, renal dysplasia, and the adrenal hyerplasia. A portion of BWS patients also develop Wilms' tumors, a tumor of the kidney, but this is not observed in mutant mice, suggesting that mice may not be a good model for this tumor. Mice carrying a mutation in the *WT1* gene, another tumor suppressor for Wilms' tumor, also fail to develop kidney tumors *(65)*. Nevertheless, the similarity of $p57^{Kip2}$-mutant mice to the premalignant condition of BWS individuals suggests that $p57^{Kip2}$ may be exerting a tumor-suppressive effect that is more vital in humans than in mice *(64)*.

In contrast to the previous CDK inhibitor mutant mice, mutant mice that carry a targeted mutation that eliminates both $p16^{Ink4a}$ and the overlapping gene, $p19^{Arf}$, provide clear evidence for the tumor-suppressive role of the $p16^{Ink4a}$ gene, even without treatment with carcinogens *(66)*. Homozygous mutant mice were viable and fertile, but developed tumors, mostly sarcomas and lymphomas, starting at 18 wk after birth. Treatment with DMBA and UVB irradiation accelerated tumor development. Cells from $p16^{Ink4a}$–/– mice also proliferated faster in culture. Furthermore, introduction of the Ha-ras gene into fibroblasts derived from –/– mice, but not from +/+ or +/– mice, resulted in focus formation in soft agar, again demonstrating the tumor-suppressive effects of $p16^{Ink4a}$ and $p19^{Arf}$ (66).

Mutations in this set of genes provide strong evidence for their role in regulating cellular proliferation in diverse tissues, and demonstrate that this is an important and perhaps central role of TSGs.

## 5. ATAXIA TELANGIECTASIA

Ataxia telangiectasia (AT) is an autosomal recessive disease with diverse clinical manifestations, including progressive neurodegeneration, immunodeficiency, and lymphoreticular malignancies, as well as several other components. The gene responsible for this disease, *ATM,* encodes a protein with kinase activity that appears to function upstream of *p53* in the cellular response to ionizing radiation. Upon targeting the *ATM* gene, homozygous mutant mice display many phenotypic features of the human disease, including the development of lymphomas *(67,68).* The relationship between *p53* and *ATM* has been studied in considerable detail, and the results and conclusions obtained thus far are complex, which is not surprising, given the tremendous variety of human cancer.

Tumor formation, consisting mostly of lymphomas, is accelerated in *ATM–/–p53–/–* mice, (but not in *ATM/p21$^{Cip1/Waf1}$* double mutants), compared to either single mutant *(69,70).* p53 protein levels are usually elevated in thymuses of mice exposed to γ-irradiation, but this failed to occur in *ATM–/–* mice *(71).* Lymphoma formation in *ATM* mice may therefore result from a failure to respond to DNA damage appropriately, especially in cells in which the damage is not lethal, but sufficient to cause malignant transformation.

Mouse embryo fibroblasts, cultured from *ATM–/–* mice, grew poorly, and underwent early senescence, ascribing a role to ATM in the maintenance of proliferation *(69,70).* This early senescence was rescued in both *ATM/p53* or *ATM/p21$^{Cip1/Waf1}$* double-mutant cells *(70,72).* The observation that p21$^{Cip1/Waf1}$ protein was elevated in *ATM–/–* cells, probably because of increased stabilization of the protein since mRNA levels were actually reduced in mutant cells, led Xu et al. *(70)* to suggest that elevated p21$^{Cip1/Waf1}$ was responsible for the early senescence. In addition, irradiated mouse embryo fibroblasts from *ATM–/–* mice continued to enter S phase, rather than arresting at the G1–S checkpoint, as is normally observed *(70,71).* This defect is more severe in both *ATM/p53* and *ATM/p21$^{Cip1/Waf1}$* double-mutant cells, indicative of a correlation between defective G1–S checkpoint control and early senescence. Together, these results suggest that *ATM* and *p53* cooperate in regulating passage through the cell cycle, and that failure to properly regulate this pathway leads to tumorigenesis.

Additional studies demonstrated that *ATM* is likely to regulate both *p53*-dependent and -independent pathways. γ-irradiation-induced thymic apoptosis, a *p53*-mediated event, occurred normally in *ATM–/–* mice in vivo, but was suppressed in *ATM/p53* double-mutant mice *(69,71).* In vitro, thymocytes from *ATM–/–* are also somewhat resistant to γ-irradiation-induced apoptosis, but thymocytes from *ATM/p53* double-mutant mice are completely resistant *(69,71).* These results confirm that the apoptotic response to irradiation is regulated through *p53*-dependent pathways, but some of these pathways may not involve *ATM.*

Although thymocytes in *ATM–/–* mice were relatively radiation-resistant, other tissues, the gastrointestinal (GI) tract and skin, in particular, were markedly more sensitive to γ-radiation treatment than wt *p53–/–* mice *(68).* Presumably, this response involves more than simple apoptosis, but also demonstrates that results in one tissue cannot be generalized to the whole organism. This extreme tissue radiation sensitivity was identical in *ATM/p53* double mutants *(73),* again contrasting with the protective effect observed with thymic apoptosis, suggesting that radiation-induced tissue damage

is not mediated through *p53*. Thus, although it is clear that *ATM* and *p53* are both intimately involved in the determination of cell proliferation and survival, the interrelationships of the specific pathways are complex.

## 6. COLON CANCER

The *APC* gene first came to notice in mice under the guise of the *Min* gene, originally defined as a dominant mutation identified in a screen of ethylnitrosourea-treated mice, which predisposed them to multiple intestinal neoplasia *(74)*. *Min* mice develop multiple tumors throughout their intestinal tract that resemble various forms of adenomas, which in some cases become invasive, but not metastatic. This phenotype bears a strong resemblance to the human condition of adenomatous polyposis coli, in which afflicted individuals develop hundreds of polyps of the colon that can progress to carcinoma. *APC* was identified to be the gene harboring mutations in humans with this disease, and it was subsequently found that the *APC* gene was mutated in *Min* mice (now designated $APC^{Min}$) *(75)*. This provides an important demonstration that mouse models of TSGs genes can result from experiments other than standard gene targeting (although other mutations in the *APC* gene have subsequently been introduced using that methodology). Indeed, the introduction of more drastic mutations in the *APC* gene has revealed that homozygous mutants do not survive embryogenesis, which ascribes a developmental role to APC *(76)*. Further study of APC has demonstrated that it plays an important role in signaling pathways related to transcriptional activation of certain genes by the LEF-1 TF, and possibly to cell adhesion mediated by β-catenin and E-cadherin *(77)*.

Adenomas in $APC^{Min}$+/– mice have lost the remaining wt allele of *APC,* consistent with findings in humans with colon carcinoma *(78,79)*. This was also true of mice carrying targeted mutations in the *APC* gene *(76,80)*. In the latter case, adenomatous cells were found to have lost the wt allele at the microadenoma stage, suggesting, as is also suspected in humans, that the loss of *APC* signifies an early event in the development of colon carcinomas *(76,80)*. Another study, that involved a different targeted *APC* mutation, utilized a novel approach for conditional gene targeting by first using homologous recombination to place *lox* site-specific recombination sites (an alternative to the previously discussed *Frt*), flanking exon 14 of the *APC* gene *(81)*. Because the *lox* sites did not interfere with expression of *APC,* mice homozygous for the targeted allele were derived, and were normal. An adenoviral vector encoding the cre recombinase, an enzyme that deletes sequences between two lox sites, was then introduced into the intestine transrectally. Subsequently, infected cells that had deleted a portion of the *APC* gene gave rise to adenomas *(81)*. As with the previously discussed conditional targeting of the *Rb* gene, this conditional approach allowed the study of *APC*–/– cells, while avoiding the problem that *APC*–/– embryos do not survive embryogenesis.

It was also shown that the cyclooxygenase 2 gene is activated during polyp formation, and that a targeted mutation in the cyclooxygenase 2 gene suppresses polyp formation in $APC^{\Delta 716}$ mice, possibly by affecting angiogenesis *(82)*. This observation may provide an insight regarding recent reports that aspirin, a cyclooxygenase inhibitor, may suppress colon polyp formation in humans *(83,84)*. $APC^{Min}$ mice have also been crossed with *p53*-mutant mice. $APC^{Min}$+/–*p53*–/– mice developed lymphomas and sarcomas similar to *p53*–/– mice, but also developed pancreatic dysplasia *(85)*. In some

cases, the pancreatic dysplasia could be classified as an adenocarcinoma, in which the wt *APC* allele was lost. Despite the increase in pancreatic abnormalities, no increase was observed in intestinal malignancy caused by absence of *p53*. This was surprising, given that *p53* is clearly mutated in tumors of the human colon, and that the *p53* mutation had also been shown to protect against irradiation-induced apoptosis in mice. These results may be indicative, as discussed previously, that *p53* may be involved in protecting against certain types of chromosome insults, but not the mutational events that finally lead to adenoma formation in $APC^{Min}$ mice.

Study of $APC^{Min}$ mice also provided a window through which to examine another important aspect of how animal models can contribute to the identification of novel TSGs. The degree of polyp formation in *Min* mice is heavily dependent on the genetic background: C57BL/6 is the strain on which tumors appear most rapidly *(74)*. This observation implies that other genes interact with *APC* to affect the tumor phenotype, which are polymorphic between different stains of mice. The mouse genome project has produced a fine genetic map of the mouse that allows the mapping and eventual cloning of so-called "modifier genes," based on differential phenotypes between strains *(17)*. One such gene has been identified and designated *MOM* (modifier of *Min*) *(86,87)*. A candidate gene found in the *MOM* locus is intestinal phospholipase A2, though it remains unexplained how this particular enzyme may effect rates of adenoma formation *(88,89)*. The pursuit of new TSGs and their genetic modifiers promises to develop into a major field of study. As discussed in a recent review by Balmain and Nagase *(90)*, it is now possible use the fine genetic map of the murine genome to begin to identify loci that control inbred mouse strain-dependent predisposition to the development of various tumors, with the expectation of identifying novel TSGs.

Recently, gene knockouts of two *SMAD* genes have provided what should prove to be very informative models for intestinal tumorigenesis *(91,92)*. SMADs are intracellular proteins that are involved in transducing signals from the transforming growth factor β (TGF-β) receptor. *SMAD4* (also known as *Dpc4*) maps to the 18q locus, a chr segment often deleted in human colon carcinoma. The *DCC* gene was originally cloned as a candidate tumor suppressor from this locus, although this now appears not to be the relevant TSG. Homozygous mutant *Smad4* mice do not survive embryogenesis, but heterozygotes appear normal *(91)*. When *Smad4* heterozygotes were crossed with $APC^{\Delta 716}$ mice, however, the double heterozygotes developed more malignant tumors than were observed in simple $APC^{\Delta 716}$ heterozygotes, including adenocarcinomas that invaded the submucosa *(91)*. As before, tumors had lost the wt alleles. In tumor cells that underwent LOH, it appeared that the chr containing mutant alleles of both *APC* and *Smad4* had subsequently been duplicated, restoring diploid levels of all other genes, *(91)*. Because murine homologs of the *APC* and *Smad4* genes are on the same chr, separated by approx 30 cM, this experiment first required mating single heterozygotes and screening for mice that had both mutant alleles on the same chr as a result of meiotic recombination. This indicated that *APC* and *Smad4* were probably the only TSGs involved in colon tumor formation on that chr.

A striking phenotype was obtained upon targeting of a second Smad gene, *Smad3*. *Smad3–/–* mice are viable and fertile, but develop metastatic colorectal adenocarcinomas between 1 and 6 mo of age, implicating TGF-β signaling in the development of tumors of the GI tract, and providing the most authentic model of human colon carci-

noma in a genetically engineered mouse obtained to date *(92).* These tumors extended into the submucosa, and metastasized to lymph nodes. APC was not lost in these tumors, and appeared to be expressed in tumor tissue. Along with the more malignant-appearing lesions, other abnormal tissue was observed, so that future analysis of these mice should reveal whether they progress through similar genetic changes that occur in human malignancy. Whether tumor formation in Smad-mutant mice relates to a growth-suppressive property of TGF-β, or an as-yet unknown function, remains to determined. The present mouse models should provide ample experimental material for these studies.

## 7. DNA REPAIR GENES

Genes involved in DNA repair comprise an important group of tumor suppressors, and mice with mutations in these genes have provided models for human syndromes such as xeroderma pigmentosum and hereditary nonpolyposis colon cancer (HNPCC). Three classes of genes have been studied, chiefly because of their involvement in human cancer-predisposition syndromes. The first class includes the DNA mismatch repair (MMR) genes, *Mlh1, Msh2, Msh6, Pms1,* and *Pms2;* the second class contains two genes involved in nucleotide excision repair, *Xpa* and *Xpc;* and the final class is comprised of the nucleotide repair gene that encodes o(6)-methylguanine-DNA methyltransferase.

HNPCC is a relatively common cancer predisposition syndrome in humans that results from mutations in DNA MMR genes, most often *mlh1* and *msh2,* and less commonly *pms1* and *pms2. Msh2–/–* mice are viable, and develop GI, skin, and lymphoid tumors *(93,94).* In *Msh2–/–* mice, expression of the APC protein appeared to be lost in their adenomas, presumably contributing to the neoplastic progression *(94).* Furthermore, accelerated intestinal tumorigenesis occurred in *Msh2–/–Apc$^{Min}$+/–* mice *(95).* LOH at the *Apc* locus in adenomatous tissue always occurred in *Apc+/-* mice, but was sporadic in *Msh2–/– Apc$^{Min}$+/–* mice *(95).* This observation suggests that the absence of *Msh2* activity leads to loss of *Apc* function, through structural mutations, rather than loss of the *Apc* chromosomal locus by large-scale deletions.

As might be expected in animals deficient in DNA repair genes, an increased DNA mutation rate can be detected in intestinal tissue of *Mlh1* and *Pms2* homozygous mutant mice, although not in *Pms1* mutants *(96). Mlh1*-mutant mice develop GI tumors, but *Pms2* mutants only develop lymphomas and sarcomas, despite the increased mutation rate in the intestine *(96,97).* Similar to *Msh2* mice, however, *Pms2–/– Apc$^{Min}$+/–* mice gave rise to 3–4× the number of intestinal adenomas as *Apc+/–* mice, further implicating MMR genes in intestinal tumorigenesis *(98).* An additional MMR gene, *Msh6,* which has recently been shown to be involved in human colorectal tumors *(99),* also appears to function as a colon TSG in mice. *Msh6–/–* mice developed both GI and lymphoid tumors, suggesting that it may be important to further examine the involvement of this gene in human cancer *(100).* Hence, not all DNA MMR genes are of equal significance in tumorigenesis, and further study of these mutations may provide insight into their respective functions.

Xeroderma pigmentosum is a rare autosomal recessive skin disease characterized by increased sensitivity to ultraviolet radiation (UV), and frequent development of skin tumors. Enzymatic defects in nucleotide-excision repair also define this disorder, and

eight complementation groups have been defined in humans. Two of the known XP genes, *Xpa* and *Xpc,* have been mutated in mice, and, in both cases, homozygous mutant mice were highly susceptible to UV-induced squamous skin carcinomas *(101–103).* With the exception of some solid organ adenomas in older *Xpa–/–* mice, spontaneous tumors were not observed in these mice *(101–103).* Prolonged treatment of *Xpa+/–* mice with UV did not result in an increased incidence of tumors, suggesting that LOH at this locus is a rare event *(104).* As observed with other mutations, *Xpc/p53* double-mutant mice experience an accelerated rate of skin tumor formation, compared with the *Xpc* mutation alone, implicating *p53* in pathways that protect against UV-induced DNA damage in the skin *(105).*

The O(6)-methylguanine-DNA methyltransferase gene *(Mgmt)* encodes an enzyme that repairs mutagenic alkylation of the $O^6$ position of guanine. Transgenic mice were generated that specifically overexpressed this enzyme in skin cells, and this overexpression was found to be protective against DMBA/TPA-induced skin tumors *(106). Mgmt–/–* mice have also been generated, and were found to have significantly lower $LD_{50}$s upon treatment with the mutagen, methylnitrosourea (MNU) *(107).* Mutant mice developed thymic lymphomas and lung adenomas *(107).* When *Mgmt*-mutant mice were crossed with *Mlh1* mutants, the resultant double homozygotes were resistant to MNU-induced lethality, although they still developed frequent lymphomas in response to MNU *(108).* These results are consistent with the hypothesis that MMR results in double-strand breaks at sites of alkylation-related mutation, leading to cell death. Cell death, in turn, prevents cells with mutations from giving rise to tumors, hence, the high lymphoma rate in MNU-treated *Mgmt/Mlh1* double mutants, in which apoptotic elimination of cells with alkylation-related mutations does not occur. Mice with mutations in DNA repair genes should continue to serve as valuable models for the mechanistic study of mutagen-induced tumorigenesis.

## 8. *BRCA 1* AND *2*

*BRCA1* and *BRCA2* are TSGs involved in human breast cancer, which have recently received considerable attention. The function of the two encoded proteins remains unclear, although BRCA1 may associate with p53 *(109),* affecting its transcriptional regulatory function; BRCA2 associates with Rad51 *(110),* suggesting a function in DNA repair. Mouse embryos deficient in either *BRCA1* or *2* arrest early in development, around E7.5–8.5, with apparent defects in cell proliferation *(111–113).* In neither case did heterozygous mice develop tumors *(112,113). BRCA* mutant mice have been crossed with *p53* mutants, again without the development of new tumors; the absence of *p53* did slightly prolong survival of both *BRCA1* or *2* homozygous mutant embryos, suggesting a possible functional association *(111,114).* Recently, mice carrying a targeted *BRCA2* mutation were derived, which resulted in production of a truncated BRCA2 protein. Most homozygous mutant embryos arrest during development, but, among the few homozygous animals that are born, lethal thymic lymphomas develop at about 12–14 wk of age *(115,116). BRCA2–/–* fibroblasts obtained from mutant embryos have defects in proliferation and DNA repair that can be related to overexpression of p53 and $p21^{WAF1/CIP1}$ (116), and further study of these cells and animals may provide more insight into the role of *BRCA* genes in mammary tumorigenesis.

## 9. TUBEROUS SCLEROSIS

The Eker rat develops renal cell carcinomas with dominant pattern of inheritance, which is consistent with the inheritance of a dominantly acting oncogene, or an inactive TSG. The *Tsc2* gene, which is mutated in the human condition, tuberous sclerosis, was found to be the responsible gene *(117–119).* The additional observation that this locus underwent LOH during tumor development indicated that *Tsc2* was likely to be functioning as a TSG *(117,120).* Transgenic rats, in which a wt *Tsc2* gene was inserted, did not develop tumors *(121). Tsc2* encodes a membrane-associated guanosine triphosphatase-activating protein designated "tuberin" *(122).* This large protein has also been found to have transcriptional regulatory activity *(123,124),* and efforts are underway to identify potential target genes *(125).* Members of the AP1 family of TFs are overexpressed in renal carcinomas from Eker rats, and may constitute one set of targets for regulation by *Tsc2 (126).*

## 10. TSG MUTATIONS NOT RESULTING IN MURINE NEOPLASMS

Several human TSGs, not discussed in previous subheadings, have been targeted in mice, without yielding a model for tumor formation. Some of these genes have been found to be homozygous lethal, including *WT1* and *Vhl.* Mice heterozygous for targeted mutations of these genes do not develop tumors, indicating either that the LOH at their respective loci is rare or that they do not function as TSGs in these animals.

Von Hippel-Landau (VHL) disease predisposes affected individuals to multiple tumor types, including hemangioblastomas and renal cell carcinoma. The *Vhl* gene encodes a protein that associates with transcriptional elongation proteins, although it remains unclear whether this is the major function of this protein. The VHL protein associates intracellularly with the extracellular matrix protein, fibronectin, and fibronectin secretion and matrix assembly appeared to be diminished in *Vhl–/–* cells and embryos *(127).* Since the abnormal interaction of cells with the extracellular matrix is an important aspect of tumorigenesis, this association with fibronectin assembly provides one possible mode through which the mutation of this gene may be involved in tumor formation. *Vhl–/–* mouse embryos do not survive beyond E12.5, because of defective placental vasculogenesis *(128).*

*WT1* encodes a zinc finger TF, and is deleted in a small fraction of Wilms' tumors, a kidney tumor that occurs in young children *(129).* Mouse embryos carrying a homozygous mutation in this gene fail to develop kidneys or gonads, with the kidney progenitor mesenchyme becoming completely apoptotic *(65). Wt1+/–* mice, on the other hand, survive to adulthood without the development of kidney or other tumors *(65).* The WT1 protein has been demonstrated to physically interact with p53 *(129a).* However, *WT1+/–p53–/–* mice still fail to develop kidney tumors, and, in *WT1/p53* double-mutant embryos, the renal phenotype is identical, with no amelioration of the apoptosis (J. Kreidberg, unpublished results).

## 11. NONMAMMALIAN MODELS

The discussion thus far has dealt with mammalian systems that have almost exclusively been models utilizing mice carrying targeted mutations. However, it is also important to acknowledge the potential contribution of nonmammalian systems to this

field. Although powerful genetic systems have been developed to analyze tumorigenesis in mice and humans, control of cell proliferation in organisms such as *Drosophila* and *Caenorhabditis* can be genetically dissected with greater throughput and at lower cost. Current analysis reveals that the molecular pathways that control cell proliferation are highly conserved between all organisms, justifying the use of nonmammalian models for the study of tumorigenesis. *Drosophila melanogaster* deserves particular mention, because flies develop tumors with important similarities to mammals *(130,131),* including induction by carcinogens, excessive cellular proliferation, morphological changes in cellular architecture, and invasiveness. Tumors can also be transplanted from fly to fly as a test of malignancy. One genetic approach described by Xu et al. *(131)* in *Drosophila* should be especially useful in the search for new tumor suppressors. Chimeric flies can be made so that some cells contain the previously discussed *Frt* site-specific recombination sites on both homologs of a chr in close proximity to the centromeres. These cells are also able to express the Flp recombinase under the control of the heat-shock promoter, which allows conditional expression at any desired timepoint, with far greater ease than is possible in mammalian systems. Induced expression of Flp in somatic cells results in mitotic recombination between chrs; such daughter cells become homozygous for the chr region distal to the *Frt* sites. If this region carries a mutation that inactivates a TSG, a tumor will develop from the chimeric cells. Further genetic analysis can identify the gene responsible. This approach has been used by Xu et al. *(132)* to identify several novel genes whose inactivation results in excessive cell proliferation.

## 12. NEW TECHNOLOGIES

Gene targeting and other transgenic technologies are powerful approaches for the study of known TSGs. New technologies are also being developed to identify novel TSGs. This chapter has already discussed the approaches proposed by Balmain and Nagase *(90),* which will attempt to map and eventually clone genes, based on genetic differences between mouse strains. Approaches based on physical differences between chrs of tumor cells and normal cells are also being developed (*see,* for examples, refs. *133–137).* Spectral karyotyping is one such technology *(138),* although several others are being developed that also detect differences between karyotypes of normal and neoplastic cells. Sets of probes that paint specific chrs are labeled with single or combinations of chromogens, so that all 19 and X, Y chrs can be distinguished from each other at once, using fluorescent *in situ* hybridization, because the unique set of chromogens will impart a different color to each chr. In tumor cells, translocations between chrs are detected, with immediate identification of both involved chrs *(138).* This approach is especially useful for mouse chrs, which are all acrocentric and close in size, making karyotyping much more difficult than in humans. Spectral karyotyping, and related approaches that allow analysis of the entire karyotype in a single experiment, should allow for more efficient screening of tumors for cancer-causing mutations.

## 13. SUMMARY

As the results summarized in this chapter amply demonstrate, the pursuit of animal models of TSGs has thus far yielded several valuable models for the study of tumorige-

nesis. Obviously, if the only result of these efforts was to demonstrate tumor formation upon loss of a specific gene, one may question the value of such an experiment. However, as evidenced by some of the earliest mutant strains that have been studied in greater detail, it is clear that these animals are providing experimental models that will provide important insights into tumorigenesis. New technologies, such as conditional gene targeting and various procedures for karyotyping tumor cells, combined with the wealth of information provided by the human and mouse genome projects, will allow even more powerful experimental approaches in the near future.

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# 2 Viral Oncoproteins as Probes for Tumor Suppressor Function

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***Contents***



## 1. INTRODUCTION

Transformation by small DNA tumor viruses requires multiple events that induce normally quiescent cells into a state of proliferation *(1)*. This is necessary because the genomes of such viruses do not carry the machinery required for the replication of its own genome nor the components necessary for the transcription of its genes *(2)*. Hence, the common strategy utilized by this group of viruses is to make use of the transcription and replication machinery used by the host cells for their own purposes. The small DNA tumor viruses such as adenovirus (Ad), simian virus 40 (SV40), and human papillomavirus (HPV), are all capable of inducing a proliferative state in quiescent host cells, by the judicious use of their transforming oncoproteins *(2–5)*.

Apparently, the viral genome is designed so that the products of their early genes are capable of inactivating the major negative regulators of mammalian cell proliferation, namely, Rb and its family members and the p53 tumor suppressor protein *(3)*. Each of the three small DNA tumor viruses have proteins that can interact with these growth-suppressive proteins, and in every situation this interaction results in an inactivation of the tumor suppressor proteins *(6–9)*. The specific interaction between such viral oncoproteins (V-ONC) and the cellular tumor suppressor proteins have been studied in great detail, and this has led to a greater understanding of the biochemical processes involved in oncogenic transformation *(10)*. An important fallout of these detailed studies is that now it is possible to utilize these interactions for diagnostic purposes, because the V-ONC act as specific probes for the functional integrity of tumor suppressor protein.

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Though the small DNA tumor viruses are not closely related evolutionarily, they all share the ability to inactivate the Rb family proteins, as well as p53 *(2,11).* In the case of Ad, the *E1A* gene product can physically interact with the functional form of Rb and its family members *(12);* at the same time, products of the *E1B* gene can bind to and inactivate the p53 protein *(13–15).* Similarly, in the case of HPVs, the E7 protein interacts with the Rb family proteins *(16),* and a separate protein, the product of the *E6* gene, binds to and inactivates p53 *(5).* The situation is slightly different in the case of SV40: Here, the large T-antigen (T-Ag) is capable of interacting with both Rb family members and the p53 protein *(4,14).* But, in all cases, the viruses carry genes that can neutralize the Rb- and p53-mediated suppression of cell proliferation.

This review is organized so that first the V-ONCs that interact with the Rb family of tumor suppressors are discussed, followed by those that bind to p53. In the last subheading, the potential use of these interactions in assessing the presence and functional status of the cellular antioncogenes is discussed.

## 2. V-ONCS INTERACTING WITH RB AND RB FAMILY PROTEINS

### *2.1. Adenovirus E1A*

#### 2.1.1. Historical Perspective

A considerable amount of work was done in the early 1980s to understand the mechanism of oncogenic transformation by the early gene products of DNA tumor viruses, especially the *E1A* gene of Ad. Two major lines of investigation were undertaken by different groups: mutational analysis of the different regions of the *E1A* gene required for transformation, and analysis of different cellular proteins associating with Ad E1A proteins. Results of these studies converged on the potential mechanisms involved in the E1A-mediated cellular transformation, and laid a solid foundation for what is known about viral oncogenesis today.

Ad E1A is a phosphoprotein expressed at the early stage of infection, and the phosphorylation of E1A has been shown to affect its function. Differential splicing gives rise predominantly to two different polypeptides of different sizes, one 243 residues long (12S E1A) and the other 289 residues long (13S E1A) *(17).* There are additional smaller forms of E1A proteins also generated by differential splicing, but the complete transformation capacity of E1A requires the regions present in the 12S E1A protein. Analysis of the E1A structure revealed three regions highly conserved among different serotypes of Ads. These conserved regions (CR) have been named CR1, CR2, and CR3 (Fig. 1). Of these, CR1 and CR2 are present on both the 12S and 13S E1A, but CR3 is present only on the 13S E1A. As can be seen from the figure, the CR3 region almost perfectly overlaps the domain spliced out in the 12S E1A.

The CR1 and CR2 regions are derived from the exon 1 of the *E1A* gene, and the CR3 region is at the junction of exon 1 and exon 2. exon 2 of the *E1A* gene constitutes the multifunctional carboxy-terminal region, and is present in both the 12S and 13S forms of the protein *(18).* This C-terminal region is involved in effecting functions like transcriptional repression, suppression of cellular transformation, and mediated susceptibility to the host cytotoxic T-lymphocyte response *(17).*

It was discovered early that the Ad *E1A* gene could modulate the gene expression of both viral and cellular genes *(2).* This was based on studies using mutant viruses, which had inactive forms of the *E1A* gene. Further studies revealed that E1A regulates

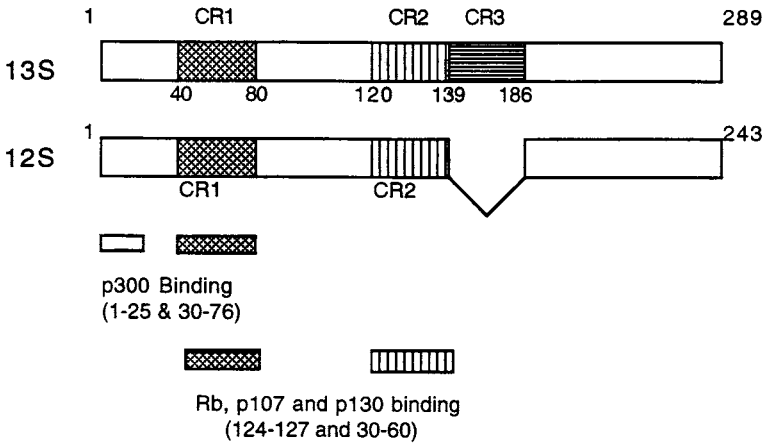


**Fig. 1.** Schematic of the Ad 5 E1A protein. Note that the CR3 domain present in the 13S protein is absent in the shorter 12S E1A. The conserved regions involved in binding to cellular proteins are also indicated.

gene expression, mostly at the level of transcription, and this is achieved through the mediation of many cellular transcription factors (TFs). E1A can induce, as well as repress, transcription from many cellular promoters, and these functions reside on different regions of the E1A protein. E1A does not bind to DNA directly, and all its cellular effects are mediated through targeting the cellular proteins of the host.

### 2.1.2. Immortalization and Transformation by E1A

It is well established that *E1A* is an oncogene, and is capable of immortalizing primary rodent cells on its own, and can effectively transform cells in cooperation with a second oncogene, such as *ras (1,2)*. The transformation function of *E1A* has been studied in detail, especially the requirements of different regions necessary for functionally cooperating with other oncogenes. Such studies have revealed that sequences contained in the exon 1 of E1A are capable of cooperating with oncogenic ras to transform primary cells *(17)*. But additional regions are necessary for transformation in collaboration with *E1B* gene or polyoma middle-T-Ag. For example, residues 140–193, overlapping with the CR3 region of the E1A protein, are required for cooperation with polyoma middle-T-Ag, and residues 266–276 in the C-terminal domain are required for cooperating with E1B in transformation.

Such detailed analysis of the regions of the E1A required for transformation helped identify three distinct domains within exon 1 sequences to be essential for transformation function. The CR1 region, as well as the CR2 regions, were absolutely necessary for transformation function, but were not sufficient by themselves. It soon became clear that the amino (N)-terminal residues, 2–25, which fell outside the span of CR1 sequences, were indispensable for transformation. Mutations within CR1 and CR2 significantly reduced the capacity of E1A to transform cells. Although the 12S E1A can bring about complete transformation in most primary cell lines, the CR3 region is essential for transformation of certain specific cell lines *(17)*.

In addition to transforming cells, E1A was found to have certain growth-suppressive properties also *(17,19,20)*. The first indication of this was obtained when it was found that

transformation of baby rat kidney cells by the 12S E1A was 100× more efficient than by the 13S E1A molecule. This raised the possibility that the CR3 domain, or other regions encoded by the exon 2, can function to suppress transformation. Further studies *(21–23)* showed that the repressive function resided within residues 237 and 283, especially in the region spanning 256–283. The molecular mechanisms by which this region mediates such suppressive properties are not yet clear, even though certain cellular proteins, such as CtBP, have been found to bind to this region. Repression of transformation by E1A exon 2 region may involve more indirect mechanisms also. For example, it is clear that E1A protein can induce the accumulation of the p53 protein, which has strong tumor-suppressive properties. E1A is thought to enhance p53 levels both by increasing the transcription of its genes, as well as by increasing the stability of the protein *(24,25).* The detailed molecular mechanisms involved in these processes are emerging now.

### 2.1.3. E1A Binding Proteins

The identification of different regions of E1A involved in cellular transformation facilitated detailed studies on the underlying molecular mechanisms. As mentioned earlier, one fruitful strategy was to identify cellular proteins that interact with E1A, and assess the potential effects of the interaction *(1).*

Early studies *(26)* identified a set of six cellular proteins that co-immunoprecipitated with Ad E1A from cellular extracts. They ranged in size from 300 to 33 kDa. The first protein to be positively identified as an E1A-binding protein was the Rb protein, the product of the retinoblastoma tumor suppressor gene. This finding was a milestone in the study of oncogenic mechanisms, because it provided an example of a V-ONC binding to and conceivably neutralizing a cellular tumor-suppressor protein. It further became evident *(12)* that both of the remaining Rb family members, p107 and p130 proteins, also bound to E1A equally well *(27).* E1A was found to bind to these proteins through the peptide sequence, LXCXE, which is present in the CR2 region. It was further found to be present in HPV E7 protein, as well as SV40 large T-Ag *(18,28).* As described in subheading 2.1.4., these oncoproteins, although derived from unrelated viruses, all appear to bind to the members of the Rb family. In the case of E1A, there appeared to be two distinct regions that made contacts with the Rb protein: the LXCXE motif in the CR2 region, and residues 30–60 of the CR1 region.

The 300 kDa protein that was found to associate with E1A has now been identified to be the transcriptional co-activator, p300/CBP *(29).* Many members of this co-activator family have been found to bind to E1A, and E1A can effectively block their transcriptional activity. It has also been reported that E1A can prevent the cyclin cyclin-dependent kinase (CDK)-mediated phosphorylation of p300. The p300/CBP family of transcriptional co-activators has indigenous histone acetylene transferase (HAT) activity, and they are associated with other HAT proteins, such as PCAF-1, in vivo. E1A is believed to affect these interactions, thus modulating the transcriptional activity of p300. Mutational studies of E1A protein had made it clear that the extreme N-terminal residues spanning 1–25 are essential for the transformation function of E1A *(30).* It is apparent that this region of E1A is involved in binding to p300, and that the subsequent inactivation is indispensable for E1A to transform cells.

The two proteins of 60 and 33 kDa have been identified to be cyclin A and CDK2. The CR2 region is chiefly involved in the binding to these proteins, and the CR1 region plays a secondary role in the interaction. Although it is established that E1A binds to

Table 1
Cellular Proteins Binding to E1A

| *Protein* | *Region of E1A required for binding* |
|---|---|
| p400 | **1–48** |
| p300 | **1–25,** 30–76 |
| Rb | **121–127,** 30–60 |
| p107 | **124–127,** 30–60 |
| p130 | **124–139,** 30–60 |
| Cyclin A, p60 | **124–127,** 30–60 |
| P33CDK2 | **124–127,** 30–60 |
| BS69 | **140–185,** 76–120 |
| CtBP | **271–284** |

cyclin A directly, it is not yet clear whether the interaction with CDK2 is direct or through cyclin A. Nevertheless, the pattern that is emerging suggests that E1A can bind to and affect the function of critical cellular proteins involved in cell cycle regulation. A list of the proteins known to bind directly to E1A, and the regions involved in the binding, is shown in Table 1.

### 2.1.4. Inactivation of Rb Function by E1A

Studies on the mechanisms involved in E1A-mediated transformation have highlighted the inactivation of Rb and its family proteins as an essential step in this process *(12).* Because Rb is a well-characterized tumor suppressor protein, the interaction between Rb and E1A has garnered maximum attention.

Rb is a nuclear phosphoprotein that plays a critical role in the progression of the mammalian cell cycle *(31).* Inactivation of Rb by phosphorylation is a necessary step for the transition of proliferating cells from G1 to S phase. It is well-established that it is the hypophosphorylated form of Rb that is functional in arresting cell proliferation, and inactivation of Rb in the mid-to-late G1 phase, by cyclins D and E and their associated kinases, facilitates the G1-to-S transition *(31–33).* E1A, as well as other V-ONCs, such as HPV E7 and SV40 large T-Ag, all preferentially binds to the functional hypophosphorylated form of Rb *(13).* It has now been shown that interaction with E1A, E7, or SV40 large T-Ag all bring about an inactivation of Rb that is equivalent to its phosphorylation by CDKs.

All three Rb family members have a central conserved domain, named "pocket domain" *(34).* The pocket domain imparts the growth-regulatory functions of the Rb protein *(35),* and almost all point mutations or deletions of the *Rb* gene found in human cancers map to this region *(36–38).* E1A binds to the functional pocket domain of the Rb protein. Studies in the past few years have established that interaction of E1A with Rb, or the phosphorylation of Rb, leads to its inactivation, which is equivalent to a deletion or mutation of the pocket domain of the gene.

As shown in Fig. 2, the pocket domain comprises two subdomains, named the A and B pockets. In the case of p107 and p130, these subdomains are separated by a spacer region *(39).* p107 and p130 are known to bind to cyclins A and E through the spacer region, through a sequence that is similar to the cyclin-A-binding domain of the

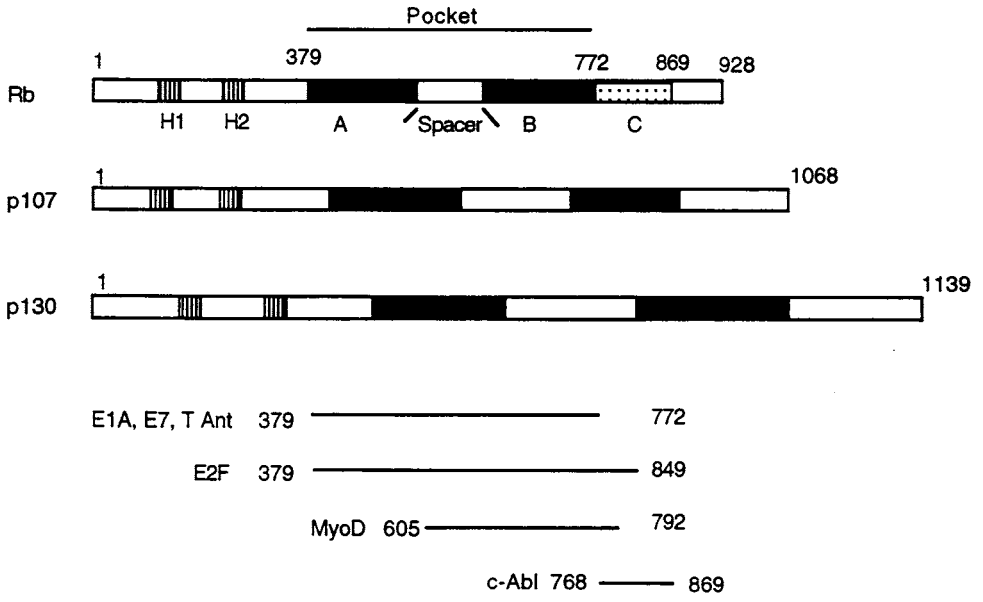


**Fig. 2.** Structure of the Rb family proteins. The conserved pocket domain is shown by the filled box; the H1 and H2 domains also share significant homology. The region 768–869 of Rb is involved in binding to c-Abl protein, and is referred to as the C box. The spacer region between A and B boxes are considerably larger and functionally distinct in p107 and p130, compared to Rb.

Waf1/cip1/p21 CDK-inhibitory protein *(40).* The spacer region is almost absent in Rb protein, although Rb can bind to the cyclin D proteins *(41).* E1A and other V-ONCs associate with Rb and Rb family members through the mediation of the A and B pockets.

The functional consequence of E1A interacting with the Rb family proteins has been elucidated. Studies in the early 1990s showed that Rb is associated with a cellular TF, E2F *(42–44),* which was originally identified as a factor necessary for the E1A-mediated induction of the Ad *E2* gene, and was found to bind to a sequence element, TTTCGCGC *(18).* There were two such elements present in the E2 promoter, and it was found that E1A induces cooperative binding of E2F to the sites, in association with the Ad E4 protein. It was also found that E1A can dissociate multiprotein complexes that contain the E2F TF *(45),* and it was already known that E1A bound to the Rb protein. Attempts were being made to identify the cellular proteins that bind to Rb as well, and such combined efforts revealed that E2F is a target for the Rb protein, and that E1A binding to Rb disrupts the interaction between Rb and E2F. The term E2F now refers to a family of six proteins, E2Fs 1–6. Of these, E2Fs 1–5 are transcriptionally active *(46),* and E2F6 is repressive in nature *(47).* Although the transcriptionally active E2Fs have similar DNA-binding abilities, they show a preference in their ability to bind to different Rb family members: E2Fs 1, 2, and 3 bind to the Rb protein *(39);* E2Fs 4 and 5 preferentially interact with p107 and p130 *(39,48,49).*

Further analysis of the functional consequences of the interactions involving Rb, E1A, and E2F showed that Rb binds to E2F, and represses its transcriptional activity *(50).* The binding of E1A could effectively reverse this Rb-mediated repression of E2F

to Rb *(50)*. Cloning of the E2F family members revealed that Rb binds to the transcriptional activation domain of E2F1, thus preventing its ability to function as a TF *(51,52)*. Recent studies have shown that Rb can block transcription from promoters containing E2F sites, by recruiting the cellular histone deacetylase enzyme, *HDAC1 (53–55)*.

In the case of E1A, it appeared that induction of E2F activity facilitated the expression of the *E2* gene. As described in later subheadings, HPV E7, as well as SV40 large T-Ag, were also capable of dissociating E2F from Rb, and thus inducing its transcription *(56)*. This was surprising, since none of the genes present in these viruses had E2F sites in their promoters. This raised the possibility that activation of E2F may be contributing to the expression of the *E2* gene in Ad, but it plays a more important role in the regulation of the cell cycle. It is now evident that the V-ONCs activate E2F to achieve a proliferative state, which is conducive for their DNA replication *(13,14)*.

It became apparent that many cellular promoters that were known to be induced by Ad E1A had E2F sites in their promoter *(57)*. Further, an analysis of the genes that are regulated by E2F showed a variety of cell-cycle regulatory genes as downstream targets of E2F. For example, genes for proteins such as DHFR, DNA polymerase α, ribonucleotide reductase, thymidylate kinase, thymidylate synthase, and so on, which are all necessary for DNA synthesis, are regulated by E2F. Further, many additional cell-cycle proteins, such as cyclins A and E, p107 CDK2, and soon were found to be regulated by E2F as well. The current model suggests that inactivation of Rb by phosphorylation, during the G1 phase of the cell cycle, releases free E2F activity, which transcribes this set of genes, required for cell cycle progression. In the case of viral infection, the products of the immediate early genes achieve the same end result, but by inactivating Rb by a direct interaction. Similarly, it may be imagined that, when the *Rb* gene is inactivated by mutation or deletion, there would be an abundance of active free E2F, which would contribute to uncontrolled cell cycle progression, and hence oncogenesis *(57)*.

The regions of E1A necessary for dissociation of E2F–Rb complexes have been worked out in detail. As discussed earlier, it is the CR2 region that chiefly mediates the interaction of 1A with Rb, but the CR1 region, especially residues 30–60 *(58),* contributes to the binding. Within this region, a tyrosine residue at position 47 is important for stable binding of the human Rb protein. There is no region in E7 or SV40 large T-Ag similar to this E1A CR1 motif. It appears that the same motifs are essential for the disruption of E2F-containing complexes by E1A. One model that has been proposed, based on extensive mutational analysis, as well as on competition experiments, is that the CR2 region would tether E1A to the Rb protein *(58)*. Once E1A is bound to Rb, the CR1 region blocks the region of Rb involved in binding to E2F, thus preventing the formation or existence of a Rb–E2F complex *(59)*. It has been proposed that cyclin D would be functioning in a similar fashion to disrupt Rb–E2F complex. Because cyclin D has an LXCXE motif, it may tether to the Rb protein and bring to its proximity the CDK4/6 kinases, which can phosphorylate Rb and disrupt an E2F–Rb interaction.

### 2.1.5. Interaction of E1A with p107 and p130

The interaction of E1A with Rb is the best-characterized interaction among all E1A-binding proteins, but all members of the Rb family interact with E1A in essentially the same fashion. It has been shown that interaction of E1A with p107, as well as with p130, can dissociate the E2F proteins associated with them *(60)*. The regions of E1A

involved in binding to p130 spans a few additional residues in the CR2 region; residues 121–127 are required for binding to Rb, as well as to p107, and the residues 121–139 are necessary for binding to p130 *(17)*.

The functional consequences of E1A binding to p107 and p130 may be the same, but it is not yet clear whether E1A must bind to and inactivate these proteins to induce cell proliferation. This is a question especially in the case of p107, in which it complexes with E2F in the S phase of the cell cycle *(61),* and hence the importance of inactivating a protein that functions past the G1–S transition point is questionable. The interaction of E1A with p130 may be more important, since Ad infects mostly quiescent cells, and p130–E2F complexes are prevalent in resting cells *(62)*. In a broader view, the interaction of E1A with either p107 or p130 appears to be less important than its interaction with Rb, simply because the role of these proteins in normal cell cycle regulation is not as significant as that of Rb. This is borne out by the fact that no mutations of the p107 gene has been reported in human cancers, and p130 has been reported to be mutated in a small subset of lung carcinomas. It may be assumed that, for the purposes of this review, the interactions of these proteins with E1A is not relevant.

### *2.2. HPV E7 Protein*

Papilloma viruses are small DNA tumor viruses that have been linked to cancers of the genital tract *(63)*. About 70 types of papilloma viruses have been isolated, and are broadly classified into low-risk and high-risk HPVs, based on the correlation between their presence in benign genital warts or malignant cervical carcinoma *(64)*. HPVs are unique in that, although they share the same pathways of transformation as Ads and SV40, the latter two are not correlated with human cancer.

The genomes of HPV types are different, but the general organization is highly conserved *(3)*. There are three distinct regions within the genome: a region containing the regulatory elements for the transcription of viral genes, a region encoding six early genes, and one encoding two late genes. The most common HPV types found in cervical carcinoma are HPV16 and HPV18; for all practical purposes, their transforming proteins are identical. As in the case of Ads, there are two early genes that are crucial for transformation: the *E7* gene, which is functionally equivalent to Ad E1A; and the *E6* gene, which is comparable to the *E1B* gene *(9)*.

It has been found that integration of the viral genome into mammalian cells causes a loss of many viral genes, but maintains the *E7* and *E6* genes. Transformation experiments have suggested that *E7* gene from the high-risk HPV types can transform cells very efficiently; the *E6* gene has a lesser capacity to do so *(65)*. Studies using the soft-agar colony formation assay have shown that E7 protein, in association with a second oncogene, can transform a variety of primary rodent and mammalian cell lines, and the continued presence of the E7 protein is required for maintenance of the transformed phenotype. The *E6* gene has a lower capacity to transform cells, but it can efficiently cooperate with the *E7* gene to transform primary human keratinocytes, which are the natural hosts for HPV. Both genes, of low-risk HPV type, have weak transformation potential, thus correlating their activity with the tumorigenicity of the HPV type.

As mentioned earlier, the E6 protein is similar to Ad E1B protein functionally, and it can interact with the p53 tumor suppressor protein like E1A. Similarly, the E7 protein, which is functionally analogous to the Ad E1A, can bind to the Rb family of tumor suppressor proteins. This interaction is dealt with here first.

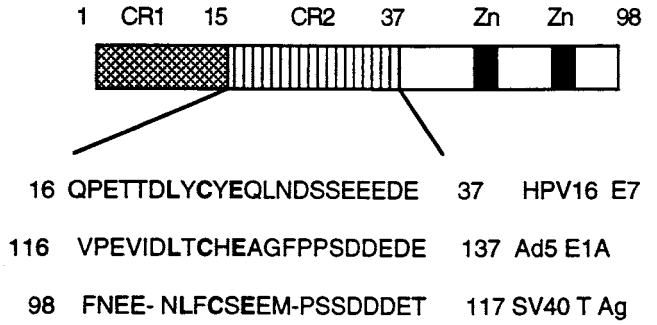


**Fig. 3.** Schematic of the HPV16 E7 protein. The conserved CR1 and CR2 domains are at the N-terminal half of the protein. The region of high-sequence homology between E7, E1A, and T-Ag is shown below, with the LXCXE motif in bold.

### 2.2.1. E7 Protein and its Interaction with Rb

The HPV E7 is a small acidic protein that localizes to the nucleus and nuclear matrix. This 98-amino-acid (aa) protein can be divided into three domains: CR1, CR2, and CR3, based on the structural similarity to the Ad *E1A* gene *(66).* CR1 and CR2 are at the N-terminal region of the protein, and the CR3 at the C-terminal end (Fig. 3). CR2 is essential for the binding to the Rb protein, and both CR1 and CR3 are required for the transformation function of E7. The CR3 region of the protein has two zinc-binding motifs, which are involved in dimerization of E7. Mutations in the zinc-binding region of E7 abolished its ability to transform cells, although it was able to bind to Rb protein very efficiently. There are two potential casein kinase sites at the C-terminus of the protein, and phosphorylation on these sites is believed to be important for the full transformation function of E7 *(3).*

The interaction of E7 with Rb family of tumor suppressor proteins has been studied in detail *(16,67,68).* In fact, the crystal structure of the CR2 region of E7, bound to the pocket domain of Rb, has been elucidated. As in the case of E1A, E7 protein binds to the pocket domain of Rb through a conserved LXCXE motif, and the binding to Rb is required for E7 to transform cells *(16).* The first indication of the correlation between Rb inactivation and the transformation by HPV E7 was obtained in 1991 *(69),* when an analysis of human cervical carcinoma cell lines revealed that those having an intact *Rb* gene had HPV E7 incorporated in the genome. In contrast, those that had a mutant *Rb* gene contained no E7. This suggested a direct correlation between the inactivation of Rb, either by mutation, or through the binding or HPV E7 protein.

Mutations in the Rb-binding motif of E7 totally abolished the ability of E7 to promote growth of primary cells in soft agar in cooperation with oncogenic *ras*. As already mentioned, low-risk HPV types have low capacity to transform cells, and, supporting this observation, HPV6 and HPV11 E7 can bind to Rb only weakly. Although the Rb-binding moieties of the high- and low-risk HPV types are similar, a comparison showed that HPV 16 E7 has an aspartic acid at position 22; HPV 6 E7 had a glycine *(3,70).* Substitution of the glycine with aspartic acid, in the HPV 6 E7, enhanced its ability to bind to Rb, and increased its ability to transform cells. This correlation does not appear universal, because certain low-risk types of HPV have an aspartic acid at

this position, and can bind to Rb efficiently. The correlation between the ability of E7 to transform cells and to bind Rb is still not clear, especially in the case of low-risk types. It has been proposed that, although the low-risk E7 proteins bind to Rb with comparable affinity, the functional consequences of the interactions may be different in the various types of HPV.

As in the case of Ad E1A, HPV E7 preferentially binds to the hypophosphorylated form of Rb. Similarly, binding of E7 could effectively dissociate Rb–E2F complexes in mammalian cell extracts. The crystal structure of HPV16 E7, bound to the pocket domain of Rb, has been solved recently, and the structure supports the earlier functional observations made on the interaction *(71)*. It was found that a 9-aa E7 peptide, carrying the LXCXE motif, binds to a highly conserved region within the B box of the Rb pocket. Both the A and B boxes seem to have structural similarities to cyclins and TFIIB, in that they all possess a five-helix cyclin fold. The LXCXE sequence was found to bind to a shallow groove in the B pocket, which was formed by three cyclin fold helices. Alternating Leu, Cys, Glu, and Leu side chains of the E7 peptide make intermolecular contacts with the B-box groove. In addition, there is a high density of van der Waals forces and hydrogen bond contacts distributed uniformly between the E7 peptide and the B box, contributing to the strength of the binding. The actual contact of the E7 peptide was to the groove of the B box, but the interphase of the A–B boxes appeared to contribute significantly to the binding. The structure also revealed a high degree of conservation of the B-box site, which binds to LXCXE motifs. Four residues that contact the backbone of the E7 peptide, Tyr709, Tyr756, Asn757, and Lys713 are identical in diverse species of Rb proteins, as well as in p107 and p130. The high level of conservation of this B-box site suggests that it plays a major role in the functioning of the Rb family proteins *(71)*.

It has been shown recently *(72)* that the half-life of the Rb protein is considerably reduced in cells stably transformed with the E7 protein *(72)*. An overexpression system showed that high levels of E7 protein can lead to an increased decay of the Rb protein, and this could be blocked by proteasome inhibitors. The degradation was limited to Rb, since there was no change in the stability of p107 or p130 proteins in response to E7. Further, this function appeared to be a specific feature of the HPV E7 protein, because neither Ad E1A nor SV40 T-Ag could affect the half-life of Rb *(72)*.

### 2.2.2. Binding of E7 to p107 and p130 Proteins

The binding of E7 to p107 and p130 has been studied in detail. Unlike in the case of E1A, there are apparent differences in the consequences of binding to Rb vs p107 or p130. First, it became apparent that E7 protein cannot dissociate p107–cyclin A–E2F complexes, unlike E1A; instead, it remains associated with the complex *(73)*. The association with the p107–cyclin A–E2F complex was also dependent on the LXCXE motif, and E7 from HPV 6 had a reduced capacity for association. It has been suggested that HPV E7 can target cellular genes like c-*myb* by targeting the p107–cyclin A–E2F complex in NIH 3T3 cells. Apparently, the expression of B-*myb* promoter in these cells correlates with the binding of distinct p107–E2F complexes at the E2F binding site, and Rb–E2F complexes do not appear to play a major role in this regulation *(74)*. It has been found that, although the inactivation of Rb family proteins and the induction of E2F activity correlates with the transformation function of E7, this alone is not sufficient. Despite the suggestion that E7 interacts differently with p107 and

p130, it is not yet clear whether the interactions of E7 with these proteins are important for its transformation function.

It may be concluded that the interaction of HPV E7 with Rb has been elucidated more clearly at the structural level, and the conclusions drawn from this can be extended to the other Rb-binding proteins, such as like E1A and SV40 T-Ag. Further, cellular proteins, such as cyclin D, may be targeting Rb through similar interactions *(71).*

## 3. V-ONCS INTERACTING IWTH p53

### *3.1. HPV E6 Protein*

The HPV E6 is an 18 kDa 151-aa basic protein that also localizes to the nuclear matrix and cell membranes *(10).* Its most notable structural feature is the presence of four Cys motifs, which can form two well-defined Zn fingers. These motifs can bind Zn in vitro, and are highly conserved between all serotypes of HPV. The E6 protein has no homology to Ad E1B or SV40 T-Ag, but can function in a similar fashion. The major common feature of these three proteins is their ability to bind and inactivate p53 tumor suppressor protein. In certain high-risk HPV-infected cells, polycistronic E6 messages have been detected, which can give raise to full-length, or to a shorter, protein, E6* *(10).*

E6 protein from high-risk HPV types associate with p53 with higher efficiency than E6 from low-risk HPV types *(5).* The binding of E6 to p53 is enhanced by a cellular protein, E6AP (E6-associated protein). It has now been established that E6 protein binding leads to the degradation of p53, thus reducing its half-life. The proteolytic degradation of p53 through the ubiquitin–proteasome pathway has been studied in detail. Consistent with these observations, cell lines that carry a high level of E6 have very low amounts of p53 protein, mimicking situations in which *p53* gene is mutated. In vitro analyses have identified two domains of E6 that are involved in the binding and degradation of p53: the C-terminal end of E6 is required for binding; the N-terminal end is required for effecting degradation. E6 protein can inhibit the transcriptional activity of p53, and this does not require the activation of the proteasome pathway. In addition to p53, a variety of cellular proteins, ranging in mol wt from 33 to 212 kDa, have been found to associate with HPV E6, but their identities are not yet known.

### *3.2. Ad E1B Protein*

The Ad 5 *E1B* gene has been studied with respect to its interaction with the p53 protein *(75).* Unlike the Rb-binding V-ONCs, there are no structural similarities between the p53-binding proteins *(8).* Thus, although Ad, HPV, and SV40 all encode proteins that can bind to p53, there are no conserved or shared domains between them. Further, the functional consequence of binding of these proteins to p53 are also different: the Ad E1B binding represses the transcriptional activity of p53; the HPV E6 protein leads to the degradation of p53 *(10).*

Ad *E1B* gene codes for two distinct protein products, one 55 kDa and the other 19 kDa in size. Only the 55 kDa E1b protein has been found to physically interact with p53. The p53-binding domain of E1B is not conserved, even in different serotypes of Ad, and E1B protein from certain strains, such as Ad12, cannot bind to p53 *(28).* Because p53 plays a major role in arresting cells in G1, in response to DNA damaging agents, or induces apoptosis if the DNA damage cannot be successfully repaired, it is believed that the V-ONCs that target p53 lead to a suppression of the cell death pro-

gram *(16)*. E1B proteins are believed to suppress cell death programs initiated by DNA damage, as well as by other viral proteins like E1A *(15,77)*. Thus, the V-ONCs that bind to p53 cause distinct functional effects than those binding to Rb and facilitating G1–S transition.

The functional characterization of p53 has shown that it is a TF, possessing distinct DNA-binding and activation domains. Further, it is very well established that p53 induces a wide variety of cellular genes, while repressing certain other genes. Studies on the functional consequences of E1b binding to p53 revealed that the 55 kDa protein targets the activation domain of p53, and thus inhibits p53-mediated transcriptional induction. This could influence the expression of vital cell cycle genes, such as the $p21^{Waf1/Cip1}$ proteins and Mdm2, which is a regulator of p53 itself. Detailed mutational analysis has shown that the ability of E1B 55 kDa protein to repress p53-mediated transcriptional activation strongly correlated with its ability to transform cells. Ad12 E1B 55 kDa protein, which was unable to bind to p53, was effective in blocking p53-mediated transcriptional activation. Conversely, the interaction between p53 and E1B was necessary, but not sufficient, for transcriptional repression, as well as transformation functions of E1B *(78,79)*. This conclusion is based on the finding that a single aa insertion at residue 443 abolishes the ability of E1B to bind p53, but it was effective in transcriptional repression and transformation. The C-terminal end of E1B outside the p53-binding region was required for its transformation function; phosphorylation at three sites within this region was also essential for the transformation function *(78,80)*.

The region of E1B 55 kDa that binds to p53 also is involved in binding to the Ad E4 protein Orf6 *(78)*. This led to the suggestion that the repressive properties of E1B are facilitated by the presence of E4Orf6. In addition, one study has shown that E1B, in cooperation with E4Orf6, modulates not only the transcriptional activity of p53, but also the levels of p53 protein. Because E4Orf6 has also been shown to bind to p53, it is thought that collective interactions among E1B55kd, E4Orf6, and p53 lead to modulation of the levels, as well as the activity, of p53.

It has been reported that the E1B 19 kDa protein can also affect p53 function, but that this does not require a direct interaction. Unlike E1B 55 kDa protein, the 19 kDa protein was unable to block the transcriptional activity of p53; since p53 is also known to repress the transcription of certain cellular genes, it is believed that E1B19 kDa protein affects the transcriptional repressive properties of p53.

### *3.3. SV40 T-Antigen*

SV40 is an oncogenic DNA tumor virus that was originally discovered in rhesus monkey kidney cells. The oncogenic property of this virus resides in two early gene products, the large (T) and the small (t) tumor Ag *(4)*. The large T-Ag can transform cells on its own, but the small t-Ag cannot; the latter can enhance the transformation potential for the large T-Ag. The transformation function of the T-Ag was found to require its interaction with the Rb family tumor suppressor proteins, as well as the inactivation of p53. SV40 thus differs from Ad and HPV in having one protein that can inactivate Rb, as well as p53 pathways; these functions reside in separate proteins in the latter two. Further, SV40 T-Ag is also capable of binding to DNA, unlike E1A or E7 proteins *(81)*.

The SV40 T-Ag is a polypeptide of 708 aa, and is considerably larger than the other oncoproteins discussed thus far (Fig. 4). There are several distinct functional domains

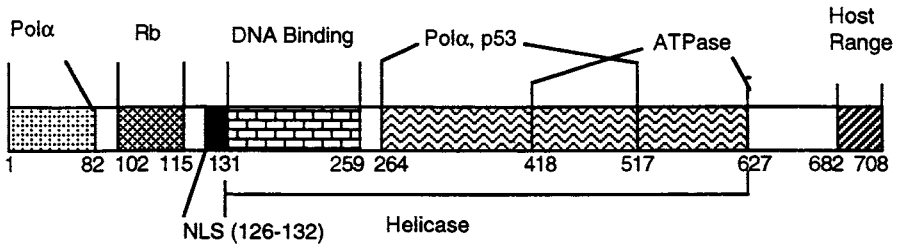


**Fig. 4.** Structure of the SV40 large T-Ag. The various functional and protein-binding domains are indicated.

that have been extensively characterized. A schematic of the T-Ag domain structure is shown in Fig. 4; as can be seen, the N-terminal end (1–82) and an internal domain is involved in binding to DNA polymerase α-primase. The Rb-binding domain carrying the LXCXE motif spans residues 102–115. Other well-defined domains include, progressively toward the C-terminal end, a NLS (126–132); a DNA-binding domain (131–259); a finger motif (302–320); a second DNA polymerase α-primase-binding domain (259–517), which partly overlaps with the p53-binding domain (275–517); and an adenosine triphosphatase (ATPase)/ATP-binding domain (418–627). A helicase domain extends from residue 131 to 627. There is a cluster of phosphorylation sites on the C-terminal region, which has been reported to be critical for T-Ag function. A small domain that defines the host range of (55)SV40 resides at the extreme C-terminal end, spanning residues 682–708. The ability of T-Ag to perform such a diverse array of functions enables it to facilitate all stages of viral replication and propagation *(4)*.

### 3.3.1. Interaction with Rb Family Members

The interaction of SV40 large T-Ag with Rb protein has been well-characterized, and the inactivation of Rb is essential for T-Ag to transform cells *(82)*. As mentioned earlier, the Rb-binding domain of large T-Ag has the conserved canonical LXCXE motif. The integrity of this motif is essential, but not sufficient, for the oncogenic activity of T-Ag. It is clear that interaction of T-Ag with Rb leads to an inactivation of Rb function, as in the case of E1A or HPV E7, leading to a dissociation and activation of E2F TF. Such studies have established that these three DNA tumor viruses all utilize common mechanisms for inducing the cells to enter S phase, creating an environment conducive to the replication of viral DNA. One difference in the case of T-Ag, however, is that it can perform considerably more functions than E1A alone, or even E1A and E1B combined *(7,83)*.

Consistent with the finding that T-Ag can efficiently dissociate Rb–E2F complexes, it was found that a human cell line, WI38-VA13, which stably expresses T-Ag, had no Rb–E2F complexes at all *(84)*. There was a reduced level of the cyclin A–E2F complex, and the loss of these complexes corresponded to an increase in the levels of free, transcriptionally active E2F. Like E1A and HPV E7, T-Ag also targets the pocket domain of hypophosphorylated Rb. Hence, biochemically, as well as functionally, the interaction of T-Ag with Rb is similar to that of E1A or E7.

It has been shown recently *(85)* that a region of T-Ag N-terminal to the conserved Rb-binding region is required for the functional inactivation of the Rb protein. This

region has been named the J-domain, because it has sequence homology to the J-domain of the DnaJ family of molecular chaperons of *Escherichia coli.* The J-domains are characterized by conserved histidine–proline–aspartate (HPD) residues, and this tripeptide is present at the N-terminus of the SV40 large T-Ag. Further, the J-domain of SV40 T-Ag, as well as certain other polyomaviruses, can functionally substitute for the J-domain of *E. coli* DnaJ chaperon.

Studies on the J-domain of T-Ag showed that it is required to overcome the G1–S arrest induced by all Rb-family members *(85,86).* In addition, it was required to reverse the repression of E2F activity brought about by Rb, as well as p130. From these experiments, it appears that, in the case of T-Ag also, the LXCXE motif tethers the protein to Rb, while a N-terminal region functionally inactivates it *(86).* This general pattern is similar to the one seen in the E1A-mediated inactivation of Rb *(58,59).*

It has also been reported that the J-domain of T-Ag can affect the phosphorylation status of p107 and p130 proteins. In cells stably expressing T-Ag, there is a reduced amount of phosphorylated forms of p107 and p130. There was also a faster turnover of p130 protein, which could be a result of its aberrant phosphorylation status. These effects of T-Ag appear to be J-domain-dependent, because point mutations in the HPD motif abolished these changes. Further, replacement of the N-terminal J-domain of T-Ag with J-domain motifs from cellular proteins restored this ability. Similarly, the transformation function of T-Ag also appeared to require a functional J-domain. The biochemical basis for the functioning of the J-domain is not yet clear, but it appears to be as important as the LXCXE motif in the Rb-binding domain for full T-Ag function.

### 3.3.2. Interaction of T-Ag with p53

As in the case of other p53 binding vONCs, the region of T-Ag that is involved in binding to p53 is large and not conserved. The biochemical effect of the binding of T-Ag to p53 is also different from the binding of E6 or E1B. One interesting facet of this interaction is that phosphorylation of both T-Ag and p53 appear to be necessary for binding in murine cells. In addition, the p53-binding region of T-Ag overlaps with the binding sites for DNA polymerase α and ATP, raising the possibility that T-Ag–p53 complexes may affect the viral functions regulated by T-Ag *(4).* This notion is supported by the finding that a single point mutation (Pro to Leu), at position 584 of T-Ag, resulted in a loss of p53 binding, along with changes in the ATPase activity of T-Ag, as well as its ability to oligomerize. This mutation also resulted in reduced stability of T-Ag, and resulted in defective replication and reduced transformation functions.

It has been found that wild-type murine p53 can block the binding of T-Ag to DNA polymerase α; in addition, wild-type p53 could effectively reduce the replication of viral origins, but mutant p53 molecules were unable to do this *(6).* This could possibly result from p53 competing for the binding to T-Ag to DNA polymerase α or another cellular protein involved in replication. One other interesting aspect of the interaction between T-Ag and p53 is that the phosphorylation, as well as the stability, of the latter increase, upon T-Ag-mediated cellular transformation. The relative importance of p53 binding in T-Ag-mediated transformation of primary murine cells was highlighted in one study, in which it was found that the N-terminal region of T-Ag, up to residue 250, was not necessary for this function. This ruled out a role for Rb-binding, nuclear translocation, and DNA binding abilities of T-Ag. In that study, residues 251–626 were

found to be vital for immortalization, suggesting that p53 binding and inactivation is indispensable for the transformation function of T-Ag *(87)*.

The modulation of p53 by T-Ag extends beyond enhancing the stability of the former. It has been shown that T-Ag can block the DNA-binding activity of p53, which correlated with T-Ag inhibiting the transcriptional activation functions of p53. One recent study showed that the N-terminal domain of T-Ag, which is not involved in DNA binding nor binding to p53, could effectively repress p53-mediated transcription. This suggests that the N-terminal region of T-Ag may be affecting p53-mediated transcription indirectly, through other cellular factors involved *(6)*.

Overall, it may be summarized that, although the interaction of V-ONCs with Rb family members has been characterized in great detail, ambiguity still exists as to the nature of their interactions with p53, and its functional consequences, partly because the p53-binding domains of the V-ONCs are large and not very well defined, and they do not share extensive homology. Despite these drawbacks, it appears to be a fruitful endeavor to study the interactions of these proteins with p53 in greater detail.

## 4. V-ONCS AS PROBES FOR RB FUNCTION

The observations described above make it clear that V-ONCs interact with Rb family members specifically and with high affinity. The most notable aspect of the interaction is that the V-ONCs specifically target the active form of Rb and Rb family members, which leads to an alteration of their normal function. These features make the V-ONCs efficient probes for assessing the functional status of the Rb protein in a given cell.

The functional effects of V-ONCs on Rb have been studied more extensively in Ad E1A, but the recent structural studies on HPV E7 peptide would make it a more adaptable probe for Rb function. The 9-aa peptide derived from E7 appears to bind to Rb efficiently, and may provide a good model for designing custom probes for Rb function *(88,89)*. Since V-ONCs bind specifically to the functional, wild-type, hypophosphorylated form of Rb, the binding itself may be considered as a measure of the functional status of Rb. In addition, because the binding of such V-ONCs leads to a perturbation of Rb activity, methods could be designed to evaluate such changes in Rb function. Many novel methods have been developed recently to measure protein–protein interactions in vivo in living cells *(90),* mostly using fluorescent probes *(91,92)*. Such methods generally measure changes in the fluorescence properties of the tagged protein or peptide when it interacts with another component. It may be imagined that a tagged E7 peptide, or one derived from Ad E1A, would be able to detect the functional status of the Rb protein, using such methods.

The tagged V-ONCs may be used to detect functional Rb proteins in biopsy samples of human tumors, or to evaluate whether the Rb protein is expressed in its functional form after being introduced into cells for gene therapy purposes *(93)*. Although constitutively active, phosphorylation site mutants of Rb are expected to be used for this purpose, the assay using V-ONCs would be of immense help in assessing the amounts, as well as the functional status, of the protein. The advantage of such an assay system is that it would be able to detect functional Rb protein, even in single cells, and the assay may be modified easily to an in vitro diagnostic system very efficiently.

The V-ONCs would be especially useful for detecting functional Rb protein, because, unlike the p53 protein, there are no good antibodies that can distinguish

between a functional form of the Rb protein and a mutated protein. Further, because even small peptides derived from the oncoproteins can specifically interact with functional Rb makes this approach feasible and attractive. It may be imagined that such specific biological probes would be of value in assessing the functional status of the vital growth regulatory proteins.

## 5. V-ONCS AS PROBES FOR P53 FUNCTION

The use of V-ONCs to assess the levels or the functional status of p53 tumor suppressor protein is not as attractive or feasible as in the case of the Rb family proteins. The chief reasons for this are that the regions of V-ONCs interacting with p53 are large, and they do not specifically interact with the functional form of p53 alone. In contrast, there are excellent immunological reagents available that can distinguish between the functional and inactive forms of p53. But it still remains an option, which could be utilized in circumstances in which antibodies may not be effective or accessible. Again, one of the many recent techniques for detecting protein–protein interactions in vivo and in vitro may be modified for this purpose. It would appear that polypeptides derived from the Ad E1B 55 kDa protein or the HPV E6 protein would be more suitable for this purpose.

It appears an exciting possibility that the oncoproteins of DNA tumor viruses may be harnessed to detect and quantitate cellular proteins that can prevent cell proliferation and oncogenesis. This would be a valuable addition to the repertoire of modern techniques to combat cancer.

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# 3 Tumor Suppressors in Metastasis

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***Contents***



## 1. INTRODUCTION

Recent advances in cancer development studies showed tumorigenesis and metastasis to be complex multistep processes. In the past decade, it became evident that cancer cells have multiple genetic alterations, including point mutations, gene amplification, recombination, gene deletion, reduced or overexpression, and loss of heterozygosity (LOH). Those affected genes include tumor suppressors, oncogenes, and a panoply of genes involved in cell cycle, motility, and adhesion. This review emphasizes the tumor suppressor genes (TSGs) and metastasis suppressor genes, and their alteration at different levels in metastasizing cells.

Metastasis is a major cause of cancer patients' deaths, but cures are often possible for patients whose tumors remain localized. Thus, understanding the genetic and physiological alterations leading to metastasis formation should be of value in defining new diagnostic techniques and therapeutic approaches. The invading tumor cells must accomplish a multistage process to metastasize to distant sites. They commonly invade the adjacent tissues, and enter the lymphatic and/or circulatory system. They disseminate, and adhere at a second site, either by binding to specific ligands or by physical limitations. Thereafter, they either proliferate in the vasculature or extravasate the endothelium and proliferate in the host tissue or organ, once conveniently irrigated. Each of the steps included in this pathway must be completed by an originally invading cell, to be able to form a micrometastasis, and a secondary or tertiary tumor may then form.

Many of the TSGs for which a role in metastatic progression was shown are discussed herein.

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## 2. TUMOR SUPPRESSOR GENES

TSGs encode proteins in which their absence, inactivation, or mutation promotes oncogenesis, or prevents apoptosis at various levels in the cell, because of their diverse functions. They have the ability to inhibit or reduce tumorigenicity when transfected in transformed cells. Most of the TSGs were originally identified by the occurrence of mutations in families with hereditary predisposition to develop certain cancer types.

### *2.1. p53 Gene*

The *p53* gene product was originally identified based on its ability, when phosphorylated, to associate with the large T-antigen (T-Ag) in SV40-infected cells *(1).* Studies then focused on the oncogenic potential of *p53,* because its mutant form cooperates with the *ras* oncogene in transforming rat embryo fibroblasts *(2).* Later transfection studies showed that *p53* is a TSG *(3–9)* whose expression and structure is altered in several cancers *(6,10–18),* and that earlier studies were performed with a mutant product. *p53* was then shown to mediate apoptosis *(19–22),* DNA damage repair *(23–30),* and cell cycle control *(31–34).*

By transcriptionally activating the expression of $p21^{WAF/CIP1/sdi1}$, an inhibitory protein to cell cycle kinases, p53 can inhibit the cell cycle in the G1–S transition *(35,36).* Also, *p53*-dependent upregulation of 14-3-3 σ is proposed to play a role in the inhibition of G2–M phase progression *(37).* On the other hand, p53 transcriptional regulation and binding to Mdm2 protein has been shown to regulate *p53* activity, and to play a role in uncoupling S phase from mitosis *(38–44).* The p53-deficient mice were shown to develop normally, but were prone to the spontaneous development of a variety of tumors *(45–51).* Similar observations were reported for *p53*-deficient cells *(52,53).*

Several studies showed the mutation of p53 in a variety of cancers. In breast cancer(BC), *p53* overexpression, mutation, and LOH are common mutations *(54,55).* Recent cohort studies revealed the presence of overexpression and/or structural alterations of *p53* gene in 38.1% of chondrosarcomas of bone *(56)* and 54% of squamous cell carcinoma *(57).* A statistically significant correlation was observed between overexpression or alteration of the *p53* gene and both the histologic grade of the tumor and the presence of metastasis. The probability of local recurrence-free, metastasis-free, and overall survival was shown to be significantly higher for patients with no overexpression or alteration of *p53* than for patients with *p53* overexpression or alteration, in the case of pediatric adrenal cortical tumors, small cell lung cancer, oropharyngeal carcinoma, BC, and several other tumors *(14,54,58–63).* The observation that *p53* overexpression is associated with a poor outcome for the patients in these studies may be explained by the accumulation of genetic alterations in the downstream targets of *p53,* which mediate cell cycle or apoptosis. Also, the overexpression of *p53* by different types of tumor cells suggests that *p53* immunity may be useful for tumor immunotherapy.

A recent study *(64)* showed that p53-immunized mice acquired resistance to tumor metastases. Therefore, an anti-idiotypic network built around certain domains of p53 seems to be programmed within the immune system, and immunity to p53 can be associated with resistance to tumor cells *(64–66).*

Data are further complicated by the fact that different domains of p53 are involved in the control of apoptosis and cell cyle. A polyproline region in p53 mediates apopto-

sis induction, but not cell growth arrest *(67).* Therefore, it is important to investigate the molecular alterations of *p53* in primary and metastatic tumors. In most cases, the description of *p53* mutation in primary tumor is indicative of a poor prognosis *(15,16,57,68–70).* Mutations in the metastatic sites correlate with those in the primary tumors, even if their frequency is generally higher *(68,71),* and suggest that *p53* status is indicative of the aggressiveness of the tumors *(58,72).* A recent study *(73)* characterized *p53* mutations in 39/58 cell lines analyzed from the National Cancer Institute (NCI) anticancer drug screen. Cells containing mutant *p53* were deficient in γ-ray induction of CIP1/WAF1, GADD45, and Mdm2 mRNA and the ability to arrest in G1 following γ-irradiation *(73).*

Further studies, using transgenic mice that overexpress a mutant *p53* gene, show its ability to promote tumorigenesis *(74,75)* and the cellular resistance of a variety of hematopoietic cell lineages to γ-radiation *(76).* These observations provide direct evidence that *p53* mutations affect the cellular response to DNA damage, by increasing either DNA repair processes or cellular tolerance to DNA damage. Thus, deletion, overexpression, or mutations of the *p53* gene seem to be important predictors of aggressive clinical behavior.

### *2.2. Retinoblastoma Gene*

The hereditary form of retinoblastoma (RB) is a dominant autosomal disease characterized by an ocular tumor in childhood. Heterozygotes carrying one mutant and one normal *Rb* allele develop RB with 90% penetrance. The Rb protein is a nuclear protein variably phosphorylated during the cell cycle. The unphosphorylated form is characteristic of the G1 phase; the phosphorylated form is present during the S, G2, and M phases *(77). Rb* was recently shown to play an essential role in DNA damage-induced arrest at the G1–S phase checkpoint *(78).*

The cell cycle inhibition activity of Rb protein was shown by transfection in many different cell lines *(79–82),* and the phosphorylation of Rb by overexpressed cyclins E and D suppresses its growth-inhibition activity *(80,81).* Several studies *(83–88)* have shown the tumor suppressor activity of *Rb* in RB, osteosarcoma, and bladder carcinoma cells, either by in vivo or in vitro transfection. Nullizygous mice for Rb are nonviable, and die between 10 and 14 d gestation, and show increased levels of both cell division and cell death by apoptosis in the hematopoietic and nervous systems *(89,90).* However, heterozygous and chimeric mice are viable, and exhibit several tumors arising from the pituitary gland *(91–93).* Rb transgenic mice, carrying several copies of the human *Rb* gene, were smaller than the wild-type (wt) ones *(94).* The degree of dwarfism correlated with the copy number of the transgene and the corresponding level of Rb protein *(94).*

Mutations and/or LOH of the *Rb* gene were found in several types of cancer, including osteosarcoma, breast carcinoma, prostate carcinoma, and small cell lung carcinoma *(95–97).* Although reduced expression, LOH, and mutations were shown in several BC cells *(98,99),* conflicting results have been found concerning the correlation between the loss of *Rb* expression and the presence of lymph node metastasis *(63,100–103).* Borg et al. *(102)* found that the LOH of *Rb* does not reflect a reduced expression of Rb protein; Spandidos et al. *(101)* and Naka et al. claimed a higher incidence of lymph node metastasis in tumors expressing *Rb*. However, most of these

data show that loss or aberrant expression of Rb is one of the most common molecular alterations in invasive tumors.

### 2.3. E-Cadherin–Catenin Genes

The E-cadherin–catenin complex, an organizer of epithelial structure and function, is commonly disturbed in invasive cancer. E-cadherin, a transmembrane protein with an extracellular and an intracellular domain, is one of the key players involved in cell–cell adhesion. The function of E-cadherin in preventing metastasis in tumor development is believed to be dependent on intracellular catenins *(104–112).* Transfection studies have shown the ability of E-cadherin to decrease cell growth and invasiveness of human carcinoma cells *(104,112),* and to suppress the development of osteolytic bone metastases from BC cells in an experimental metastasis model *(113).* Byers et al. *(109)* showed that E-cadherin-negative BC cells, which had been transfected with E-cadherin, exhibited large increases in adhesion strength, only if the expressed protein was appropriately linked to the cytoskeleton. The data also show that E-cadherin-negative tumor cells, or cells in which the adhesion molecule is inefficiently linked to the cytoskeleton, are more likely than E-cadherin-expressing cells to detach from a tumor mass in response to shear forces equivalent to those exerted in a lymphatic vessel or venule.

Several studies have shown that downregulation of E-cadherin *(106,108,111,114–120)* and catenins *(121–123)* correlate with a poor outcome of the patients. When E-cadherin and α-, β-, and γ-catenins were analyzed as one group, a significant association was seen between reduction in immunoreactivity of at least one of these four proteins and the presence of metastases *(110).* These data indicate that if one of these proteins is downregulated, the function of the others in suppressing tumors and metastasis is altered. Also, a significant association was seen between lobular invasive tumors and β-catenin expression *(110).*

Another study *(124)* on laryngeal squamous cell carcinoma (LSCC) suggested that the aberrations in the function of α-catenin, the anchoring protein of E-cadherin, may cause dysfunction of the cadherin–catenin complex, leading to disturbed cell–cell adhesion. Patients with cytoplasmic presence of α-catenin appeared to have a trend toward poor overall survival, which suggests that cytoplasmic localization of α-catenin is associated with aggressive behavior and metastatic phenotype of LSCC *(124).*

However, specific E-cadherin peptides (histidine-alanine-valine [HAV]) were shown to inhibit cell aggregation and disturb the epithelial morphology, and were able to stimulate invasion of cells expressing E-cadherins *(125).* This suggests that E-cadherin functions are inhibited by homologous proteolytic HAV-containing fragments, which may promote cancer invasion. Therefore, it seems important to assess E-cadherin–catenin status in primary tumors, as a prognostic factor.

### 2.4. MMAC/PTEN Gene

The tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was isolated by different groups *(126–129),* based on its mutation in several human cancers. The gene product was shown to possess an intrinsic protein tyrosine phosphatase activity *(128).* The abundance of its transcription is altered in many transformed cells. In transforming growth factor β (TGF-β)-sensitive cells, PTEN expres-

sion is rapidly downregulated by TGF-β *(128)*. The transfection of wt PTEN into glioma cells, expressing endogenous mutant alleles, caused growth suppression, but was without effect in cells containing endogenous wt PTEN *(130)*. Tamura et al. *(131)* have shown that overexpression of PTEN inhibited cell migration; antisense PTEN enhanced migration. Integrin-mediated cell spreading and the formation of focal adhesions were downregulated by wt PTEN, but not by PTEN with an inactive phosphatase domain *(131)*.

PTEN was recently shown to dephosphorylate focal adhesion kinase (FAK) and to inhibit integrin-mediated cell spreading and cell migration *(132)*. This suggests that a general function of PTEN is to downregulate FAK and Shc phosphorylation, Ras activity, downstream mitogen-activated protein kinase activation, and associated focal contact formation and cell spreading *(132)*. Also, a role for PTEN in regulating the activity of the phosphoinositol 3-kinase pathway in malignant human cells was shown *(133–136)*. The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells *(134)* and the expression of PTEN in primary astrocytes reduced the levels of 3′ phosphoinositides and inhibited protein kinase B (PKB/Akt) activity *(135)*. Homozygous PTEN-deficient mice were found to die by d 9.5 of development, and to show abnormal patterning and overgrowth of the cephalic and caudal regions *(137)*. Embryonic fibroblasts from PTEN-deficient mice exhibited decreased sensitivity to apoptosis stimuli and constitutively elevated activity and phosphorylation of PKB/Akt *(137)*.

Several studies gave evidence of LOH, reduced expression, and alteration of PTEN in several types of aggressive cancer, including prostate, breast, endometrium, lung and ovary *(127,138–155)*. Furthermore, Furnari et al. *(130)* showed that ectopic expression of mutant PTEN alleles, which carried mutations found in primary tumors, and have been shown, or are expected, to inactivate its phosphatase activity, caused little growth suppression. Taken together, these observations suggest that PTEN could serve as a prognostic factor in human cancers, and that its mutation or loss of expression is predictive of poor prognosis.

### *2.5. Deleted in Colorectal Cancer Gene*

The deleted in colorectal cancer *(DCC)* gene is one of several genes altered during tumorigenesis. It encodes a receptor for netrin-1, a chemoattractant involved in axon guidance *(156–159)*. When wt cDNA was transfected into nitrosomethylurea (NMU)-transformed tumorigenic human papilloma virus-immortalized human epithelial cells that had allelic loss and reduced expression of *DCC,* it suppressed tumorigenicity, but truncated *DCC* did not *(160)*. However, *DCC*-deficient mice displayed defects in axonal projections, but were not affected in growth, differentiation, morphogenesis, or tumorigenesis of the intestine *(161)*. On the other hand, Mehlen et al. *(162)* have shown that DCC induces apoptosis in the absence of netrin-1 binding, but blocks apoptosis when engaged by the ligand.

Although mutation of *DCC* gene was rare, a high incidence of LOH and decreased mRNA expression in colorectal cancer, especially with hepatic metastasis, was observed *(163)*. Different studies indicate that LOH or decreased *DCC* expression are closely associated with liver and lymph node metastasis of gastric, colorectal, breast, and testicular germ cell cancers *(164–171)*. The farther away the lymph node metasta-

**Table 1**
**Molecular Alterations of Some tumor suppressor Genes in Primary and Invasive Human Tumors**

| *Gene* | *Invasive tumors* | *Molecular alterations* | *Refs.* |
|---|---|---|---|
| *BRCA1* | BC and ovarian cancers and squamous cell carcinomas of the esophagus | LOH, deletion, reduced expression and/or mutation | *(318–324)* |
| *BRCA2* | Female hereditary and male sporadic BC | LOH, deletion and/or mutation | *(320,322,325,326)* |
| *Nf1* | Neurofibromatosis type 1, neurofibrosarcoma, leukemia, melanoma, shwannoma | Deletion, reduced expression, mutation, splicing alteration | *(196,197,327–345)* |
| *Nf2* | Neurofibromatosis type 2, meningiomas, schwannomas, mesotheliomas | Deletion, reduced expression, mutation, splicing alteration | *(198,199,346–358)* |
| *MTS1* | Melanomas, sarcomas, esophageal, hepatocellular and lung carcinomas, prostate, cervix, breast, and oral cancer. | LOH, deletion, reduced expression, mutation, methylation | *(98,187,359–368)* |
| *APC* | Familial polyposis coli, colorectal tumors, hepatocellular adenoma, oral and ampullary carcinomas, fibromatosis, thyroid tumors | Deletion, LOH, mutation, reduced expression | *(210–212,369–380)* |

sis was from the primary tumor, the higher the frequency of allelic deletions became *(165)*.

Furthermore, DCC is a caspase substrate, and mutation of the site at which caspase-3 cleaves DCC *(162)* suppresses the proapoptotic effect of DCC completely. This data suggests that DCC may function as a tumor-suppressor protein by inducing apoptosis in physiological conditions in which netrin-1 is unavailable. This function depends on caspase cascades by a mechanism that requires cleavage of DCC. Assessment of this parameter in primary lesions may thus find predictive application.

The *p53* gene, the retinoblastoma gene, the E-cadheringene, the *MMAC/PTEN* gene, and the *DCC* gene constitute the five most-reported TSGs for which a correlation between mutations and/or deletion and metastasis was reported. Other functional studies characterized additional putative tumor suppressors, including *BRCA1 (172–177), BRCA2 (178–185), MTS1 (98,186–191), Nf1 (192–197), Nf2 (198–204),* and *APC (205–212)* genes (Table 1).

However, as discussed above, the metastatic process is a multistep phenomenon, and the causal event of tumorigenicity may be of different sources. Thus, it is important to focus on the latest steps leading to metastasis, and to try to isolate genes, that have the ability to suppress metastasis, and to study their mechanisms of function.

## 3. METASTASIS SUPPRESSOR GENES

In the past decade, four such genes were identified. Here the authors review one of the most functionally studied among them, *nm23,* which was identified in this lab.

### *3.1. nm23-H1*

*Nm23-H1* was identified through a differential colony hybridization between related low and high metastatic potential murine melanoma cell lines, in which *nm23* mRNA expression was found to be reduced in the high-metastatic-potential cell line *(213).* Presently, the human *nm23* family of genes consists of five members: *nm23-H1, nm23-H2, nm23-DR, nm23-H4,* and *nm23-H5 (213–218).* These isoforms are closely related, having identity to Nm23-H1 of 94, 76, 59, and 30%, respectively. Homologs of this family are studied as abnormal wing disks *(awd)* in *Drosophila* development *(219,220)* and nucleoside diphosphate kinase (ndpk) in other species *(221,222),* and are highly conserved through evolution. When compared to Nm23-H1, mouse and rat homologs have 88–95% identity; the *Drosophila awd* has 87% identity; *Xenopus, Arabidopsis,* yeast, and *Dictyostelium* are 82–89, 78, 73–75, and 70–76% identical, respectively. On the other hand, several strains of *Escherichia coli* contain genes displaying more than 60% identity to *nm23.* The evolutionary conservation of this protein suggests that the *nm23* gene is pivotal for cellular survival, and that the signaling pathway involving this protein may be held intact from lower to higher organisms.

Among the family members, the *Nm23-H1* isoform has been repeatedly linked to metastasis, and, therefore, is the focus of the rest of this chapter. Reduced *nm23*-H1 mRNA or protein expression levels have been observed in highly metastatic tumor cells among a variety of *in vitro* monmdel systems, including N-NMU-induced rat and human mammary tumors, mouse mammary tumor virus-induced mammary tumors and Ras or Ras+ adenovirus 2 E1a-transfected rat embryo fibroblasts *(223–226).* In some other model systems, differential *nm23* expression was not apparent *(227).* This inconsistency is indicative of tumor heterogeneity, whereby multiple molecular pathways may underlie the metastatic process.

Regarding human *in vivo* analyses, breast tumor cohort studies showed a correlation of reduced Nm23 expression with indicators of high tumor metastatic potential, such as reduced patient survival *(228–233),* positive lymph node metastases *(234–237),* or poor differentiation state *(238).* No mutations were found in the coding sequence of *Nm23-H1* among 20 tumor specimens sequenced *(239).* Most recently, *Nm23-H1* was reported to be an independent prognostic factor for disease-free survival, upon multivariate analysis of a cohort of patients with node-negative breast tumors *(228).* Similar expression relationships have been reported in cervical *(240–243),* hepatocellular *(244–246),* ovarian carcinomas *(247–250),* and melanoma *(251–255).* Again, not all cohort studies reported this expression association; however, the data, although not unanimous, indicate that reduced *nm23* expression accompanied high tumor metastatic potential in several cancer cell types, which is characteristic of a causal relationship.

Reduced RNA and protein expression can sometimes be the result of chromosomal aberrations. LOH of the *nm23-H1* locus has been detected in breast carcinomas, lung adenomas, and kidney and colon carcinomas *(256,257).* However, an infiltrating breast tumor cohort study, conducted comparing Nm23-H1 protein expression with allelic deletion at the *nm23-H1* locus, and patient disease-free survival, showed that, although allelic deletion of *nm23-H1* was observed, protein levels in these specimens were not

uniformly low. In fact, the best correlate of poor patient survival (metastatic potential) was low Nm23-H1 expression, regardless of deletion status *(239).*

Loss of Nm23-H1 expression may contribute to metastatic progression in many cancers, but overexpression of *nm23* RNA predicts advanced-staged neuroblastoma tumors. In 20% of these advanced cases, a mutated form of Nm23, a serine (Ser) to a gylcine (Gly) change, at amino acid (aa) 120, was present *(258–260).* A similar occurrence was cited for metastatic colon carcinomas, in which high expression of nm23 was associated with the more aggressive tumors, and these tumors were more susceptible to *nm23* mutations *(256).* Although possible, inactivating Nm23 protein through mutations seems to be relatively rare. In contrast, loss of expression of this gene is a consistent property of aggressive tumors.

### 3.1.1. Metastasis Suppressor Activity

Nm23 function was assessed by the transfection of murine *nm23-1* cDNA and subsequently human *nm23-H1* cDNA into the highly metastatic murine K-1735 TK melanoma and human MDA-MB-435 breast carcinoma cell lines, respectively. Clonal cell lines constitutively overexpressing *nm23* exhibited a 50–96% reduction in tumor metastatic potential *in vivo,* with no effect on tumor growth *(261,262). In vitro, nm23* transfectants were deficient in soft agar colonization and motility to serum, insulin-like growth factor, and platelet-derived growth factor, compared to control transfectants, but exhibited comparable proliferation rates *(261–263).* Four independent laboratories subsequently reported *(264–267)* that transfection of *nm23* into melanoma or mammary carcinoma cell lines resulted in significantly reduced tumor metastatic potential *in vivo* (Table 2). Additionally, *in vitro* suppression of cell motility was replicated in metastatic MDA-MB-231 breast carcinoma cells transfected with *nm23-H1 (264).* Nm23-H1 seems to control metastasis independent of primary tumor size, thereby suggesting that nm23 is not a tumor suppressor, but a metastasis suppressor.

How could Nm23-H1 be inhibiting metastasis? Based on the observation that reduced or mutated *Drosophila awd* expression correlated with multiple developmental abnormalities postmetamorphosis *(219,220),* it is reasonable to postulate that *nm23* may function in differentiation in higher organisms. Indeed, *nm23-H1* transfectants of the MDA-MB-435 human breast carcinoma cell line were found to recapitulate aspects of normal mammary differentiation in three-dimensional culture, including formation of ascinar structures, production of the basement membrane proteins and sialomucin, and growth arrest *(268).* Independent laboratories reported *(269,270)* that transfection of PC12 cells with *nm23* induced neuronal differentiation, and antisense to *nm23* reversed TGF-β—induced differentiation of colon carcinoma cells. Taken together, these results suggest that Nm23-H1 plays a role in the development/differentiation aspect of cells, which may be an important underlying biological mechanism affecting cell motility and overall metastasis behavior.

### 3.1.2. Mechanisms of Nm23

The biochemical mechanism whereby Nm23 overexpression suppresses tumor metastasis, or induces differentiation, is still under investigation. Site-directed mutagenesis was utilized to correlate Nm23-H1 sequence and biological function in the highly metastatic human MDA-MB-435 breast carcinoma cell line *(271).* Mutations were pro-

**Table 2**
**Transfection Experiments Reporting and in vivo Metastasis Suppressor Activity for Nm23-H1**

| *Cell line* | *% inhibition* | *Ref.* |
|---|---|---|
| K-1735 TK Melanoma | 58–96 | Cell 65:25, 1991 *(261)* |
| MDA-MB-435 Breast carcinoma | 65–90 | Oncogene 8:2325, 1993 *(262)* |
| B16F10 Melanoma | 93 | Int. J. Cancer 60:204, 1995 *(265)* |
| B16FE7 Melanoma | 80–87 | Cancer Res. 55:1977, 1995 *(267)* |
| MDA-MB-231 Breast carcinoma | 44–46 | Br. J. Cancer 78:710, 1998 *(264)* |
| MTLn3 Mammary carcinoma | 44–52 | Int. J. Cancer 65:531, 1996 *(266)* |
| DU 145 Prostate carcinoma | 60–90 | Cancer Lett. 133:143, 1998 *(381)* |

duced at various aas, and the control, wt, and mutant constructs were transfected into MDA-MB-435 cells, and assayed for one aspect of the tumor metastatic process, motility in Boyden chambers *in vitro* toward serum, or a defined chemoattractant (ATX) *(272).* MDA-MB-435 clonal cell lines, transfected with a control vector, exhibited dose-dependent chemotaxis; clones expressing wt *nm23-H1* were 44–98% less motile to serum, and 86–99% less motile to ATX. Three mutations at two aa positions failed to inhibit chemotaxis: Mutation of proline 96 to Ser (P96S) is the conditional dominant killer of prune *(k-pn)* mutation in *Drosophila awd,* which causes developmental defects; serine 120 has been reported to be mutated to glycine (Gly) in 6/28 stage IV human neuroblastomas *(258),* and may be subject to autophosphorylation *(273).* Additionally, serine 120 lies two aas carboxy to a histidine (His) autophosphorylation site involved in the nucleoside diphosphate kinase (NDPK) and His PK activities of Nm23, and contributes to the structure of the active enzymatic pocket *(274).* Mutation of Ser 120 to Gly (S120G), or, to a lesser extent, to an alanine (Ala) (S120A), resulted in high levels of motility. In contrast, mutation of the autophosphorylation site Ser 44 to Ala (S44A) resulted in motility comparable to that of wt *nm23-H1.* The significance of this data is twofold: It indicates that the two sites of Nm23-H1, P96 and S120, are essential domains for Nm23-dependent regulation of cell motility; and it provides the first structure–function correlation for Nm23 in tumor metastasis.

How does Nm23-H1 signal a cell? Although this question has not been definitively answered, the Nm23 proteins have been reported to possess many biochemical functions, including a nonspecific (NDPK) activity *(222,275),* His PK activity *(276–279),* Ser PK activity *(280,281),* autophosphorylation on both Ser and His residues *(273,282–284),* and binding to multiple G proteins, *(285),* RZR orphan receptors *(286),* β-tubulin *(287),* glyceraldehyde-3-phosphate dehydrogenase *(281),* and vimentin *(288).* Several of these activities are debated in the literature. In order to determine if any known biochemical activity correlated with biological function in breast carcinoma motility suppression, the wt and site-directed mutant Nm23-H1 proteins were produced in *E. coli,* and purified to apparent homogeneity *(279).* The S120G/A and P96S mutations exhibited unique deficiencies in aspects of His dependent protein phosphotransferase activity: The S120 mutants exhibited reduced His autophosphorylation levels, which resulted in reduced downstream transfer to Sers and reduced His PK activity to three in vitro substrates; the P96 mutation exhibited normal levels of autophosphorylation, but reduced transfer of phosphate to three substrates in a His PK assay. The

Table 3
Site-Directed Mutagenesis Studies of Nm23-H1

| | *Autophosphorylation* | | | | |
|---|---|---|---|---|---|
| *Mutation* | *His* | *Ser* | *NDPK* | *His PK* | *Cell migration (Mean ± SEM)* |
| Wt | + | + | + | + | 1.0+/–0.7 |
| P96S | + | + | + | – | 17.5+/–3.7 |
| S120G | ± | ± | + | ± | 42.2+/–6.6 |
| S44A | + | ± | + | + | 1.0+/–0.6 |

+ = activity; – = no activity; ± = reduced activity.

mutant Nm23-H1 proteins exhibited levels of NDPK activity comparable to wt. The data permit the hypothesis that a His dependent protein phosphotransferase activity is responsible for the motility suppressive effect of *nm23-H1* (Table 3; *279*). The importance of the Nm23 His phosphotransferase activity, but not its NDPK activity, was replicated in *Drosophila* development *(289).* To date, however, the nm23-dependent signal transduction pathway has not yet been defined.

### 3.1.3. Translation Studies

In most cases, cancer patients succumb to complications arising from metastatic disease. One way to lengthen a patient's life-span may be to halt or eliminate the metastatic capacity of the tumor. Utilizing the knowledge acquired about *nm23-H1,* two clinical possibilities arise: First, based on the evidence that many metastatically competent tumors exhibit reduced Nm23 expression, and that overexpression of Nm23 results in decreased tumor metastatic potential *in vivo,* it is possible that elevation of the Nm23 expression of overt or micrometastatic tumor deposits in BC or other cancers may be of therapeutic benefit to limit metastatic colonization and dissemination. Second, Nm23 could be used as a marker of tumor metastatic potential, to identify compounds with preferential antiproliferative activity against metastatic breast carcinoma and melanoma cells.

The first strategy utilizes the observations that low nm23-H1 expression seems to be a limiting feature in the regulation of metastasis and cell motility. These findings lead to the hypothesis that transcriptional and translational regulation of *nm23-H1* expression may be important determinants of high vs low expression levels. Thus, if one could turn on the transcription of nm23, thereby increasing RNA and subsequent protein levels, the tumor cells' metastatic capability may be hindered or reversed. A study *(290),* substantiating the clinical potential of increasing nm23 expression, reported that Nm23 transfectants of four different cell lines exhibited increased sensitivity to cisplatin (a clinical chemotherapy [CT] drug) to inhibition of proliferation *in vitro,* and that a more pronounced reduction of metastatic colonization occurred upon cisplatin administration in *in vivo* experiments using control and *nm23* transfectants of the K-1735 TK murine melanoma cell line. In addition, a recent ovarian cohort study *(247)* supported this finding. This suggests that, if agents are identified that can increase the Nm23 expression of micrometastatic breast carcinoma or other cancer cells *in vivo,* the combination of these agents with alkylating agents may be a potential therapeutic regime.

In order to identify factors that could elevate Nm23-H1 expression, the transcriptional regulation of *nm23-H1* promoter is being studied. The *nm23-H1* promoter has been cloned by several laboratories *(291–293).* Deletion constructs of the *nm23-H1* promoter identified a 248-bp fragment that mediates high vs low expression levels (unpublished data). Current research is attempting to define the transcriptional elements (i.e., transcription factors, methylation) that are responsible for its higher expression in nonmetastatic cells.

The second translational approach also utilizes the fact that nm23 expression is reduced in highly metastatic cells; however, in this case, *nm23-H1* expression was used as a marker for metastatic potential. In this analysis, Nm23 expression levels in the breast carcinoma and melanoma subset of the panel were determined by densitometry of Western blots, and correlated to *in vitro* sensitivity to CT agents. Through a screen of antiproliferative activity of compounds on a 55-cell-line panel, performed by NCI's Developmental Therapeutic Program, growth-inhibitory potential of drugs can be correlated to a molecular event that varies among the cell lines (in this case, Nm23-H1), using the COMPARE program (in this case, Nm23-H1). Among 177 standard CT compounds that are currently in clinical use, no agents were identified that were preferentially growth-inhibitory to low Nm23-expressing, high metastatic breast carcinoma and melanoma cell lines. However, a compound known as NSC 645306 (resynthesized as NSC680718) was identified from a 30,000 compound repository, with such *in vitro* activity *(279).*

Tumor-growth-inhibitory potential was demonstrated *in vivo* by a hollow fiber assay in five tumor cell lines historically difficult to treat by conventional CT: two metastatically competent breast carcinoma cell lines, a melanoma cell line, and colon carcinoma and non-small cell lung carcinoma cell lines *(279).* NSC680718 also possesses antiangiogenic activity in vivo, using a rat aorta assay, with 50 and 98% inhibition of angiogenesis at 5 and 10 μ*M,* respectively (unpublished data). The compound did not elevate Nm23 expression, indicating that this was simply a marker in these studies. Taken together, the data define an approach for identifying novel compounds that may exhibit preferential inhibitory capacity for metastatically competent breast and other tumor cells, and which may become important standard CT in the future *(279).*

### *3.2. KAI1 Gene*

*KAI1/CD82* is a metastasis suppressor gene on human chromosome 11p11.2 that encodes a glycoprotein of the transmembrane four superfamily *(294).* Reduced KAI1 expression associates with malignant progression of human tumors, including prostate, breast, lung, bladder, ovary, and pancreatic cancers *(69,295–299).* The transfection of KAI1 into invasive melanoma or colon cancer cells reduced or suppressed their invasive and metastatic potential *(300,301).* Reduced KAI1 mRNA in tumor cells seems to influence their metastatic ability, and thereby enhances their malignant potential *(296,297,302–305).*

In addition, several studies suggest that KAI protein may undergo posttranslational modifications (N-linked glycosylation) in invasive cells. Other members of the transmembrane four superfamily were cloned, and their expression seems also to inversely correlate with the aggressive behavior of tumor cells. The expressions of *MRP-1/CD9, CD81,* and *KAI1/CD82* genes were found to be useful indicators of a poor prognosis in BC patients *(299,306).* It was recently suggested that KAI1 expression may be directly activated by p53 *(69).* A cohort study of prostate tumors showed a direct correlation

between p53 and KAI1 expression. This suggests that the loss of p53 function, which is commonly observed in many types of cancer, leads to the downregulation of the *KAI1* gene, which may result in the progression of metastasis *(69)*. Therefore, a simultaneous survey of p53 and KAI1 expression in human cancers should be of interest.

### 3.3. KiSS-1 Gene

The *KiSS-1* gene encodes a predominantly hydrophilic, 164-aa protein with a polyproline-rich domain indicative of an SH3 ligand (binds to the homology 3 domain of the oncoprotein Src) and a putative PK C-α phosphorylation site *(307,308)*. When transfected into C8161 melanoma cells or MDA-MB435 cells, the full-length *KiSS-1* cDNA suppressed metastasis in an expression-dependent manner *(307,309,310)*. Its expression seems to occur only in nonmetastatic cells *(307)*. The data suggest a mechanism whereby *KiSS-1* regulates events downstream of cell–matrix adhesion, perhaps involving cytoskeletal reorganization. However, more studies are needed to address the function of *KiSS-1* and its expression in human cancers.

### 3.4. TSP1 Gene

Another example of a metastasis suppressor gene is provided by the thrombospondin 1 *(TSP1)* gene *(311–314),* encoding an extracellular matrix protein. Transfection of *TSP1* into human breast carcinoma cells reduces their metastatic potential and angiogenesis *(315)*. However, *TSP1* also inhibits cell growth by a different mechanism *(315–317),* and additional mechanistic studies are needed to address the role of TSP1 in cell growth and motility. Therefore, this gene may be a good prognostic factor, when combined with other TSGs, for human cancers.

In addition to those genes, numerous growth factor and hormone receptor families have been shown to affect the tumor progression and metastasis. Despite these findings, it is still unclear whether all those factors independently or synergistically affect the metastatic progression in human cancers. Further studies on the cooperation between TSGs and/or metastasis suppressor genes, in controlling the metastatic phenotype, will raise new insights into human cancer diagnosis and gene therapy.

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# 4 Apoptosis

## *Machinery of Cell Death in Development and Cancer*

*Jinyan Du and David E. Fisher, MD, PhD*

***Contents***



## 1. INTRODUCTION: CELL DEATH AND THE GOAL OF CANCER THERAPEUTICS

Apoptosis, or programmed cell death, plays a critical role in development, tissue homeostasis, and disease. Characterized by nuclear condensation, membrane blebbing, and DNA fragmentation *(1),* this suicide program allows cells to die without inciting a local inflammatory response, and is thought to play a central role in both cell death during normal homeostasis and in response to stress. One of the most important observations with respect to apoptosis is the recognition that this death pathway is commonly disrupted within cancer cells. This observation led to the realization that cancer is more than a disease of uncontrolled proliferation: It is also usually a disease of ineffective cell death.

Through the study of cell death in cancer, insights have been made that directly impact not only on tumorigenesis, but also on cancer treatment. For example, signifi-

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cant information has been learned about the manner in which p53 regulates the apoptotic response to DNA damage. This in turn has been found to correlate with clinical prognosis (i.e., virtually all malignancies currently curable with chemotherapy remain wild-type for p53). Dismantling of the apoptosis cascade appears to be remarkably common in cancer, e.g., loss of p53, overexpression of bcl-2 or family members, and loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (upregulation of the Akt survival pathway). Although it is too soon to see direct clinical benefits from understanding of apoptotic machinery, these investigations may permit the construction of productive and well-targeted screens for cancer drug discovery.

## *2. Apoptotic Machinery*

The basic apoptosis machinery appears to be evolutionarily conserved. The first detailed genetic description of this death pathway came from studies of Horvitz et al. *(2–3),* who described cell death mutants in the nematode *Cenorhabditis elegans* during development. These mutants, termed CED genes (for *C. elegans* death), were identified as positive or negative death regulators. In *C. elegans,* the core cell death machinery consists of three proteins, CED-9, CED-4, and CED-3. CED-9 protects cells from apoptosis; CED-4 and CED-3 promote cell death *(2–3).* Under physiological conditions, CED-9 forms a complex with CED-4 and CED-3, and prevents the activation of CED-3 by CED-4 *(4–6).* With apoptotic stimuli, CED-9 dissociates from the complex, and CED-3 is activated to execute the cell death program. Recently, a novel protein, Egl-1, was found to antagonize CED-9's function *(7):* It binds to CED-9 through its BH3 domain, disrupts the interactions between CED-9 and CED-4, and thereby promotes CED-4-mediated CED-3 activation *(8).*

Molecular counterparts of all these gene products have also been identified in mammals. CED-3 is homologous to mammalian caspases, a cysteine protease family that cleaves after aspartic acid residues *(9–10).* Apaf-1 is a mammalian counterpart to CED-4 *(11).* Caspases exist in proenzyme forms (zymogens), which may themselves be activated by other caspases. Following release of cytochrome-c from mitochondria, cytochrome-*c* binds to Apaf-1, and Apaf-1 recruits procaspase-9, stimulating its proteolytic processing *(12–13).* Both CED-9 and Egl-1 are related to Bcl-2 family proteins, which are alternatively activators or inhibitors of apoptosis *(3,7,14,15).*

Multiple stimuli can trigger apoptosis. Several of the apoptotic pathways have been well studied at a mechanistic level. In one pathway, DNA damage and other stresses activate p53 through posttranslational protein stabilization, which triggers apoptosis through both transcription-dependent and -independent mechanisms *(16–17).* In the transcription-dependent pathway, p53 stimulates a variety of target genes, which include certain ones capable of producing apoptosis. For example, p53 can upregulate Bax expression *(18–20).* Bax drives cytochrome-*c* release from mitochondria *(21–22),* and thereby caspase-9 activation *(12).* Caspase-9 activates caspase-3, and stimulates subsequent cell death *(12).* Another pathway is initiated by death receptor ligation. Adaptor proteins bind to oligomerized Fas/CD95 *(23)* and recruit procaspase-8 to form (DISC) *(24),* a protein complex that modulates death signaling from this receptor. At high local concentration, the intrinsic protease activity of procaspase-8 is sufficient for

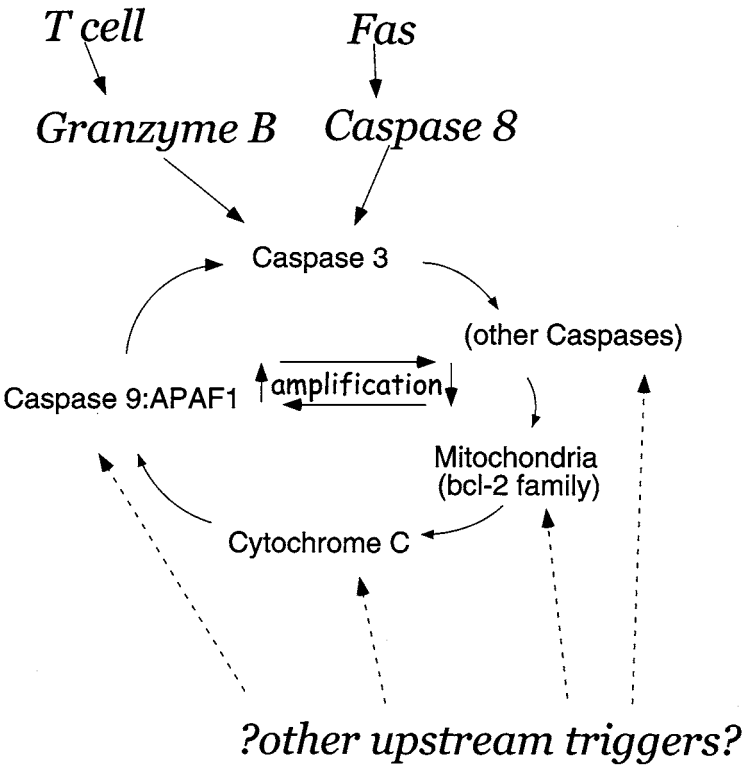


**Fig. 1.** Apoptosis triggers and amplification loop. Several pathways of apoptosis activate caspases at known stages. Others remain to be discovered, and could potentially target multiple sites within the caspase cascade. Because of interactions among caspases and mitochondria, an amplification loop may contribute to the strength of the death signal.

its activation by intermolecular proteolyic cleavage *(25).* Subsequently, caspase-8 activates the caspase cascade.

Like many signaling pathways, apoptotic pathways may undergo crosstalk, amplification, or homeostasis. One important example appears to be a role for mitochondrial cytochrome-*c* release as a means of amplifying an apoptotic signal. For example, mitochondrial damage downstream of caspase activation may result in further cytochrome-*c* release, Apaf-1 caspase-9 activation, and caspase-3 stimulation. In addition, caspase-8 can directly cleave Bid *(26,27),* a bcl-2 family member located in mitochondria, thereby causing cytochrome-*c* release, caspase-9 activation, and caspase-3 induction (Fig. 1).

Deregulated apoptosis may lead to diseases. Too little apoptosis may occur in cancer or autoimmune disease (related to incomplete removal of autoreactive lymphocyte clones), and too much apoptosis in neurodegenerative disease. Efficacy of cancer therapy can be dramatically affected by the status of apoptotic regulators. For example, tumors with p53 mutations, or wild-type p53 inactivated by oncogenes, tend to respond poorly to treatments, but highly curable cancers, such as childhood acute lymphoblastic leukemias, typically retain wild-type p53, which can be activated by irradiation or chemotherapy drugs *(17,28–30).*

## 3. SIGNALING FOR DEATH *(31)*

Fas-induced apoptosis and the perforin–granzyme degranulation pathway are the two major pathways in cytotoxic T-cell-mediated target cell killing *(32)*.

### *3.1. Fas and FasL*

Fas-mediated apoptosis helps to eliminate autoreactive T- and B-cells in the immune system *(33)*. FasL binds to Fas, a transmembrane receptor, and induces receptor oligomerization *(33,34)*. (FADD) and caspase-8 are recruited into the multiprotein DISC complex *(23,24)* through protein–protein interactions involving a discrete death domain. Recruitment of caspase-8 into such complexes is thought to lead to its autoproteolytic cleavage, and, thereby to activation *(25)*. Activated caspase-8, in turn, activates downstream caspases, which execute apoptosis (Fig. 1). Alternatively, weaker activation of caspase-8 may cause proteolytic activation of Bid in mitochondria, which subsequently stimulates cytochrome-*c* release from mitochondria *(26,27)*. Cytochrome-*c* interacts with Apaf-1, and procaspase-9 is recruited into the complex for activation *(12,13)*.

### *3.2. Tumor Necrosis Factor*

Paradoxically, Tumor necrosis factor (TNF) typically promotes cell survival, except when protein synthesis is inhibited *(31)*. One of its receptors, TNFRI, trimerizes upon TNF binding *(34)*. (TRADD), an adaptor protein that interacts with the TNFR death domain, is recruited to the receptor *(35)*. TRADD may recruit FADD and caspase-8 to induce apoptosis *(36–38)*. Alternatively, it may recruit receptor interacting protein (RIP) *(39,40)* and TNF receptor-associated factor 2 (TRAF2) *(36,41),* which activate jun-N-terminal kinase (JNK) or nuclear factor-κB (NF-κB) pathways to promote cell survival. The blockage of NF-κB makes primary glomerular mesangial cells more sensitive to TNF-α-induced apoptosis, but it has no effect on Fas-stimulated cell death *(42)*. A feedback loop has been suggested, in which activated TNFRI upregulates TRAF1 transcription through NF-κB, and, conversely, TRAF1 modulates TNFRI-induced NF-κB activation *(43)*.

Another TNFR, TNFRII, can promote cell death or survival by recruiting TRAFs *(44)*. Association of TRAFs to TNFRII activates JNK and NF-κB, and induces the survival or apoptosis pathways *(45,46)*. TNFRII lacks a death domain. It may function in apoptosis by producing endogenous TNF, which can activate TNFRI *(47)*.

TR6, a new member of TNFR family, is expressed in lung tissues and colon adenocarcinoma. TR6 specifically interacts with Fas ligand and LIGHT (herpes virus entry mediator), and may negatively regulate apoptosis induced by these two ligands *(48)*.

### *3.3. Granzyme B*

Granzyme B, a serine protease, plays a critical role in cytotoxic T-lymphocyte killing. In murine granzyme B, arginine 226 is the critical determinant of its substrate specificity *(49)*. Its translocation into a target cell is perforin-dependent and adenosine triphosphate (ATP) independent. The translocation precedes nuclear apoptotic events, such as DNA fragmentation and nuclear envelop breakdown. Bcl-2 and caspase inhibitors can block these events *(50)*. The unique feature of granzyme B is its preference for proteolytic cleavage after aspartate, which mimicks caspase substrate speci-

ficity. In this way, it is thought that granzyme B may directly activate caspases to induce apoptotic death of target cells (*51;* Fig. 1).

## 4. SIGNALING FOR SURVIVAL

MMAC/PTEN, a phosphatidylinositol phosphatase, is implicated in the oncogenesis of prostate cancer. It may regulate apoptosis and growth arrest through separate pathways. In PTEN-deficient LNCaP cells, expression of exogenous PTEN blocks Akt activation, and induces growth arrest or apoptosis. Overexpression of Bcl-2 only rescues cells from PTEN-mediated apoptosis, but has no effect on PTEN-induced growth suppression *(52).*

The kinase, protein kinase B PKB/Akt, protects cells from apoptosis, following a variety of triggers. Mechanisms for the protective effect appear to include phosphorylation of Bcl-2 proteins, caspases, and forkhead transcription factors. PKB/Akt controls association/dissociation of Bcl-2 proteins by phosphorylation. Phosphorylation of Bad at Ser 136 ablates its apoptosis-promoting function, because phosphorylated Bad is translocated from mitochondria to cytosol, and can no longer interact with Bcl-XL. TNF induces Akt-mediated Bad phosphorylation through the PI3K pathway *(53–55).* Akt can also phosphorylate caspase-9, to block its proapoptotic function *(56).*

The forkhead transcription factors, FKHR, is translocated in the pediatric tumor, alveolar rhabdomyosarcoma. It is found that PKB/Akt can phosphorylate FKHR1 at Thryonine 24 and Serine 253 in vitro *(57).* Upon phosphorylation by activated Akt, FKHR becomes associated with 14-3-3 proteins, and is retained in cytoplasm. FKHR is dephosphorylated after survival factor withdrawal. Its dephosphorylation leads to its nuclear translocation, to target gene activation, and, thereby, to cell demise. Thus, Akt may promote cell survival by inhibiting FKHR *(58).*

It has been shown that several growth factors can suppress apoptosis by activating the Akt pathway. For example, epidermal growth factor can rescue T47D breast adenocarcinoma and HEK293 embryonic kidney epithelial cells from Fas-induced apoptosis, in an Akt-dependent manner *(59).* Also, vascular endothelial growth factor is able to activate Akt and protect endothelial cells from loss of anchorage-dependent survival *(60).*

## 5. BCL-2 FAMILY PROTEINS *(14,61)*

Bcl-2 family proteins are either proapoptotic or antiapoptotic. To date, at least 16 members have been identified *(62–64).* Bcl-2, the first family member identified, was found to be misexpressed by t(14;18) chromosome translocation in low-grade B-cell lymphomas *(65–67).* It is homologous to *C. elegans* CED-9 *(3,68).* Overexpression of Bcl-2 extends cell life-span; antisense treatment accelerates apoptosis *(15,69–73).* Bcl-2 docks on the mitochondrial outer membrane, as well as endoplasmic reticulum and nuclear envelope, through its C-terminal hydrophobic domain *(64,74–76),* although the membrane-docking is not required for its survival-promoting function *(77,78).* The Bcl-2 family is divided into two subfamilies, based on their activities: prosurvival and proapoptotic *(14).*

All Bcl-2 proteins contain at least one of four Bcl-2 homology domains (BH1–BH4) *(14).* BH3 appears to be essential for cell-killing functions of the proapoptotic family members *(7,79,80).* Proapoptotic Bcl-2 proteins are further divided into the Bax fam-

ily, whose members contain BH1, BH2, and BH3 domains, and BH3-only proteins, which possess only BH3 (but no other BH) domain *(14).*

Bcl-2 proteins may execute their functions by dimerization with homologous proteins, association with nonhomologous proteins, or formation of ion channels/pores *(61).* It has been reported that prosurvival Bcl-2 can heterodimerize with proapoptotic Bax *(81),* and their ratio determines the cell fate *(82).* Bcl-2 and Bax can also function independently as regulators of cell death and survival *(61).* There are mutants of both proteins that cannot heterodimerize with each other, but are still able to suppress apoptosis and cell survival, respectively *(83–88).* Also, in Bcl-2 or Bax knockout mice, each protein is functional in the absence of the other *(89).* Nevertheless, the dimerization between Bcl-2 and Bax provides an important generic mechanism by which numerous members of this family may regulate their activity. The BH3 domain is critical for the dimerization among Bcl-2 proteins. Mutagenesis and structural study of Bcl-XL elucidated the structural basis of the dimerization. The amphipathic α-helix in the BH3 domain inserts into the hydrophobic cleft created by the BH1, BH2, and BH3 domains, and binds to the pocket through the hydrophobic surface of the helix *(79,90–92).*

In addition, Bcl-2 proteins may regulate apoptosis by interacting with nonhomologous proteins *(61).* Bcl-2 and Bcl-XL can interact with a wide range of cellular proteins. Their partners include Apaf-1 *(93,94);* death effector domain (DED) containing proteins Bap31 and MRIT *(95,96);* Raf-1 *(97,98);* the guanosine triphosphatase, R-Ras *(99);* calcineurin *(100);* chaperone regulator BAG-1 *(101);* p53-binding protein, 53BP-2 *(102);* prion protein *(103);* membrane protein BI-1 *(104);* the spinal muscular atrophy protein, SMN *(105);* and adenine nucleotide translocator located at the mitochondrial inner membrane *(106).* Interaction of Bcl-XL with Apaf-1 prevents the association of Apaf-1 with pro caspase-9, suppressing procaspase-9 proteolytic activation *(14,94,93).*

Bcl-2 family proteins may also insert into intracellular membranes, and function without dimerization with other family members *(61).* The three dimensional structure of Bcl-XL resembles pore-forming bacterial proteins, diphtheria toxin, and colicins *(91).* It has been reported that Bcl-2, Bax, and Bcl-XL are able to form ion channels with different conformations and ion selectivity in synthetic lipid bilayers *(107–111).* The membrane-docking domain of proapoptotic Nip3 is essential for its activity, but its BH3 domain is dispensable *(112–113).* Other indirect evidence supports the importance of channel-forming capacity for Bcl-2 proteins. Upon exposure to apoptotic stimuli, cytoplasmic localized Bax becomes resistant to alkaline or high-salt extraction, consistent with translocation to intracellular membranes *(108).*

More than one mechanism has been proposed to explain cytochrome-*c* release from mitochondria during apoptosis. Normally, cytochrome-*c* resides between the inner and outer mitochondrial membranes. Apoptosis signals promote the translocation of Bax from cytosol to the outer mitochondrial membrane. Channels formed by Bax may allow cytochrome-*c* to escape into the cytoplasm *(61).* Alternatively, loss of the electrochemical gradient (Dψ) across the inner mitochondrial membrane during apoptosis may promote the opening of mitochondrial PT pores, which consist of ANT and voltage-dependent anion channel (VDAC). The opening of PT pores leads to the loss of an ion gradient, influx of water into mitochondria, and cytochrome-*c* release. Expanding of the matrix space eventually results in organelle swelling and membrane rupture *(61).*

It is known that Bcl-2 proteins can interact with mitochondrial PT pores, and control their conformation. Bax has been co-purified with the PT pore complex. Bcl-2 has been shown to be able to close PT pores in reconstituted liposomes. Bcl-2 family proteins may interact with mitochondrial VDAC, to modulate cytochrome-*c* release during apoptosis. PT pore opening seems to cause cytochrome-*c* release, rather than being a result of cytochrome-*c* release. A study using reconstituted liposomes shows that proapoptotic Bax and Bak promote VDAC opening and cytochrome-*c* passage through the channel; antiapoptotic Bcl-XL suppresses VDAC opening and blocks cytochrome-*c* release *(114).*

Cellular localization of Bcl-2 proteins is regulated by the association/dissociation between family members. Hypophosphorylated Bad can interact with Bcl-XL to promote cell death. Upon phosphorylation by PKB/Akt or Raf-1, Bad is no longer able to bind to Bcl-XL, thereby abolishing its proapoptotic function *(53,54,115).* The locations of Bcl-2 proteins can also be controlled by proteolysis. Removal of N-terminus of Bid by caspase-8 promotes its translocation to the mitochondrial outer membrane *(26,27).*

## 6. MITOCHONDRIA AND CYTOCHROME-*C (76)*

Mitochondria may promote cell death through multiple mechanisms. Apoptotic stimuli disrupt electron transport *(116)* and ATP-production mitochondria *(117).* In addition, caspase activators, such as cytochrome-*c (118,119)* and apoptosis-inducing factor are released into the cytoplasm *(120,121).* Also, reactive oxygen species are produced by mitochondria during apoptosis *(122).* Release of mitochondrial cytochrome-*c* is thought to activate a proapoptotic complex consisting of caspase-9 and Apaf-1 (mammalian homolog of *C. elegans* CED-4), resulting in stimulation or amplification of downstream caspase activity (Fig. 1).

## 7. CASPASES *(123,124)*

Caspases belong to a family of cysteine proteases that cleave after aspartate residues *(125).* Fourteen caspases are now known, and many have been shown to be involved in apoptosis *(123).* Caspases are synthesized as inactive precursors, and their activation is tightly regulated. A procaspase consists of four domains: an N-terminal prodomain, a large subunit, a linker, and a small subunit. Proteolytic activation removes the prodomain and linker *(126,127),* then two large subunits and two small subunits form the active enzyme. Each tetramer contains two active sites, which are composed of residues from both the large subunits and small subunits *(128–130).*

Caspases are classified as initiators and effectors. Initiators become activated earlier in the cascade. They have long prodomains that contain either DED or caspase recruitment domains (CARDs). Initiators can cleave themselves when clustering (autoactivation), and process downstream caspases as well. Effectors are activated downstream in the cascade, and have short prodomains. The variability of prodomains may facilitate the regulation of caspase activation by different upstream pathways *(123,124,126).*

Only a subset of cellular proteins is inactivated or processed during apoptosis. Thus, caspases have strict specificity. Caspases cleave after aspartate residues at what is called the substrate P1 site. Four amino acids N-terminal to the P1 site specify substrate preferences. The P4-site amino acid is the primary determinant of substrate specificity *(131,132).*

Cellular targets of caspases fall into two categories: apoptosis regulators and structural proteins *(123).* Multiple cytoprotective proteins are inactivated by caspases during apoptosis. DNA endonuclease, DFF40/CAD, is responsible for chromatin collapse and DNA degradation during apoptosis. An inhibitor of DFF 40/CAD (ICAD), binds to CAD to repress its activity in nonapoptotic cells. In apoptotic cells, ICAD is cleaved by caspases, and dissociates from CAD. The freed CAD is now able to function as a nuclease *(133,134).* Bcl-2 proteins are also targets of caspases *(14,135,136).* Caspases can directly disassemble cell structures as well. Nuclear lamina is a cytoskeletal structure involved in chromosome organization. Its major component, nuclear lamin, is cleaved by caspase-6 *(137).* Actin, as well as some actin-regulatory proteins, are also targeted by caspases during apoptosis *(138–142).*

Inactive procaspases are constitutively expressed in cells. Caspase activation must be regulated in a stringent manner. Effector caspases are usually processed by initiator caspases. Initiator caspases are typically activated by a different strategy: induced proximity. When caspase co-factors become active upon exposure to apoptotic signals, these co-factors may recruit caspases into complexes, producing high local concentrations. Low intrinsic proteolytic activity of caspase precursors is sufficient for their processing under conditions of close proximity. Alternatively, initiators may exist in a conformation that forbids autocleavage. Binding to co-factors may then cause conformational changes in caspases, to permit autoprocessing *(123,143).*

Ordering of the cytochrome-*c*-initiated caspase cascade (Fig. 1) has been examined in cell-free systems, using immunodepletion. Cytochrome-*c* released from mitochrondria binds to Apaf-1. The complex recruits procaspase-9, and clustering of caspase-9 stimulates its autoproteolytic cleavage. A recent study showed that cytochrome-*c*-mediated caspase-9 activation requires dATP-dependent apaf-1 oligomerization. In the absence of dATP, apaf-1 interacts with cytochrome-*c* as a monomer. dATP hydrolysis drives apaf-1 oligomerization. After oligomerization, the apaf-1–cytochrome-*c* complex can recruit and activate caspase-9, then mature caspase-9 is released from the complex to further activate downstream caspases *(144).* Another study showed that procaspase-9 may recruit procaspase-3 to the apaf-1–caspase-9 complex through the WD-40 repeats in apaf-1 *(145).*

Following activation, caspase-9 may dissociate from the complex to initiate the caspase cascade, or, alternatively, downstream caspases may be recruited to the complex through adaptor proteins. In either case, caspases-7 and -3 become activated next in the cascade. Caspase-3 further activates caspases-6 and 2. Finally, caspase-8 and caspase-10 are activated by caspase-6. Caspase-1, -4, and -5 failed to be processed in this system. A positive feedback loop was revealed in the cascade. Caspase-9 initiates the processing of caspase-3, and, conversely, caspase-3 activates caspase-9 *(147).* In the context of living cells, it is possible that this entire pathway reflects an amplification loop. By this model, upstream apoptotic signals may trigger mitochrondrial injury (possibly through caspase action), resulting in cytochrome-*c* release, apaf-1/caspase-9 activation, caspase-3 activation, further mitochondrial damage, further cytochrome-*c* release and so on. The precise role of such an amplification loop (Fig. 1) in the overall life–death decision of a cell remains to be determined.

Fas has been shown to activate caspase-3, not only by promoting autoproteolytic activation of caspase-8, but also by stimulating denitrosylation of the catalytic cysteine of caspase-3. Procaspase-3 is S-nitrosylated in unstimulated cells. Its denitrosylation

upon Fas activation correlates with an increase in intracellular caspase activity. Together, nitrosylation/denitrosylation may provide an additional mechanism to control caspase activity *(146).* Caspase activity is also regulated by inhibitors. A subset of inhibitors of apoptosis (IAPs) suppress apoptosis by blocking caspase activation.

Caspase-independent apoptosis has also been described in the literature. For example, granzymes A and B are loaded into target cells through perforin in cytotoxic T-lymphocyte-killing pathway. Granzyme B-mediated apoptosis depends on caspase activation; granzyme A-induced cell death is not impaired by caspase inhibitors *(148).* Overexpression of Bax-like proteins has also been suggested to induce cell death without caspase activation *(149–151).*

## 8. *P53 (17)*

The *p53* gene encodes the most commonly mutated tumor suppressor in human cancer. The protein contains 393 amino acids, and displays transcription factor function. Through a domain located at the N-terminus, *p53* interacts with additional factors to stimulate transcription *(152,153).* The region following the transactivation domain contains five SH3-binding motifs, and is crucial to the tumor-suppressing function of *p53 (154).* The central domain functions as both a DNA-binding and a protein-binding motif *(155–163).* The C-terminal region is involved in tetramerization and nonsequence-specific DNA binding *(164–166).*

*p53* is thought to modulate two discrete functions, both in response to stress: cell cycle arrest and apoptosis (*see* Fig. 2): Both activities may play significant roles in its tumor-suppressive function. Considerable information has been learned about the pathways leading from stress (particularly DNA damage) to *p53* upregulation. This pathway involves a series of kinase activities, thought to include DNA-dependent protein kinase and the product of the ataxia telangiectasia gene *(ATM).* The consequence of this signaling cascade is thought to be phosphorylation and stabilization of p53 protein. p53 stability may also be regulated by viral products. For example, human papillomavirus E6 protein triggers ubiquitin-dependent proteolysis of p53 *(167).* E1A is thought to upregulate p53 protein levels, through a pathway in which it induces expression of the alternative reading frame (ARF) at the $p16^{Ink4a}$ locus, which in turns inhibits the action of Mdm2, an oncoprotein that triggers degradation of p53. Via this pathway, p53 levels can be downregulated by loss of ARF or amplification of Mdm2, two events that occur in a significant number of human cancers.

p53 upregulation produces either cell cycle arrest or apoptosis, depending on cellular context. In fibroblasts, oncogenic transformation is associated with, p53 inducing apoptosis; the untransformed state is associated with p53 inducing cell cycle arrest. These alternative outcomes (arrest vs death) represent a potential therapeutic index in the clinical scenario, permitting a potential treatment or stress to produce selective toxicity (Fig. 2). Many human cancers are associated with mutations in p53 or p53 regulators *(17,28–30,168).* In this light, it is noteworthy that p53 loss is common in adult, often incurable, tumors, but it is much less commonly mutated or deleted in the more highly curable pediatric malignancies *(169).*

p53 is thought to regulate apoptosis through both transcription-dependent and -independent mechanisms. It may control the expression of several key regulators of apoptosis, such as bax *(20),* although the in vivo significance of this regulation to

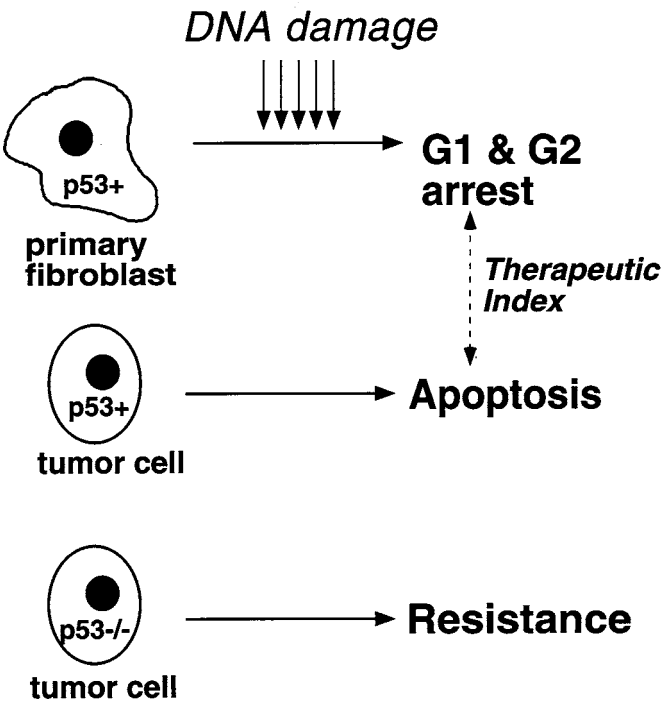


**Fig. 2.** Response to DNA damage is modulated by p53. Induction of p53 results in growth arrest at G1 and G2-M in untransformed (primary) fibroblasts; identical treatment of oncogene-transformed fibroblasts (tumor cells) results in apoptotic death. The differential responses of arrest vs, death produce a potential therapeutic index. Loss of p53 in tumor cells is associated with treatment resistance.

p53-dependent apoptosis remains unclear. It has been found that 14 p53-induced genes are activated by overexpression of p53 in the colorectal cancer line, DLD-1 *(170).* These proteins probably function in response to oxidative stress. p53 may also transcriptionally target the death receptors, Fas *(171–174)* and DR5 *(175),* insulin-like growth factor binding protein-3 *(176),* and p53-activated gene 608 *(177),* to repress antiapoptotic pathways and promote cell death. The human homolog of *Drosophila sina,* SIAH-1, was recently identified, and its expression is induced by p53 during apoptosis. SIAH-1 may function as an apoptosis inducer and tumor suppressor *(178).*

In some contexts, however, the transactivation function of p53 may not be required for p53-mediated apoptosis. In transcription-independent apoptosis, p53 may be able to act as a transcriptional repressor, or p53 may induce cell death through protein–protein interactions *(17).*

## 9. IAPS AND DEATH-ASSOCIATED PROTEINS *(179,180)*

IAPs were first found in baculoviruses *(181,182).* Two defining motifs exist in almost all IAPs: The first is the (baculoviral inhibitor of apoptosis repeat domain *(181,182),* which consists of a putative Ser/Thr phosphorylation site and arrays of DX3CX2C and HX6C, which may be metal coordination sites; the second is the interesting new gene zinc finger motif *(183),* which contains two Zn coordination sites

*(184).* This domain may be involved in DNA–protein interactions, and is critical to the antiapoptotic function of baculoviral IAPs *(185).* The CARD motif has been found in two human IAPs *(186).*

IAPs can protect cells from diversity of apoptotic triggers, including death receptor ligation, viral infection, growth factor withdrawal, chemotherapy drugs, and irradiation *(179).* The ability of IAPs to block apoptosis is cell-type-specific *(179).* IAPs may inhibit cell death by blocking caspases or activating NF-κB. It has been shown that IAPs can directly inhibit caspase-3 and -7 *(187–189).* Alternatively, they may block caspase-9 activation by cytochrome-*c (190).* Some IAPs are able to activate NF-κB. Conversely, NF-κB can induce IAPs in multiple cell lines *(179).* One of the human IAPs, survivin, may be involved in malignancy. Survivin, which is normally expressed only in fetal tissue, has been found in most cancer cells tested *(191–193),* where its expression has been suggested to correlate with shorter disease survival *(194–179).*

The technical knockout strategy is an ingenious method that was designed to identify rate-limiting genes in apoptosis. HeLa cells are transfected with an antisense cDNA library. Transfected cells are selected against apoptosis-triggering agents, such as interferon γ. cDNA fragments are rescued from interferons γ-resistant cells, and used to identify the genes that are responsible for the resistance *(195).*

Five death-associated proteins (DAPs) have been identified through this approach *(180).* Among them, DAP-2 (DAP-kinase [DAPK]) is a $Ca^{+}$/calmodulin (CaM)-dependent protein kinase. Its N-terminus contains a kinase domain followed by a CaM-binding domain. The 3′ -end of the protein is analogous to the death domain *(196).* DAPK can autophosphorylate or phosphorylate other substrates in vitro. It is associated with microfilaments in vivo *(197).* Overexpression of DAPK induces apoptosis in a kinase-activity-dependent manner. Moreover CpG islands of the DAPK gene are hypermethylated in many B-cell malignancies *(198).* This kinase may be involved in Fas-mediated apoptosis, since DAPK antisense treatment or overexpression of the DAP-kinase death domain protects cells from Fas-induced apoptosis. Thus, DAPK may function downstream of the Fas receptor, and its apoptosis-inducing activity requires the death domain *(199).* Recently, DAPK2, a homolog of DAPK, was found in mice. Localized in the cytoplasm, DAPK2 also stimulates apoptosis, when overexpressed *(200).*

## 10. MYC *(201–203)*

The proto-oncogene c-*myc* not only signals proliferation, but also sensitizes cells to apoptosis. Deregulated c-*myc* expression leads to apoptosis in the presence of cell growth arrest signals, cell differentiation signals, or cytotoxic agents. A large number of proteins have been found to associate with c-*myc,* in the process of attempting to uncover the mechanism(s) underlying the ability of this oncoprotein to regulate proliferation and death.

The C-terminus of c-*myc* contains a basic/helix-loop-helix/leucine-zipper (b/HLH/Zip) domain. HLH and leucine zipper motifs mediate protein–protein interactions, and the adjacent region rich in basic residues recognizes E-box DNA sequences in the major groove of DNA *(204–207).* A transcriptional activation domain (TAD) is located at the N-terminus of Myc *(208).* The TAD contains two Myc boxes: MB1 and MB2. MB2 is crucial to the transcription repression function of myc *(209–212).* Max *(213–216),* YY-1 *(217),* AP-2 *(218),* BRCA-1 *(219),* TFII-I *(211,220,221),* and Miz-1

*(222)* interact with the C-terminal b/HLH/Zip domain; p107 *(223,224),* Bin1 *(225),* Myc modulation 1 (MM-1) *(226),* protein associated with Myc (Pam) *(227),* transformation/transcription domain-associated protein (TRRAP) *(228),* and associate of c-Myc-1 AMY-1 *(229)* can associate at or near the N-terminal TAD.

Max possesses a b/HLH/Zip domain highly homologous to Myc, and a C-terminal nuclear localization signal. Max dimerizes either with itself or with Myc, under physiological conditions, and their heterodimerization appears to be essential for Myc-dependent function, including DNA binding, transactivation, transformation, and apoptosis *(214–216).* Max's dimerization and DNA-binding activity are regulated by phosphorylation and alternative splicing *(58,230–233).* Max resides in the center of two competing pathways: one in which it dimerizes with Myc to produce proliferation, and one in which it dimerizes with Mad proteins to promote differentiation. Max:Mad dimers repress transcription by recruiting histone deacetylases through the co-repressor, mSin3 *(234,235).*

Association of YY-1 with c-*myc* prevents Max binding *(217).* Myc antagonizes transcriptional activation and repression of YY-1 by interfering with its binding to TBP and TFII-B *(236).* Correspondingly, YY-1 may be able to affect c-*myc* activity indirectly *(237).* Transcription factor AP-2 inhibits c-*myc* function by competitively binding to the same DNA sequence as the Myc/Max complex, and AP-2 has been found to be capable of suppressing c-*myc*-mediated apoptosis *(203).* The tumor suppressor, BRCA-1, can associate with c-*myc* in vitro and in tissue culture cells. Overexpression of BRCA-1 inhibits c-*myc*-mediated transactivation *(203).* Transcription factor TFII-I recognizes both E-box and the pyrimidine-rich Inr sites *(238).* The binding of the c-*myc*/TFII-I complex to both promotor elements results in transcriptional repression of various target genes *(220,221).* Miz-1 is a Zn finger protein. c-*myc*/Miz-1 association is required for c-*myc* to inhibit Miz-1-mediated growth arrest *(203).*

p107, a member of the Rb family, interacts with MB2 of c-*myc,* through its pocket domain, to inhibit c-*myc*-mediated transactivation *(223,224,239).* Bin-1 is an adaptor protein that may act as a tumor suppressor *(203).* It may be a significant regulator of c-*myc*-induced apoptosis. MM-1 is a nucleocytoplasmic protein that inhibits transactivation by c-*myc (203).* TRRAP may be a co-activator of c-*myc*-mediated transactivation *(203).* The yeast homolog of TRRAP, Tra1, is a component of the chromatin remodeling complex, SAGA *(240).* Pam binds to c-*myc*'s N-terminal domain, both in vitro and in vivo *(227),* but the physiological role of Pam/c-*myc* remains uncertain. Amy-1 interacts with the MB2 motif of c-*myc (229),* but only binds to certain phosphorylated isoforms of c-*myc,* in a cell-cycle dependent manner. This association may regulate Amy-1 translocation into the nucleus *(203).*

c-*myc* targets various genes, although their products are not yet known to be essential for Myc-mediated apoptosis. Its genes, identified as potential transcriptional targets, include *cdc25A (241);* α-prothymosin *(242);* lactate dehydrogenase A *(243); MrDb (244); DEAD* box family RNA helicase, which functions in RNA processing and translational control; ornithine decarboxylase *(245,246);* and growth arrest and DNA damage-inducible gene *(gadd45) (247).*

Inappropriate c-*myc* expression under restricted growth conditions induces apoptosis in interleukin-3-dependent 32Dcl3 cells *(248),* Rat-1 fibroblast cell lines, primary rat embryo fibroblasts *(210),* mouse embryo fibroblasts *(249),* quiescent renal epithelial cells *(250),* hepatocytes, and lymphoid cells *(249,251).* Deregulated c-*myc* expression

also stimulates apoptosis in M1-Myc cells in the presence of differentiation signals *(252).* On the other hand, Myc expression is not required for apoptosis in 32Dcl3 cells and TGFβ/p53ts myeloid leukemic M1 cells *(253,254),* suggesting that Myc overexpression is sufficient, but not necessary for tumor cell apoptosis.

Release of cytochrome-*c* from mitochondria appears to occur downstream of Myc-induced apoptosis. A recent study showed that anticytochrome-*c* antibody can block c-*myc*-mediated apoptosis *(255).* Currently, it is unclear how direct this regulation of mitochondrial function is within cells.

Two models have been proposed for c-*myc*-mediated apoptosis: the conflict model, and the dual signal model *(249,256).* According to the conflict model, c-*myc* directly regulates only proliferative signals. Conflicts between such proliferative signals and distinct growth arrest signals, presented simultaneously in the cell, generates the unique biochemical context resulting in caspase activation and apoptosis. By the dual signal model, c-*myc* fundamentally triggers both proliferative and apoptotic signals. Apoptosis occurs in the absence of survival factors; proliferation dominates in the presence of such factors. There is evidence for and against each model. Abundant experimental data have produced their own set of conflicting support for these models. For example, in certain Myc-deregulated cell systems, there is little correlation between growth arrest and apoptosis *(257).* In addition, insulin-like growth factor 1 and platelet-derived growth factor can protect cells against Myc-mediated apoptosis, without inducing cell proliferation *(258,259).* Clearly, more mechanistic detail is needed regarding Myc's biochemical activities in regulating both proliferation and apoptosis in vivo.

## 11. PROSPECTS

The discovery that death can be more than chaotic disruption of cellular metabolism has dramatically changed modern biology. Adding to the examination of growth and differentiation in terms of cell cycle and tissue-specific gene expression, survival signals have gained prominence in regulating key steps in development. Diseases, especially cancer, have been mostly recategorized, not only in terms of growth dysregulation, but also in terms of death dysregulation. For cancer, this probably is important both during the process of tumorigenesis and in the context of cancer therapy. One of the striking features of apoptosis is the recognition of how commonly it occurs under both physiologic and pathologic conditions. Indeed, it is rare to observe descriptions of nonapoptotic death in the current biological literature. Nonetheless, it is clear that nonapoptotic death plays an important role in much of human pathology (in contrast to apoptosis, which also occurs in the course of normal cellular homeostasis). It is likely that the explosion of information regarding the ordered, efficient events in apoptosis will permit the identification of therapeutic targets of importance, and, hopefully, novel treatments. In this manner, manipulation of apoptotic events may permit both up- or downregulation of cellular survival in a manner that could impact on an enormous number of human diseases.

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# 5 Drug Discovery in Oncology

*Alex Matter, MD*

***CONTENTS***



## 1. INTRODUCTION

Drug discovery in oncology has undergone profound changes over the past 20 yr; the rate of change has markedly accelerated over the last 5 yr, and it is therefore appropriate to take stock of these changes, and to ask what the next steps in this evolving landscape of concepts, skills, and technologies are likely to be. More than ever, drug discovery in oncology finds itself at the crossroads of academic research, industrial research and development (with a growing share by the biotech industry), clinical research, regulatory authorities, and public health, including major partners, such as the National Cancer Institute (NCI). All of these partners are driven, more than ever, by the forces related to productivity, i.e., a relentless drive for quality at manageable cost, within minimal time frames. These forces are behind the technological revolution that is still taking place, the drive to secure competitive patent positions, the drive to be faster on the market through streamlined R&D processes, the drive for a more efficient approval process and flexible handling of market access by

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health authorities, and the importance of pharmacoeconomic aspects for the payors at large, even in disease states, such as advanced cancer, in which the medical need is undisputed.

This chapter concentrates mostly on technical aspects of drug discovery; keep in mind, however, that a almost invisible force field is exerting a powerful effect on all aspects of this complex endeavor. Considering the wide field of drug discovery in oncology, the chapter limits itself, for reasons of space, to the discussion of research and some early development activities, excluding late-stage development aspects, and therapeutic modalities directed against cancer itself, to the exclusion of chemopreventive regimens, as well as supportive therapy.

Drug discovery in oncology has undergone changes in two major and separate aspects:

- Development of novel technologies and processes: How does one approach a particular drug target? The technological revolution and the impact of novel technologies, processes, concepts, and rationales on the whole drug discovery process is briefly summarized in Subheading 3.
- Development of concepts and rationales: Which target should one tackle? Molecular epidemiology and a much deeper understanding of the pathophysiology of cancer at the molecular level are at the roots of new concepts and rationales. An evaluation of possible targets, taking these elements into account, is proposed in subheading 4.

Some examples of modern approaches in anticancer drug discovery are illustrated in subheading 5. Key parameters for the selection of one or another treatment modality are briefly explained in subheading 6. Subheading 7 deals with some of the issues related to the predictive quality of drug screens. Subheading 8 proposes some principles for the organization of efficient drug screens. Subheading 9–11 list key success factors for various stages of drug discovery, and Subheading 12 briefly deals with a few major elements of project management.

## 2. DEVELOPMENT OF NOVEL TECHNOLOGIES AND PROCESSES

Rapid technological change has occurred on many levels.

### *2.1. Chemistry (1–4)*

One major development in synthetic chemistry over the last 10 yr is the emergence of combinatorial techniques to produce large number of compounds in a relatively short time frame. Combinatorial chemistry comprises many different approaches, requiring either deconvolution or allowing direct or indirect analysis, based on an encoding strategy of single compounds (one bead–one compound). Vigorous efforts are underway to make these libraries more informative, using more efficient synthetic strategies and more sophisticated analytic techniques.

Computer-assisted molecular modeling, structural chemistry, two-dimensional (2-D)- and three-dimensional (3-D)-quantitative structure activity relationship (2-D- and 3-D-QSAR) analysis have become commonplace tools of the trade of the modern synthetic chemist. Comparative molecular field analysis is a 3-D-QSAR technique in which the steric and electrostatic fields surrounding a set of molecules are sampled at discrete grid points, then correlated with biological activity. These techniques have acquired a high degree of predictive quality, i.e., in many cases, the predictions of the

modeler are borne out by the hard-copy synthetic compound. Combining the above technologies, intelligent combinatorial libraries, based on multivariate design and multivariate (QSAR) analysis, are expected to provide high chemical diversity with a reasonable number of compounds *(3).*

Another important development in chemistry is the fact that more and more complex structures have become feasible: complex chiral compounds, antisense chemistry (including, e.g., the phosphorothioate and ribonucleotide variations), polysaccharide synthesis, and the elaborate synthetic pathways of natural compounds, are some prominent examples *(77,84).* Bioreactions (enzymatic reactions) are often introduced to complement classical synthetic chemistry (e.g., for enantioselective hydroxylation or the synthesis of other enantiomerically pure products).

### *2.2. Analytics (5–29)*

The bewildering variety of established and emerging analytical technologies can be grouped conveniently into the following:

1. Nuclear magnetic resonance (NMR) technologies have developed into a panel of highly versatile techniques for the analysis of compounds in solution or attached to solid supports. Both structure and ligand–receptor interactions can be studied simultaneously, in automated fashion. A variety of refinements has evolved for various applications: magic angle spinning-NMR, nuclear Overhauser effect, $^{13}C/^{1}H$-NMR, affinity NMR for the observation of ligand binding to a macromolecule (for review, *see* ref. *20*). These technologies are now more and more combined with high-throughput analytics needed in combinatorial chemistry, genomics, and proteomics. Bio-NMR (in tissues and animals; *see* subheading 2.12) is a further addition measuring e.g., $^{31}P$-NMR spectra (markers of cellular energetics and metabolism).
2. Mass spectroscopy (MS) technologies: liquid chromatography coupled with MS or tandem-MS, matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF MS) *(14),* electrospray/MS/MS for the rapid and precise measurement of molecular mass of (macro)molecules; high-throughput MS using fully automated MS equipment *(11),* affinity selection MS in combination with combinatorial chemistry (looking, e.g., at peptide substrate specificity of enzymes/receptors), capillary electrophoresis coupled with MS to study protein–protein and protein–ligand interactions.
3. Optical spectroscopy (OS) comprising technologies such as circular dichroism, infrared-OS, Raman, UV, and fluorescence; fluorescence polarization (FP) for the study of ligand–receptor interactions.
4. X-ray crystallography for structural studies (30–33), more and more frequently utilized also for the analysis of the interaction between drug candidates and drug targets at the atomic level.

This list is by no means exhaustive. In many instances, these technologies are applied in concert, in order to reap maximal information.

### *2.3. Assay Technologies*

Measurement of ligand–receptor, enzyme–substrate, and antigen–antibody (Ab) interactions has been performed by classical methods, including radioimmunoassays, enzyme-linked immunosorbent assay, and many variations thereof. More recently, a number of novel assay technologies have been developed in order to cope with the need to reduce time and cost of assays, while preserving their robustness, simplicity,

and sensitivity. New assay techniques that reduce the number of processing steps and the time intervals of incubation and analysis are needed, particularly to cope with the vast number of compounds produced by combinatorial chemistry.

Homogeneous assays (in which components are all in the same solution) and non separation assays (in which the components are bound to a solid phase) are increasingly introduced in high-throughput screens. A widely used example of the latter is the scintillation proximity assay (SPA) of Amersham. SPA is a convenient technology that uses scintillant-containing microspheres (fluoromicrospheres or SPA beads), which are chemically treated to enable the coupling of macromolecules. The assay uses radioisotopes with low-energy radiation ($^{3}$H-emitting β particles, $^{125}$l-emitting Auger electrons with very short path lengths). If a molecule labeled with one of these isotopes is bound to the bead surface, it is in close proximity for the emitted radiation to produce a measurable light signal; the energy of isotopes that are not bound to the beads (attached to unbound ligand) is dissipated in the aqueous environment. This leads to a simple, homogeneous assay format, avoiding separation steps, and reducing manipulations to a minimum, which is particularly suitable for high throughput screening (HTS).

A major drive toward homogeneous assays, avoiding radioisotopes, has led to assays based on FP (*see* below), and to new assay formats such as homogeneous, time-resolved fluorescence resonance transfer (FRET) *(15)*. This latter assay format combines the advantages of radioisotope assays (sensitivity) with the advantages of fluorescence (safety, stability of labels). The principle of this technology entails the use of europium ($Eu^{+3}$), a rare lanthanide that is known to demonstrate a slow decay of its fluorescent signal. The trapping of $Eu^{+3}$ in a macropolycyclic complex, such as the cryptates, allows formation of a highly efficient fluorescent complex ([Eu]K), which avoids fluorescence quenching by common assay components, such as oxygen, water, and proteins. (Eu)K represents therefore an efficient donor that can be used for labeling macromolecules. A further refinement of this assay format is the use of an acceptor molecule, such as a modified allophycocyanine (XL665), for the efficient energy transfer between donor and acceptor in the same assay. With the combined use of these two fluorescent labels, it is possible to analyze fluorescent signals without quenching, in the homogeneous assay format, and numerous applications have already been designed to make full use of this attractive technology. Numerous variations of this technology have been devised that cannot discussed here.

As mentioned, FP is used as an alternative in modern HTS, to measure an increase of polarization in labeled ligands, upon binding to relevant receptors. A number of applications have been described (reviewed in ref. *22*), which show the feasibility of this approach and its suitability for HTS.

### *2.4. Biosensors*

Biosensors constitute a new and rapidly growing family of established and emerging technologies. Established technologies are based on surface plasmon resonance, which is valuable for measuring protein oligomerization and receptor–ligand-binding affinities. Novel applications, such as planar wave guide sensors and evanescent-field fluorimetry, are emerging as useful components in current drug screens. Future trends comprise, e.g., miniaturization of sensors, detection by Ab capturing of analytes, development of sensor chips, and so on. This field is rapidly broadening into a major technology bundle.

### *2.5. Vectors*

Plasmids, and retroviral, adenoviral, and other vectors are now routinely used to transform cells.

### *2.6. Genomics/Pharmacogenomics*

Description of the changes in gene expression following the administration of drug candidates or drugs in cellular and animal screens, as well as in clinical trials, has certainly become a major activity in drug discovery. Five different methods are commonly used: expressed sequence tag sequencing, subtractive cloning, differential display/representational difference analysis cDNA microarray hybridization, and serial analysis of gene expression (SAGE).

cDNA microarray hybridization technologies for the detection of differential gene expression are expected to become dominant technologies for the measurement of relative mRNA levels of multiple genes in a tissue or cell line. mRNA expression levels are determined after isolation of polyA$^+$ mRNA and synthesis of the cDNA label through hybridization to oligos on different chips (experimental, control). Quantitative fluorecence measurements of both chips are then compared, and the ratio on each spot yields the relative expression level. Expression levels of up to 6500 genes can be analyzed simultaneously, and it is to be expected that performance of these chips will increase dramatically. Affymetrix and Synteni appear to be in the lead at present in a field of about 20 companies directly involved in these technologies *(21)*.

An alternative approach, the SAGE technology *(6,26,62)* has also become a broadly applicable approach for the detection and comparison of thousands of transcripts, particularly low-abundance transcripts. It is estimated that a transcript expressed at an average of three copies per cell can be detected with 92% probability *(75)*. In this publication, studying approx 45,000 different genes of gastrointestinal tumors, transcripts of about 1% of the genes were expressed at significantly different levels in normal and neoplastic cells.

Methods for high-throughput target identification exploit modified yeast two-hybrid systems, which allow screening of any newly identified protein (bait) against a cDNA library (prey), in order to find the native binding partner. The prey cDNA library is expressed in a large panel of individually cloned yeast strains that are formatted into microtiter plates, and are used repeatedly against the different baits, similar to a compound collection that can be used in different HTS assays. For each new screen, the bait code is inserted next to a DNA-binding domain, and this yeast strain is then mated individually with the panel of prey clones. In case of an interaction between bait and prey, transcription is activated and a positive readout generated by the β-galactosidase reporter enzyme. This system has been refined and automated in some places to a high efficiency, allowing rapid and successful identification of novel bait proteins.

An alternative method for rapid detection of relevant drug targets in yeast *(Saccharomyces cerevisiae)* has been devised and called synthetic lethal screening *(9)*. This method screens for second site mutations that, by themselves, are not lethal, but, in combination with a primary defect (such as a defect in the DNA repair machinery), produce lethality. Such gene products that produce lethality in cells with primary genetic lesions would be attractive anticancer drug targets.

A highly sophisticated approach, aiming also at high-throughput target identification, has been developed in mammalian cells. This approach consists of a flow chart of

technologies, starting with a combinatorial retroviral gene library to infect cells that are transfected with a green fluorescence protein *(GFP)*/BFP reporter gene, under the control of a promoter that is giving a readout related to a cellular function of interest. Peptide sequences with inhibitory function can then be isolated and tested for their function in secondary cellular screens. Inhibitory peptides can then be used as baits in a yeast two-hybrid screen to detect their intracellular binding partners. The feasibility of this approach has been documented *(10).* This approach has also been used to identify inhibitors of enzymes, such as caspases, in living cells, in a combinatorial mode *(88).*

### *2.7. Proteomics (12)*

This is usually understood to represent high-resolution 2-D gel electrophoresis coupled with computerized image and data analysis, followed by MALDI-TOF MS or nanoelectrospray. Several gels of the same preparation can be run to average the spot images and improve the signal-to-noise ratio. Changes in abundance (≥2) or position of proteins in the 2-D electrophoresis pattern are then correlated with functional changes induced by altering experimental variables. A 2000 protein/gel resolution is achievable; for higher resolution, so-called "zoom gels", focused on narrow pH zones, are proposed. Estimates are that proteins with more than 400 copies/cell will then become detectable, a level of detection that far outstrips the currently achievable sensitivity of >10,000 copies/cell that are detectable.

### *2.8. (Engineered) Cell Lines and Transgenic Cell Lines*

Cell-based assays have become an integral part of modern drug screens. Classically, these were used in a relatively simple manner to measure proliferation rates, growth, and cytotoxicity of drug candidates; in a more sophisticated way, biochemical markers have been studied, such as autophosphorylation patterns of surface receptors, enzymatic activity of members of signal transduction pathways, and expression of transcription factors by Northern, or Western blots.

More recently, with the introduction of reporter gene technologies, the output signal to any drug exposure has become more refined. In such systems, transcriptional activation or inhibition of a particular reporter gene can be measured following, e.g., ligand binding to a surface receptor or interference with a particular element of a signal transduction pathway leading to the reporter gene. These systems are amenable to high-throughput analysis and automation, and several readout techniques are now available *(22):* β-galactosidase (bacterial), luciferase (from the firefly), alkaline phosphatase (human placenta), β-lactamase (also in living cells, by loading a substrate intracellularly as a membrane-permeant ester *(29),* and (jellyfish).

Another invention concerns the introduction *of fusion genes, such as (GFP),* fused to any protein of interest; the readout is green fluorescence, but different mutant proteins, with blue and yellow emission spectra, are now also available. This technology can also be used to study protein–protein interaction by FRET.

Transgenic cell lines are becoming more and more available, in parallel with the emergence of transgenic animals (*see* subheading 2.10.); gene targeting via homologous recombination (exploiting the Cre/loxP system) is now widely available, in order to produce knockout animals in which the gene of interest is missing *(43,44).* These animals can then be used to generate cell lines containing the identical genetic defect. A further refinement allows replacement of the knocked-out gene by other genetic

material, such as the human homolog of the gene under study. These animals and the derivative cell lines are gradually introduced into drug screens, with obvious benefit in terms of relevance of the model situation.

The logic of knockout animals/cell lines has been carried to the level of mRNA in generating cell lines expressing ribozymes mediating a knockdown of the mRNA of interest. Last, a functional, knockout can be achieved via intracellular single-chain Abs. (sFv), which are directed against the gene product of interest *(38).*

### *2.9. Cell and Organ Culture Techniques*

These methods have seen gradual refinements, with the introduction of matrices, feeder layers, defined tissue culture media, soft agar cell culture, and spheroid cultures.

### *2.10. Novel Animal Screens*

Among many developments, a few are particularly striking and relevant for the drug discovery process in oncology:

1. Xenografts employing human tumor tissue or cell lines.
2. Orthotopic murine tumor models, which are particularly useful in the study of metastatic processes.
3. Transgenic mice with mutations in relevant oncogenes (such as *ras (37), myc (42), E2F1 (45),* or *erbB-2/neu (47),* or tumor suppressor genes *(p53) (49),* which have seen many important improvements with the introduction of the (inbred-strain-derived) embryonic stem cell technology, conditional knockouts and targeted transgenesis (e.g., introduction of dominant mutant tumor suppressor genes or viral inactivators of p53/Rb (SV40 LT) *(41).* For more information, the reader may wish to consult the database of the Jackson labs (Transgenic and Target Mutation Database, http://tbase.jax.org) *(46,48).*
4. Quantitative angiogenesis assays *(40).*

### *2.11. Microscopy*

Cell sorting and confocal microscopy are now introduced in most drug discovery units. Classical histological techniques, autoradiography, and immunohistochemistry have been complemented more recently by new methods, such as *in situ, hybridization techniques* comprising comparative genomic hybridization and fluorescence *in situ* hybridization (FISH) *(25,28).* FISH has become a very widely applied technique. An interesting development concerns laser-assisted microdissection *(5),* allowing the molecular biology study of isolated cells in mixed tissue samples.

### *2.12. In Vivo Imaging*

Two groups of technologies are particularly worth mention:

1. *Magnetic resonance spectroscopy and magnetic resonance imaging (MRI)* are now well established tools in the drug discovery process. Besides a merely anatomical, noninvasive high-resolution imaging technique, MRI also allows study of blood flow and tissue perfusion, using an intravascular contrast agent (Endorem); the use of extracellular contrast agents (GdDOTA, Dotarem) provides information on vascular permeability. Functional MRI (fMRI) is a further step whereby a control image is subtracted from the image after an experimental intervention, allowing one to map precisely in 3-D the changes that occurred after a stimulus (e.g., drug administration, electrical stimulation, and soon).

2. Fluorescence imaging of whole animals, using GFP technology (*see* subheading 2.8.), or using the luciferase reporter gene system coupled with advanced CCD cameras to detect gene expression with high sensitivity *(35,36),* yields images of high resolution, and allows noninvasive imaging of whole animals over the time-course of an experiment. These techniques may offer great value in the future, and effectively compete with MRI technologies.

### *2.13. Tracking of High-affinity Drugs In Vivo*

Prerequisites for application of such technologies are high potency of the drug under study, possibility of labeling the drug, and that the signal is not entirely absorbed by the tissue. Currently, only techniques of nuclear medicine have this type of sensitivity:

1. Planar γ camera: 2-D image.
2. Single photon emission computer tomography (SPECT): 3-D image.
3. Positron emission tomography (PET): requires a proton source (cyclotron), and produces 3-D images.

#### 2.13.1. PET

Drug concentrations of about $10^{-12}$ *M* may be measured, i.e., <1 μg are usually sufficient for PET studies in humans (tracer dose). Frequently used radionuclides are carbon-11 (with a half-life of 20 min) and fluorine-18 (half-life 110 min). Biodistribution and pharmacokinetic studies are fields of application for this expensive and not user-friendly technique.

#### 2.13.2. SPECT

Technetium-99m (half-life, 6 h) for perfusion studies, iodine-123 (half-life, 13 h), and iodine-131 (half-life, 8 d), are useful for drug labeling, and are among the radionuclides that can be used for SPECT. This is a technology that more easily allows labeling of drugs; it has, however, less spatial resolution (5 mm), and is less sensitive than PET.

### *2.14. Engineering*

Automation, robotization, and miniaturization have been developed to a degree at which efficient handling of large sample numbers has become commonplace. We are now at a stage at which ultrahigh-throughput screening technologies (e.g., 9600-well format) in very small assay volumes (1–2 μL or less) seems feasible, and at which introduction of these technologies into routine screening activities may be achievable over the next few years.

### *2.15. Information Technology*

High-volume data handling and the management of large databases, including networks, have become major challenges. Vigorous efforts are underway to develop efficient tools for data mining that are based on automated applications of a set of algorithms, to explore and model complex relationships between variables and objects. The challenge is to identify interesting compounds. Several tools have been developed that are able to analyze patterns in very large databases. In the opinion of experts, the identification of domain-specific, interesting patterns is still in its infancy, and a completely unsupervised automatic application of data mining tools without domain knowledge and without statistical background knowledge is not feasible at present.

Many data-mining tools attempt to extract frequent patterns from data; the identification of interesting rare patterns is not yet achieved.

Integration of these technologies and processes in an efficient manner, at reasonable cost, into a logical flow of drug screens, remains a major challenge, even for the most experienced drug discovery organizations.

## 3. SELECTION OF DRUG TARGETS *(50–75)*

Three major factors appear to play a crucial role in the increasingly refined process of target selection: relevance, validity, and feasibility. Usually, the selection of a novel drug target is triggered primarily by its discovery in biology, usually at an academic center. Only subsequently is its relevance and validity challenged by epidemiological and clinical considerations. Often, the excitement that is generated by the discovery of an apparently important new target molecule is a powerful motor to trigger investments; the slower and less exciting work to substantiate its relevance and validity is often neglected or postponed, many times at a substantial cost, and sometimes at the cost of failure.

Relevance of a drug target means primarily its relative frequency in various cancer types, realizing that high frequency is not necessarily linked with causality in terms of tumorigenesis or maintenance of the tumorigenic state/progression; careful distinction should also be made between familial and sporadic cancers, because relevance of a drug target in familial cancer (found by any positional cloning efforts) may not translate into relevance for sporadic cancer: one classic example is *BRCA1*. Determination of its relevance will allow determination of patient populations with respect to age, sex, race, geography, and the residual medical need after treatment with the best available therapy. These are minimal parameters that are most important for forecasting the market value of any prospective drug candidates, and are also the basis for an assessment of the risk/reward structure of a project.

Validity of a drug target relates to the fact that any target manipulation by drugs or other modalities will lead to a sustainable and desired change in the biology of a given cancer, and, ultimately, after appropriate development, to a favorable outcome for the cancer patient. One is dealing here with issues such as redundancy of pathways and resistance of cancer to manipulation of any kind, in the broadest sense. An example of such difficulties is, for instance, the finding that bcl-2 overexpression in biological terms is deemed to be a target for inhibition, since its well-documented antiapoptotic effects would appear to indicate an important role in tumor progression. In epidemiological terms, the contrary seems to be true *(74),* and bcl-2 overexpression in breast carcinomas correlates with a number of favorable prognostic factors, such as ER/PgR expression, low-grade histology, and other parameters. Another example is seen with farnesyl transferase inhibitors (FTIs), which inhibit Ras processing. K-Ras processing can proceed via farnesylation (inhibited by FTIs) or geranylgeranylation (which is not inhibited by FTIs). In A549 cells *(90),* for instance, K-Ras processing is resistant to both FTIs and inhibitors of geranylgeranylation. Yet, either type of compound is able to effectively inhibit tumor growth of A549 tumor cells in mice. It must then be speculated that alternative, unknown targets are hit by these compounds. These two examples illustrate the complexity of the biological systems, and explain to some extent why so many rational hypotheses are not substantiated in the context of a living animal, not to speak of heterogeneous patient populations.

An issue that is also important to consider is genetic instability of cancers, as highlighted in a recent review *(60).* Clearly, any anticancer drug could become easily ineffective through resistance mechanisms that are driven by genetic instability. In practice, this may be particularly disturbing in fast-dividing tumors, such as leukemias; the more slowly dividing epithelial tumor types may be less quickly able to develop effective resistance mechanisms. Drug candidates that are directed against elements of the host (e.g., angiogenesis, immune mechanisms) are unlikely to be susceptible to resistance caused by genetic changes: One should bear in mind, however, that numerous resistance types (e.g., MDR-1, P450 enzyme induction) do not require genetic alterations to develop. The suggestion of Lengauer et al. to consider genetic instability as a field in which novel drug targets may be found, is very interesting, and the hypothesis that numerous, if not most, cytotoxic agents may be affective because of genetic instability of the cancer cells, merits further analysis.

The validation process comprises both the relevance and validity of a given drug target, and starts with the molecular epidemiology of a given drug target and its role in the pathophysiology of the type of cancer that is under study. In many instances, tool compounds, Abs antisense molecules, and viral vectors, and so on can be used to verify the validity of a drug target at the biochemical, cellular, and intact animal level, even in the absence of any good lead or reference compounds. This work should, at the very least, accompany the drug screen; preferably, it is done prior to large investments in structural biology, lead optimization, and laborious animal screens. However, in many instances, this is hardly possibly, and risk must be carried foreward until a test compound shows its merit in advanced clinical trials. Reducing risk by early validation of a drug target must certainly be one of the primary goals in any drug screening effort. Major consideration in this respect is the predictive quality of any drug screen that must be assessed, whenever possible, using clinically used reference compounds or systems that have shown in other situations a measure of predictive quality. It is fair to say that, in oncological drug discovery, predictive quality is generally low, definitely much lower than is usually thought (*see* below).

The third major factor in drug target selection, *its feasibility,* is dependent on many parameters that are beginning to be understood quite well. It is obvious from decades of pharmaceutical research that, in general, enzymes and receptors for small molecule ligands are almost ideal targets for drug discovery efforts. This is particularly facilitated when the molecular structure of the interaction site is well described at high resolution, when there are high-affinity natural ligands, and when there are assays available that are amenable for high-throughput screens. A particular problem is posed by drug targets that are characterized by protein–protein interactions, such as cytokine receptors and transcription factors. In a few instances, there are natural compounds (e.g., cyclosporin A, rapamycin) that pave the way for a drug discovery effort, and, recently, many examples have been pioneered of total syntheses of seemingly unapproachable molecules (e.g., taxol, epothilones, discodermolide, and so on). When such natural compounds do not exist, and when high throughput screens do not provide high-affinity leads, there is only the route of *de novo* design open to obtain useful lead compounds. It is then important whether a relatively small interaction site can be defined by molecular modeling (corresponding to a few, probably not more than eight, amino acids), whether there are highly charged residues in the vicinity of the binding site (such as the phosphorylated tyrosine recognized by SH2 regions), and whether the binding site is flat or comprises one, or pos-

sibly two grooves. In general, results of such *de novo* designs have not been spectacular, despite tremendous efforts (e.g., SH2 regions, STATs, ras-raf, ras-GAP, p53/Mdm2 [*see* Fig. 1]). It may well be that, with the advance of artificial intelligence, combined with high-speed, powerful computers, this situation may change rapidly. On the other hand, low chances of success of such screens have led some to speculate that such targets should then, and only then, be approached when strong, submicromolar leads emerge from high-throughput screening. Thus, detailed validation of targets would only be done later, when principal feasibility of the target has been established. This is a new combinatorial approach to target selection.

## 4. CANCER DRUG TARGETS WITH ESTABLISHED RELEVANCE

*Drug targets can be usefully classified* in two ways: first, into targets that are uniquely linked to the genotype of cancer (more or less stable mutations, such as the Philadelphia chromosome in [CML]) that occur in all, or at least in the vast majority of, cancer cells of a given cancer type; a range of oncogenes and tumor suppressor genes have been defined governing key biological parameters, such as the regulation of the cell cycle, apoptosis, differentiation; the genomic instability of cancer cells (driven, e.g., by myc, cyclin D or ras activation, loss of *p53* or ATM function, and so on) is one of the most recent fields in which genetic alterations can now be identified with sufficient precision to build new concepts for drug discovery. Second, drug targets may be defined by host–tumor relationships (hormone-responsive cancers, angiogenesis, invasive properties, osteolysis). This type of classification will have immediate bearing on the level of molecular definition and nature of the drug target, feasibility criteria (*see* Table 2), likelihood of preexistent resistance and/or resistance induction, and size of patient population.

In Table 1, the epidemiology of some of the currently most actively investigated cancer drug targets is summarized, including some examples of drug candidates and their approximate stage of development.

At the earliest possible time-point, a putative target profile for a drug candidate shoud be established. Such a profile would indicate cancer type(s)/patient population, dosage and route of application, treatment schedule and treatment duration, possible partner drugs for combination regimens, and expected patient benefit. It is clear that at the outset of any research project these elements are not available with any degree of precision. A gradual refinement of thinking regarding these parameters is, however, important, and a steady stream of crosschecks with experts from other disciplines, particularly clinical and regulatory, is mandatory, to ensure buy-in from organizations and outside opinion leaders.

## 5. SELECTION OF TREATMENT MODALITIES

In Table 2, some major advantages and disadvantages of current treatment modalities are compared.

## 6. PREDICTIVE QUALITY OF DRUG DISCOVERY SCREENS

The following discusses the predictive quality of preclinical screens in the broad sense of clinical outcome, i.e., how predictive, e.g., a particular receptor binding test is regarding clinical outcome in patient populations, when administering the same receptor agonist.

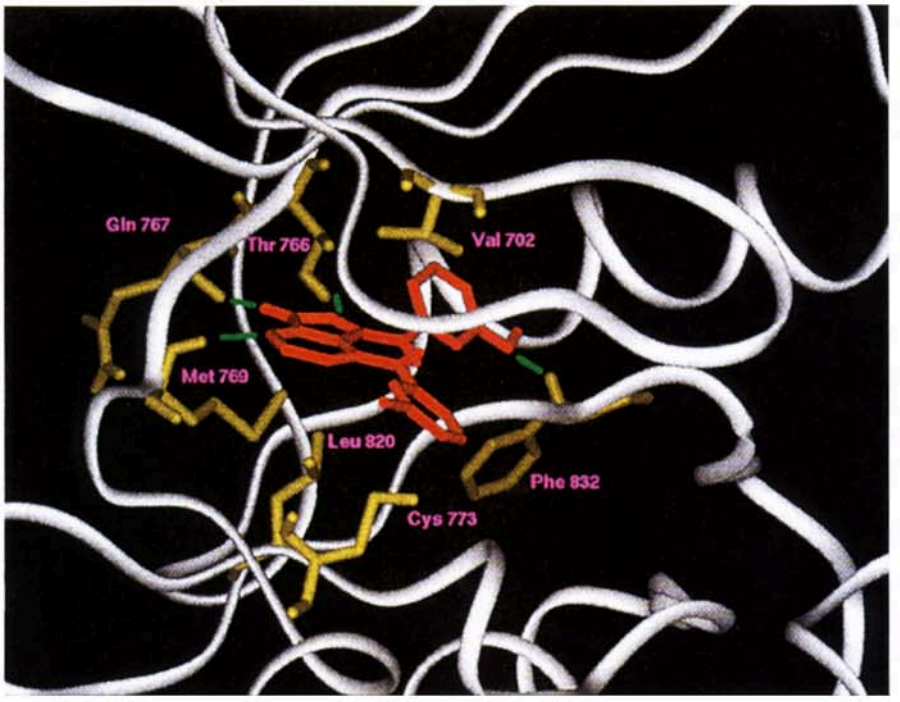



**Fig. 1.** Tyrosine kinase inhibitor (CGP 59326) modeled into the adenosine, triphosphate-binding pocket of the catalytic region of eadermal growth factor (EGF) receptor tyrosine kinase.

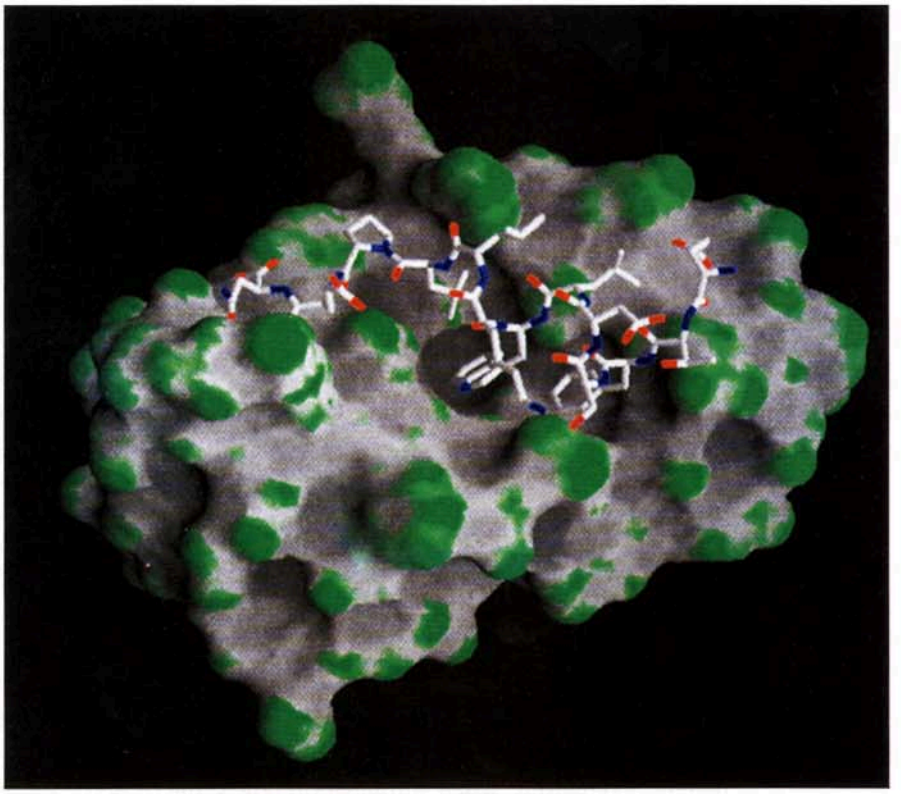


**Fig. 2.** Deep hydrophobic cleft of Mdm2 in the p53-binding region *(31)*.

Table 1

| *Target* | *Epidemiological findings in human cancer* | *Examples for drugs/drug candidates* | *Current state of development* |
|---|---|---|---|
| Farnesyl transferase (activated Ras) | Mutations of *ras* found in 90% of pancreatic, 50% of colon, and 30% of lung cancer | L-744,832; L-745,631 (Merck), B956 (Shionogi), SCH56580; SCH58450 (Schering-Plough), and many others *(78,89)* | Phase I/II and preclinical |
| EGF-receptor/erbB-2 in tumor disease | Overexpression of EGF-R<br>Constitutive activation of mutant receptor in gliomas, breast, lung | CP-358,774 (Pfizer), ZD1839 (Zeneca), SU 5271/PD 153,035 (Sugen), PD 165,557 and PD 158,780 (Parke-Davis), PKI166 (Novartis), many other compounds *(79,91,92)* | Preclinical–phase I–II |
| Bcr-abl mutations | 100% in CML, 25% of AML | STI571 (Novartis), FCE 28436 (Pharmacia-Up ohn), AG1112 (Yssum) | Preclinical–phase I |
| (PDGF)-driven tumorigenesis/ angiogenesis | Brain, ovarian, lung, prostate | SU 101 (Sugen) | Phase I/II |
| Role of protein kinase C (PKC)- and c-raf in cancer | | ISIS 3521 (antisense against PKCα), ISIS 5132 (antisense against c-raf) (both ISIS-Novartis), PKC412 (Novartis) | Phase II |
| Cyclin-dependent kinases in cell cycle events | | Flavopiridol (HMR) Staurosporine, UCN-01 (NCI-Kyowa Hakko), Butyrolactone-l, Olomoucine, Roscovitine *(81)* | Phase II<br>Preclinical |
| Metalloproteases in processes such as invasion and metastasis *(94)* | Metastatic process, invasion of tumor cells into adjacent tissue | Marimastat (BB 2516, British Biotech),<br>AG 3340 (Agouron),<br>Bay 12-9566 (Bayer)<br>MMI270 (Novartis) | Phase III<br>Phase I<br>Phase I<br>Phase I |
| Angiogenesis | Tumor angiogenesis in most solid tumors | Fumagillin and TNP-470 (Takeda), ovalicin, $\alpha v\beta_3$ ligand, angiostatin and endostatin (EntreMed) | Preclinical–phase I–II |
| Vascular endothelial growth factor (VEGF)-dependent angiogenesis | Tumor angiogenesis in most solid tumors | ZD6474 (Zeneca), SU 5416 (Sugen) PTK787 (Novartis-Schering AG) | Phase I trials ongoing |

*(continues)*

Table 1 (*Continued*)

| *Target* | *Epidemiological findings in human cancer* | *Examples for drugs/drug candidates* | *Current state of development* |
|---|---|---|---|
| PDGF-R/VEGF-R-dependent angiogenesis | Tumor angiogenesis in most solid tumors | PD 166,285 (Parke-Davis), SU 6668 (Sugen) *(79,91,92)* | Preclinical–phase I |
| Steroid receptors | Acute promyelocytic leukemia liposarcoma, other cancers? | All-*trans*-retinoic acid, Vesanoid (Roche)<br>Troglitazone (PPARγ agonist, Parke-Davis), GW 1929 (Glaxo-Wellcome) | On the market<br>On the market/preclinical<br>Preclinical |
| Transcriptional regulation of endocrine pathways (estrogen-R, androgen-R) | Hormone-insensitive prostate and breast cancer | Distamycin-related compounds, minor groove-binding compounds, hairpin polyamide design (*86*) | Preclinical |
| Apoptotic pathways | 1) p53/hdm2 antagonists<br>2) Bcl-2/Bcl-xL antagonists<br>3) Histone deacetylase inhibitors<br>4) Proteolytic activity of the proteasome | (1) No compounds published<br>(2) G 3139 (antisense, Genta); epothilones (indirectly)<br>(3) Trapoxin A, Trichostatin A<br>(4) Lactacystin A, PS 341 (Proscript/Millenium) | (1) Preclinical<br>(2) Phase I/II<br>(3) Preclinical<br>(4) Phase I |
| P-glycoprotein-mediated multidrug resistance (MDR-1 and many others) *(48)* | Pre-existant oder drug treatment-induced | PSC833 (Novartis), VX-710 and VX-853 (Vertex), GF 120918 (Glaxo-Wellcome) | Phase I–II–III |
| Telomerase | Activated in up to 90% of all cancers | Antisense molecules, ribozymes *(93),* reverse transcriptase inhibitors (e.g., ddGTP, 7-deaza-dGTP, anthraquinones, prophyrin derivatives, and so on) *(87)* | Preclinical |

Information has been adapted, with permissions from refs. *76–94.*

Table 2

| *Modality* | *Advantages* | *Disadvantages* |
|---|---|---|
| Small molecules | Feasibility, ease of manufacturing, purity, stability, patentability | Cost of manufacturing sometimes very high, often insufficient selectivity resulting in nonmechanism-related side effects, risk of mutagenicity/teratogenicity, risk of metabolism-related lack of efficacy or delayed organ toxicity, risk of lack of bioavailabilty, difficulties in formulation |
| Endocrine agentss | As above, plus the fact that usually endocrine pathways are well worked-out, and hypotheses are easily testable; in general, endocrine surrogate markers available; high selectivitys | As above, but side effect profile mostly mechanism-related |
| Natural compounds *(77,80)* | Feasibility largely dominated by the availability of expression systems and/or natural sources that are easily accessible. | Total synthesis in most cases highly or extremely difficult (sometimes impossible) and very costly; otherwise, as above (small molecules) |
| Antisense | Exquisite target specificity, at least in theory no nonmechanism-related toxicity; clearcut surrogate markers for biochemical drug effects (mRNA, protein levels) | Cost of goods, difficulties in manufacturing; risk of nonmechanism-related side effects (complement activation, coagulation pathway, proinflammatory effects); parenteral application route, lack of clinically validated models of systemic efficacy of antisense molecules |
| Peptides | Relative ease of manufacturing; purity, stability as powder, ease of formulation, instability in the bloodstream can be circumvented by unnatural amino acids/cyclization, in general high selectivity, lack of mutagenicity/teratogenicity | Parenteral drugs; can be immunogenic/allergenic; instability in the blood stream needs to be dealt with; in general, do not cross cell membranes nor the blood–brain barrier; limited applicability |
| Cytokines | Production mostly relies on recombinant organisms; Exquisite selectivity at the level of cytokine receptors; however, pleiotropic effects in most cases according to biology of the cytokine in question | Parenteral drugs; in some cases, very serious toxicity (e.g., vascular leakage syndrome), metabolic instability in the bloodstream; patent situation often complex |
| Antibodies | Humanized antibodies now generally available; high selectivity through antigen-binding sites; lack of mutagenicity/teratogenicity, good pharmacokinetic profiles in the case of humanized antibodies; Purity/stability readily achievable | Manufacturing relatively complex, mostly in eukaryotic cells; cost of goods relatively high (on a per gram basis); patent situation often complex |

*(continues)*

Table 2 (*Continued*)

| *Modality* | *Advantages* | *Disadvantages* |
|---|---|---|
| Radioligands | Technology established; imaging technology available using alternative isotopes | Clinical efficacy still unproven; complex procedure; marked organ toxicity possible, whole body exposure to high irradiation levels |
| Vaccines | More and more tumor antigens characterized; relevant epitopes becoming available; highly efficient antigen presentation modes and adjuvants available; immunological characterization of immune response types well in hand | Clinical efficacy still unproven; lack of predictive animal models; mostly effects in preclinical models based on a prophylactic regimen; clinical trial plans Based most often on adjuvant setting, resulting in large trials with high risk of failure |
| Targeted toxins by ligands, antibodies, or viruses | In theory, absence of side effects because of targeting of toxic principles; manufacturing issues addressable by fusion gene approaches; in the case of viruses, topical/local approaches possible | So far, this approach is clinically not validated; sometimes surprising amount of toxicity caused by nonspecific trapping of complexes |
| Photodynamic therapy | In principle, exquisite selectivity attainable through selective accumulation of the photosensitizer in the tumor and the geometry of excitation at the tumor level; availability of a number of photosensitizers with favorable pharmacokinetic profile and adequate accumulation in the tumor; availability of relatively cheap and user-friendly solid state lasers; clearly demonstrated patient benefit, mostly in the palliative setting | Treatment limited to superficially positioned tumors in hollow organs (brain, necessitating craniotomy) and also limited, at least with currently available sensitizers and lasers, in penetration to a few millimeters. Treatment procedure surgical in nature. Phototoxicity with first generation photosensitizers. Treatment limited to (endoscopically) visible tumors |
| Gene therapy modalities | At the outset, based on the principle of a once-and-for all correction of genetic disorders, most attractive in cancer research because of the genetic character of the disease. In principle, feasibility established based on efficient integration of new genes by retroviruses and some other viruses | Clinically, this approach is not validated, despite extensive efforts in many top laboratories and clinical institutions. The major hurdles are: low rate of transduction, low rate of integration of desired genetic information, rapid clearance of newly introduced genetic material, immunogenicity of the vector, and manufacturing issues. Nevertheless, efforts continue at a feverish rate |

It is a fair assumption that the predictive quality of any test or assay increases with the level of complexity of the screen; thus, biochemical screens, although highly accurate and reproducible, are far removed from any relevant predictive value; complex screens in the intact (tumor-bearing) animal, although less accurate and more expensive, appear to mimick much more closely the clinical situation. To a great extent, however, the notion of highly predictive animal screens has been discredited, as witnessed by several decades of experimental research. This is true for pharmacokinetic, toxicological, and therapeutic efficacy parameters. The pitfalls of predicting pharmacokinetic and toxicological parameters in man, based on animal results, are well documented, and are not be discussed further.

Therapeutic efficacy screens merit close attention because of their experimental complexity, cost, and the pain inflicted on animals. Any cancer researcher in the field will know of instances in which animal screens have not been able to predict clinical outcome. Apart from the fact that negative experiences are always more disturbing and more easily retained in one's memory, there is a consensus that predicting efficacy of a novel anticancer agent for any given cancer type, in the absence of clinically validated predecessor compounds, cannot be attained with any degree of accuracy. The lack of tissue-type predictability of the efficacy of novel anticancer agents has been amply documented by the NCI, exploiting results obtained over several decades of research. It would be wrong, however, to ascribe no predictive quality whatsoever of animal screens for the clinical setting. There are many examples of screens that are almost perfectly predictive for clinical results. Such examples comprise, for instance, cytotoxic agents, such as tubulin stabilizers, antimetabolites, or alkylating agents, tested in xenograft models; endocrine agents, such as estrogen-lowering agents (aromatase inhibitors) tested in the carcinogen (DMBA)-induced rat mammary tumor; and photodynamic therapy, tested in subcutaneous murine tumors.

There are also situations in which negative or unconvincing test results should have prompted a negative decision regarding further development, and in which, only with the benefit of hindsight, the predictive quality of efficacy screens (with negative results) was finally borne out by clinical results. Such examples comprise, for instance, immunostimulants tested in murine metastatic tumor models. There is always a tendency, also, among experienced cancer researchers, to believe more easily positive results, and to disbelieve negative results, when these contradict a personal bias.

Robust assessment of the predictive quality is only possible when solid clinical treatment results become available, usually only after pivotal phase IIb/phase III trials. There are, however, warning signs that should be taken seriously in alerting preclinical researchers and clinicians to possible failure of a drug candidate. Such signs comprise a (very) narrow therapeutic window (<2) in rodent screens; small treatment effects, such as a decrease in tumor growth rate (in the absence of complete tumor stasis or regression); lack of reproducibility of treatment results; marked activity in special tumor models only; and use of demanding routes and schedules of application.

## 7. BUILDING A FLOW CHART FOR SCREENING ACTIVITIES

Historically, anticancer drug screening was a hit-or-miss activity, testing compounds more or less at random in some murine leukemia models. Generally speaking, this approach led to only marginal successes, and was replaced or complemented by much

broader panels of tumor cell lines. With the advent of concepts based on molecular genetics, it became possible to build target-oriented drug screens that were much more efficient in coping with a sizable number of compounds. This trend has continued to this day, and it is now commonplace to test $10^5$ to $5 \times 10^5$ compounds in early lead-finding screens, in a matter of a several weeks. Bottlenecks remain in complex animal screens, and every effort is therefore made to weed out inactive compounds in less demanding screens. The principle applied is a hierarchical succession of drug discovery screens, going stepwise from a biochemical level to a cellular level, and from there to animal screens. Each screen is then adapted to the planned throughput, and quantitative decision criteria govern the flow of compounds. The parameters that minimally need to be dealt with are:

- Potency
- Selectivity
- Cellular activity/selectivity (for intracellular targets)
- Pharmacokinetic parameters ($C_{max}$, $t_{1/2}$, area under the curve, oral bioavailability)
- Efficacy
- Toxicity
- Spectrum of activity
- Scheduling
- Combinations with partner compounds
- Resistance (preexistent resistant phenotype, P-glycoprotein induction, bcl-2 overexpression, tubulin isotypes/mutations, gene amplification).

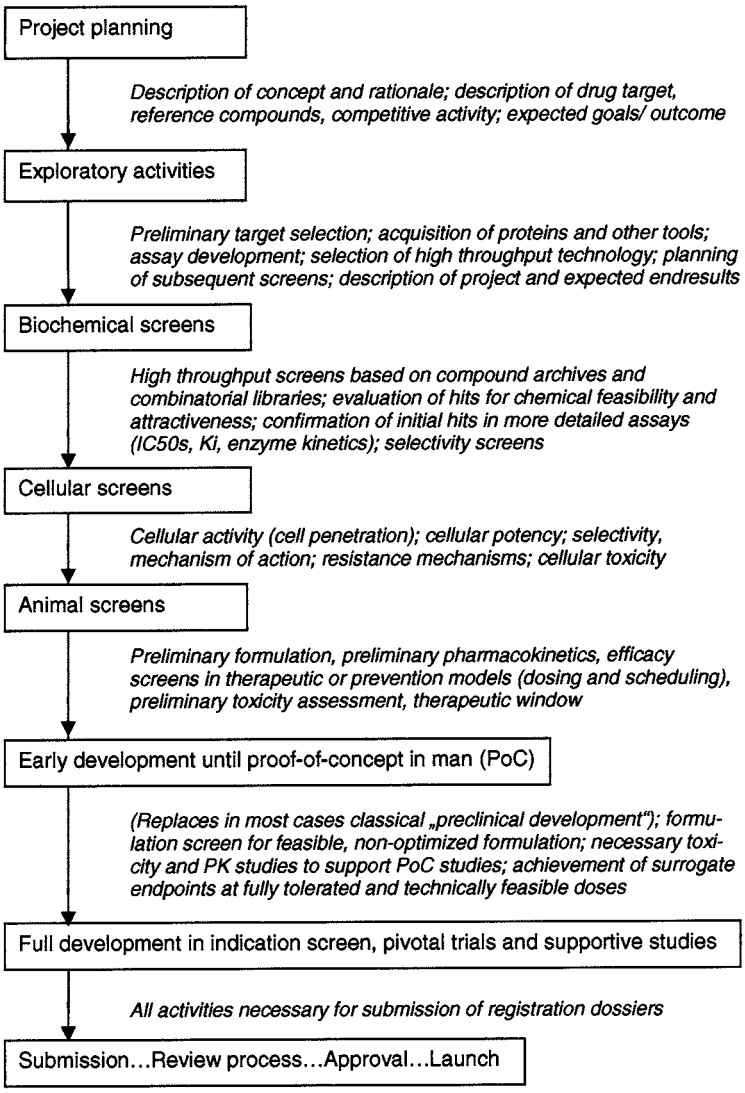
In a very simplified way, for chemistry-driven projects, such flow charts appear in Flow Chart 1.

For projects based on biologicals, such as cytokines, monoclonal antibodies, or gene therapy approaches, all high-throughput screens would be replaced by careful biological characterization of the biological entity in relevant cellular and animal systems. Usually, these projects are characterized by an early emphasis on safety and manufacturing issues, careful characterization of producer cell lines, and so on.

As mentioned above, the validation process of the drug target accompanies the entire process; early validation, even in the absence of any good leads or reference compounds, is sought by all means; a valid drug candidate is later used to characterize therapeutic efficacy at tolerated doses in animals. The next major hurdle is the PoC studies in man, and final validation is obtained, at least in the majority of the cases, at the time of submission.

The underlying principle throughout this process is early attrition, i.e. rejection of nonvalid drug targets and treatment modalities at the earliest possible time. In order to do this, the following points must be watched carefully at all times:

- Quality of screens (accuracy, reproducibility, predictive quality).
- Clearcut decision criteria with clearcut decision-making if success becomes improbable or the competitive situation becomes untenable.
- Efficiency of screening, as can and should be measured by parameters such as throughput, turnaround time, and cost.
- Creative and rapid responses to obstacles that arise during screening.
- Integrated approach to drug screening, applying state-of-the-art technologies at the right time.

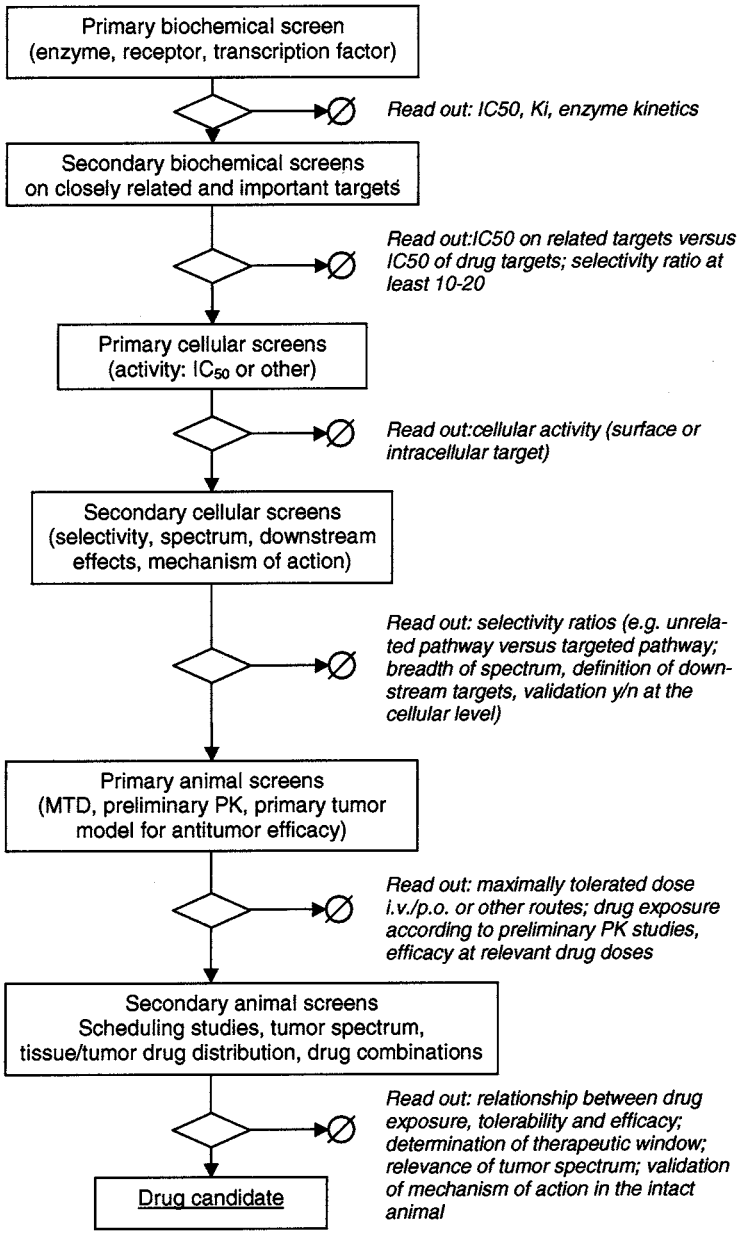



**Flow Chart 1** Activities/Measurements

- Adequate planning and preparation of downstream activities, in order to minimize interface problems.

A description of a more detailed flow chart, as used in actual drug screening efforts (again, in the context of a chemistry-driven project) is depicted as Flow Chart 2 (comprising only the research activities in a more narrow sense).

This drug candidate would then be ready for entry into early development activities until PoC in man. One activity, the development of surrogate marker technology, is missing on Flow Chart 2. This is an activity that is pursued at almost every level of the drug screen, and in which an imaginative approach is particularly important, both for early attrition rates and for the success of the PoC studies in man.




**Flow Chart 2** Preclinical Research Activities

## 8. KEY SUCCESS FACTORS FOR EARLY-STAGE DRUG DISCOVERY SCREENS

High-quality execution and effective bundling of the following technologies, putting aside the overall feasibility of a given approach, greatly determine the success rate of early research projects. A few key attributes should be mentioned.

- High-diversity compound archives, including natural compound libraries: size and diversity of the libraries.
- Cloning/expression/purification of recombinant proteins: immediate availability of a variety of expression systems, state-of-the-art protein chemistry.
- Efficient, high-throughput screens/ultrahigh-throughput screens: assay development by experts, in stages, to the point at which the assay can cope, at reasonable cost, with the desired throughput. Approximate benchmark: 300,000–500,000 single compounds should be screened, and hits confirmed in single-point assays within 2–3 mo; top performance is around 1 mo.
- Structural biology: a series of engineered expression vectors should be rapidly cloned, expressed, and purified proteins checked for propensity to crystallize, in a batch mode, in order to heighten chances of crystal formation; crystallography data are particularly (only?) useful at the time of lead optimization.
- Modeling and medicinal chemistry, including combinatorial chemistry: Although combinatorial chemistry is many times not productive in lead finding, it is most valuable in lead optimization. Creative ways of directed multiparallel synthesis greatly enhance the efficiency of the drug optimization process, provided that screens can cope with the number of compounds, and are sufficiently accurate and able to discriminate between very similar compounds.

## 9. KEY SUCCESS FACTORS FOR LATE-STAGE COMPOUND DEVELOPMENT

Pharmacological characterization of compounds is only possible if the following technologies are put in place: preliminary formulation, also for difficult-to-handle compounds; preliminary pharmacokinetics, measurements of drug exposure relating to drug action in target tissues; range and predictive quality of available in vivo models.

## 10. IMPORTANCE OF SURROGATE MARKERS

The availability of surrogate markers with high predictive quality can change entirely the risk profile of a development project, and therefore has eminent practical significance. Very determined efforts should be made to make these available, at least in a prototype assay format, at the time of the first clinical trials.

*Surrogate markers* can be divided into five categories:

- Mechanistic markers reflecting the mechanism of action of the candidate drug, e.g., EGF receptor downregulation/decrease in autophosphorylation after administration of an EGF receptor tyrosine kinase inhibitor, or decrease of relevant mRNA species in target tissue after administration of antisense compounds.
- Downstream biochemical/endocrine markers of drug action, e.g., decrease of estrogen levels in the bloodstream after administration of aromatase inhibitors, or induction of S-adenosyl methionine decarboxylase (SAMDC) in the target tissue after administration of a SAMDC-inhibitor.
- Noninvasive methods (*see* subheading 2.12.) measuring anatomical parameters (e.g., tumor size) or functional markers, such as tumor tissue blood flow or vascular permeability of the tumor blood vessels using PMRI techniques.
- Tumor markers for early signs of efficacy; examples include CA125 in ovarian cancer or Prostate specific antigen in prostate cancer.

- Toxicity markers identifying target organs and signaling tolerability problems; the most frequently occurring examples are depression of white blood cells as a consequence of bone marrow depression, liver (ALAT, ASAT) and kidney (creatinine clearance) biochemical markers, neurological markers (gait, spasms, convulsions, and so on).

## 11. TEAM MANAGEMENT IN DRUG DISCOVERY PROCESS

One of the most challenging issues is the management of a multidisciplinary process that is in itself dynamic, i.e., in which the partners are constantly changing, and the nature of the required expertise and the approaches to problem-solving are distinct for each phase of R&D. The challenges occur at different levels. There are conceptual difficulties, particularly for highly innovative projects with a speculative aspect; there are the technological hurdles, almost always including informatics as a key component; personal as well as organizational aspects; and last, the financial implications. In many cases, a geographic component complicates interactions between team members (remote teams).

Given the complexity of the tasks, it is evident that only teams of experts are able to cope with these effectively. These teams must be able to speak a common language, despite the heterogeneity of expertise and the frequently occurring geographical hurdle. This means that teams in different parts of the world need to interact efficiently; in many cases, this also means that language barriers must be overcome, which, for many technical people, can be high. The coordination of these activities is so demanding that most organizations have chosen to entrust professional project managers with the coordination of all the development activities. It is also evident that only organizations with up-to-date IT environments can remain competitive.

One major hurdle is the fact that such teams are subject to two contradictory forces: on the one hand, these teams require a certain stability over time, and team members cannot be changed too frequently without incurring delays; on the other hand, the type of work that is required is constantly changing over the normal research and development time. In these cases, it is highly profitable to minimize interfaces by establishing a sort of fluidity of the team, which means that at least the core team is never changing in its entirety, but gradually evolves over time. Such a process can be graphically depicted as in Fig. 3.

## 12. CONCLUSIONS

Drug discovery has become an exceedingly complex endeavor. The eruption of revolutionary technologies, mostly linked in some way with miniaturization, robotization, and major IT components, is posing difficult problems in terms of adequate choice of technologies for the target at hand, cost, and overall management of resources. The choice of the drug target in terms of relevance, validity, and feasibility remains tricky, despite the availability of molecular epidemiological data for most important tumor types. Nevertheless, problems can now be tackled that were unassailable just a few years ago. The speed of HTS, the complex chemical syntheses, and the sophisticated molecular screens, coupled with large databases, are examples of tremendous technical progress. On the other hand, it is worthwhile to remember that many problems are still unyielding, even to the most dedicated groups of scientists. Crystallography has many times hit high hurdles, as have efforts to produce high-affinity leads for protein–protein interaction drug targets. These and many other hurdles still exist, but the pressure

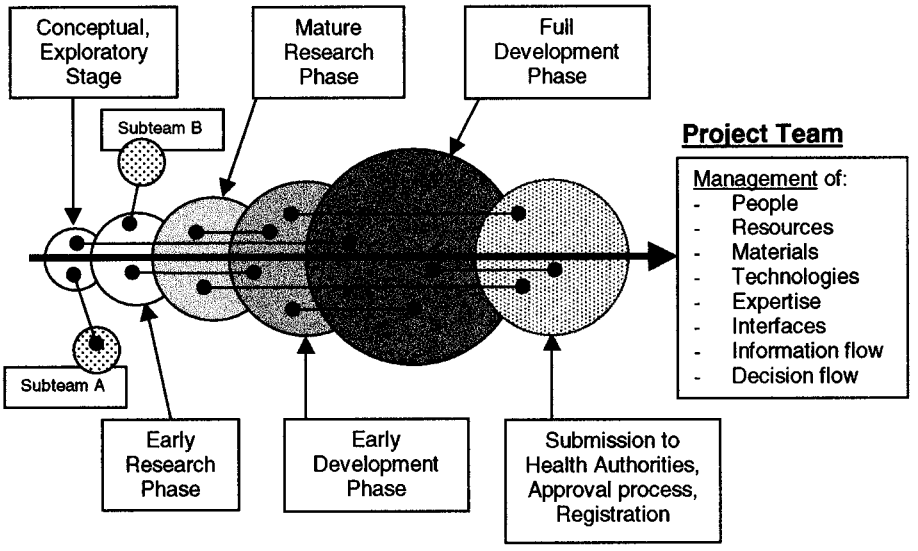



**Fig. 3.** Project team organization over time. The overlapping circles represent the size of the project team, with mostly changing team members, but some fairly constant ones (such as, e.g., a research or a clinical pharmacology representative, symbolized by a line with a bullet at either end); subteams with specialized missions (e.g., HTS, crystallography, fermentation, and so on) complement the main team. Most bigger teams, particularly in development, work as a collection of subteams.

toward increased productivity steadily increases, and with it the urgency to find technical solutions for ever-more-difficult problems.

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# II Tumor Suppressor Pathways

# 6 Retinoblastoma Protein in Growth Control and Differentiation

*Lilia Stepanova, PhD and J. Wade Harper, PhD*

***Contents***



## 1. INTRODUCTION

Proper development of multicellular organisms from a single cell requires precise control of cellular proliferation, differentiation, and apoptosis. This multistep control involves a complex interplay between numerous genes regulating spatial and temporal aspects of development and proliferation. Defects in these control mechanisms can lead to developmental defects or tumor formation. Research performed over the past decade has brought us closer to an understanding of how a family of critical growth regulators, typified by the retinoblastoma (RB) tumor suppressor gene *(Rb),* function to control cell proliferation and differentiation.

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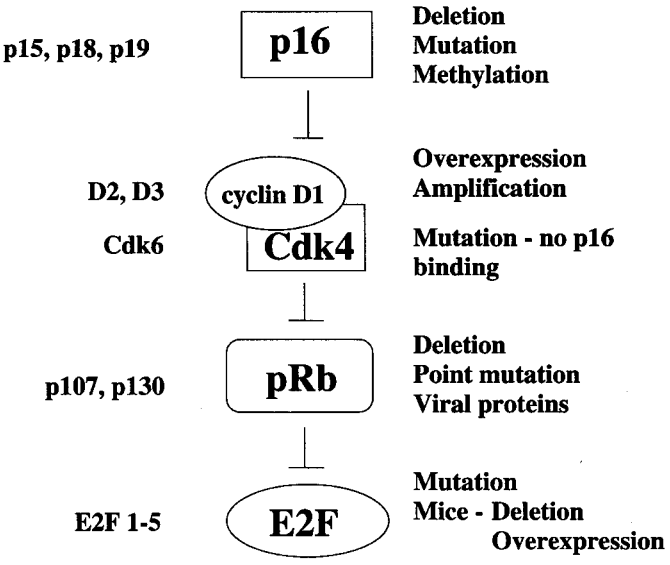


**Fig. 1.** The *Rb* signaling pathway. Protein components of the Rb pathway are shown alongside their homologs on the left. The types of mutations found in human tumors are indicated on the right. In mice, both deletion and overexpression of E2F-1 were shown to be oncogenic. In humans, frequent mutations of E2F-4 were found in gastrointestinal tumors *(230,231).*

The *Rb* gene was cloned in 1988 as a gene that, when mutant, predisposes individuals to childhood RB *(1,2).* Subsequent studies revealed that *Rb* is frequently mutated in various types of human cancers, including small cell lung carcinomas; osteosarcomas; sarcomas; breast, bladder, and prostate carcinomas; myelomas, and leukemias *(1–11).* Initial insight into the function of Rb protein came with the observation that three unrelated DNA tumor viruses had evolved a similar strategy to transform cells by binding a set of cellular proteins, including Rb *(12–15).* The central role Rb plays in the transformation by these viruses became apparent after the observation that mutations in these viral proteins, rendering them unable to bind Rb, eliminate their transforming ability *(15–20).* Since then, significant progress has been made in understanding Rb function, its upstream regulators, and downstream targets. Through analysis of *Rb*-deficient mice, as well as mice deficient in the *Rb* homologs *p107* and *p130,* the complex role played by the *Rb* family in coordinating proliferative control with the processes of differentiation and apoptosis during development is beginning to be understood. Moreover, it is now known that *Rb* is a component of a G1 signaling pathway (Fig. 1), and mutations in a number of components of this pathway have been linked to transformation. It is clear that Rb functions primarily through its ability to bind and modulate the actions of various transcription factors (TF). Negative control of transcription by *Rb* may take several forms, including passive masking of transactivation domains *(23),* active inhibition of surrounding enhancer elements *(24–29),* or interference with transcription complex assembly *(30).* Interactions with Rb are not always inhibitory, however, and a number of TFs are activated by Rb, although the mechanisms and biological significance of such interactions remain obscure. This chapter discusses recent

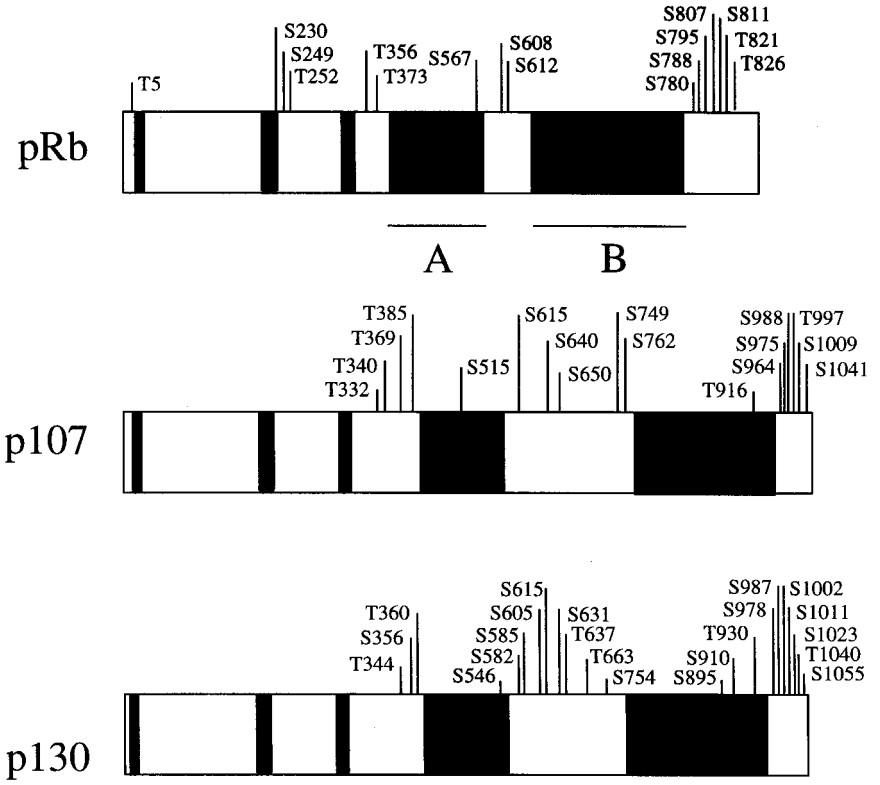


**Fig. 2.** Structural domains in pocket proteins. Conserved regions are shown as black boxes. Highly conserved A and B subdomains of A/B pocket domain are underlined. Consensus CDK phosphorylation sites are shown for each protein. Single letters refer to serine (S) and threonine (T) residues; numbers refer to the position of the amino acid in the protein.

advances in understanding how Rb controls cell cycle progression, differentiation, development, and apoptosis.

## *2. The Pocket Protein Family*

In mammals, the *Rb* family (Fig. 2) contains three members, *Rb, p107,* and *p130,* with *p107* and *p130* being more closely related, based on sequence *(31–32).* These proteins are frequently referred to as "pocket proteins," because they all share a structural element called the A/B pocket, which serves as a protein–protein interaction domain. A number of proteins have been identified that can associated with the A/B pocket of all three family members, including DNA tumor virus oncoproteins, such as E1A and T-antigen (Table 1). The interaction with the A/B pocket is typically mediated by an LXCXE motif *(74)* located in the target protein. The structure of the LXCXE peptide from human papilloma virus E7 with the *Rb* pocket has recently been solved *(75),* which provides a model for how cellular proteins may interact with the A/B pocket. In addition, all three family members bind to members of the E2F family of TFs through sequences overlapping the A/B pocket and the C-terminus *(33,76–82).* The C pocket (located at the C-terminus of *Rb*) contains sequences that

Table 1
Viral and Cellular *Rb*-associated Proteins

| *Protein* | *Function* | *Ref* |
|---|---|---|
| E2F | Transcription factor | *(23,26,33)* |
| SV40 large T-antigen | Simian virsus protein | *(12,16,17)* |
| E1A | Adenoviral protein | *(13,16,18)* |
| HPV E7 | Human papilloma virus protein | *(14)* |
| BRLF1 | Epstein-Barr virus protein | *(34)* |
| D1-3 | Cyclins | *(35)* |
| MyoD | Muscle transcription factor | *(36)* |
| Myogenin | Muscle transcription factor | *(36)* |
| Mdm2 | Oncoprotein | *(37)* |
| ATF-2 | Transcription factor | *(38)* |
| Elf-1 | Lymphoid transcription factor | *(39)* |
| HBP1 | Transcriptional repressor | *(40)* |
| AP-1 | Transcription factor | *(41)* |
| c-Jun | AP-1 family transcription factor | *(42)* |
| SP-1 | Transcription factor | *(43)* |
| E4TF1 | Transcription factor | *(44)* |
| AP-2 | Transcription factor | *(45,46)* |
| Id-2 | Transcription factor | *(47)* |
| UBF | Transcription factor (pol I) | *(30,48)* |
| TFIIIB | Transcriptional regulator (pol III) | *(49)* |
| TAF(II)250 | TFIID subunit (pol II regulation) | *(50,51)* |
| HDAC1 | Chromatin remodeling histone deacetylase | *(52–54)* |
| TFIID | TATA-box binding protein | *(55)* |
| c-Ab1 | Tyrosine kinase | *(56)* |
| Raf-1 | Serine/threonine kinase | *(57)* |
| RbAp46 | Component of mSin3 co-repressor complex | *(58)* |
| RbAp48 | RbAp46-related protein | *(59,60)* |
| Trip230 | Thyroid receptor co-activator | *(61)* |
| RIZ1 | Zinc-finger protein | *(62,63)* |
| BOG | LXCXE-protein | *(64)* |
| RIM | Leucine zipper protein with LECEE motif | *(65)* |
| CDC25A | Protein phosphatase type 1 catalytic subunit | *(66)* |
| PU.1 | Lymphoid transcription factor | *(55)* |
| c- and N-Myc | Transcription factor | *(67,68)* |
| HBRM | Disruption of nucleosome structure | *(69)* |
| Lamin A, C | Nuclear matrix protein | *(70,71)* |
| Hsc73 | Heat shock protein | *(72)* |
| hBRG1 | Transcriptional activator | *(73)* |

can interact with c-Abl *(83,84).* In addition to these interactions, which are relatively well understood, a large number of other *Rb*-binding proteins have been found (Table 1), although, in many cases, the biological relevance of these proteins to growth control has not been established. A feature that distinguishes *Rb* from *p107/p130* is that the latter contain a large spacer sequence between the A and B pockets, which allows

*p107* and *p130* to form stable complexes with cyclin-dependent kinases (CDKs) *(85–88).* Such tight interactions are not observed with *Rb,* although, as described below, *Rb* does contain cyclin-targeting sequences as well. Recently, genes encoding *Rb* homologs in *Caenorhabditis elegans* and *Drosophila* have been identified *(89,90).* These genes appear to be hybrids between the p107/p130 proteins and *Rb,* and, in *C. elegans,* there appears to be only a single *Rb* homolog, suggesting that there may be functional as well as structural overlap. The identification of *Rb* homologs and mutants of these genes, in genetically tractable organisms such as these, holds promise for defining higher-order signaling pathways that regulate development and proliferation.

The major growth-inhibitory activity of *Rb* is exerted during the G1 phase of the cell cycle, and this function is thought to reflect the interaction of *Rb* with one or more proteins. In its growth-inhibitory form, *Rb* binds to cellular proteins that normally function to promote cell division. This inhibitory function is then reversed in late G1, upon phosphorylation of *Rb* by cell-cycle-regulated protein kinases, the CDKs. Phosphorylation releases particular associated proteins from *Rb,* thereby reversing *Rb*-mediated inhibition of cell proliferation. However, currently it is not understood what *Rb* targets are most important for inhibition of proliferation, and whether there are particular phosphorylation events that regulate particular aspects of *Rb* function.

## 3. E2F TFS AS POCKET PROTEIN TARGETS

By far the best-understood targets of pocket proteins are the E2F family of TFs (Fig. 3). A detailed review of *Rb*–E2F interactions is beyond the scope of this review, and this topic has recently been covered in detail *(91).* E2F TFs coordinate the transcriptional program required for cell cycle progression, and are linked to the basic cell cycle machinery through pocket proteins *(23–26,92–96).* E2Fs contain a transcriptional activation domain, but associate with E2 sites in promoters through the DNA-binding proteins, DP-1 and DP-2. Five E2F TFs are known in mammalian cells, but it is not clear how these individual proteins may differentially regulate transcription of the more than two dozen E2F-dependent genes discovered so far. Pocket proteins associate with E2F complexes in a cell-cycle-regulated manner. Early models suggested that pocket proteins bind to E2F, and block access of E2Fs activation domain to relevant TFs. Although this may still be true in some instances, it is now known that *Rb* can function as a general transcriptional repressor that is brought to particular promoters through association with E2F (Fig. 4). *Rb*–E2F complexes are found mostly in the G1 phase of the cycle; p107–E2F complexes are found mostly in S phase, and p130–E2F complexes are prominent in G0 *(79,87,97–102).* Precisely how different pocket proteins regulate distinct E2F complexes is not well understood. Rb associates most avidly with E2F-1, E2F-2, E2F-3, and, to a lesser extent, with E2F-4; p107 preferentially binds E2F-4, and p130 can be detected in complexes with E2F-4 and E2F-5 *(76–78,103–107).* The available data indicate that removal of *Rb* or p107–p130 leads to derepression of different sets of genes *(106,108–110),* suggesting some level of specificity for repression of particular genes by pocket proteins. In addition to being regulated by pocket proteins, E2F1–3 are also regulated by association with and phosphorylation by CDKs *(111–113).* Unlike E2F1-5, E2F-6 does not contain transcriptional activation activity, but can associate with DPs in a process that represses transcription *(114–117).*

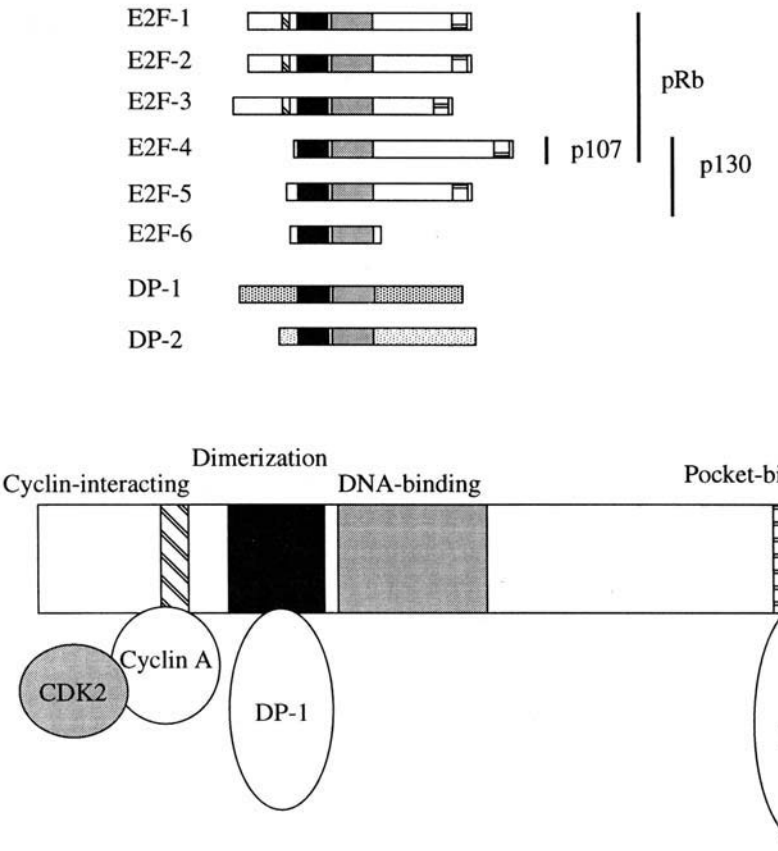


**Fig. 3.** Schematic diagram showing the structure of E2F and DP families members. E2F-6 does not have transcription activation domain, and is unable to bind pocket proteins. Domain structure of E2F-1 protein and its interacting proteins is depicted in more detail.

## 4. GROWTH CONTROL BY *RB* THROUGH REPRESSION OF POL I- AND POL III-MEDIATED TRANSCRIPTION

Genes regulated by E2F are transcribed by RNA polymerase II. Recent data also implicate Rb in the regulation of pol I- and pol III-mediated transcription (Fig. 5), which widens the potential roles of Rb in cellular function *(48,118–121).* pol I is responsible for transcription of rRNAs, and pol III transcribes small nuclear RNA, small rRNA, and transfer tRNA. *Rb* associates with the dimeric pol I TF, UBF, thereby blocking its binding to DNA. In vivo, the extent of the UBF–*Rb* interaction changes in different cellular states, such as the process of cell cycle exit, suggesting that this function is regulated in some way *(30,48).* Rb is also able to inhibit transcription of multiple pol III templates *(118–121,48).* The level of pol III transcription in *Rb–/–* primary fibroblasts was 4–5-× higher than in *Rb+/+* fibroblasts, providing genetic evidence of a general role for *Rb* in pol III transcription. *Rb* physically associates with TFIIIB *(121–148),* a component of the pol III complex, although it remains to be determined whether this interaction underlies inhibition of pol III by *Rb.* TFIIIB is responsible for

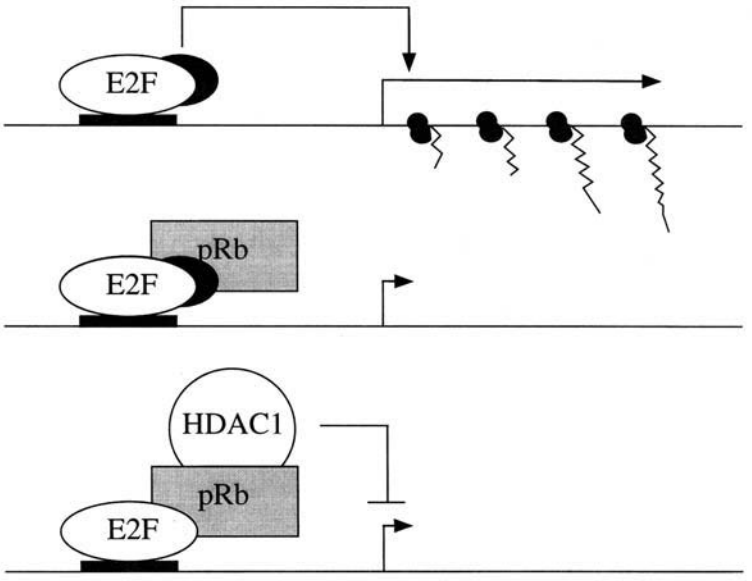


**Fig. 4.** Regulation of E2F-dependent promoters by *Rb.* **(A)** E2F activates transcription through its transcriptionactivating domain, indicated as a black domain on E2F. **(B)** *Rb* as a specific inhibitor of E2F transcription. Interaction of *Rb* with E2F blocks access of the activation domain with the transcriptional machinery, thereby blocking activation of target genes. **(C)** *Rb* as a transcriptional repressor that associates with a histone deacetylase, and which is brought to specific promoters through association with E2F. Recruitment of deacetylases to promoters by *Rb* may repress transcription in an E2F-dependent manner.

directing pol III to transcriptional start sites *(122).* The activation of TFIIIB during the cell cycle is strikingly similar to that of E2F; TFIIIB is inactive during most of the G1, and its activity increases as cells progress into S phase. Both pol 1 and pol III activities are repressed in quiescent cells, and increase in parallel upon serum stimulation *(118,123,124).*

The regulation of pol I and pol III transcription by Rb provides an interesting link between Rb and biosynthetic activity of the cell. By regulating the abundance of rRNA and tRNA, Rb may limit the rate of cell growth and protein accumulation *(30,48,49,125).* Deregulation of this control may contribute to tumor development by supplying sufficient quantities of ribosomes and tRNAs for unrestrained cell growth. Indeed, some naturally occurring mutations in Rb affect pol I and pol III regulation, which correlates with loss of growth control.

## 5. MECHANISMS OF TRANSCRIPTIONAL INHIBITION BY RB

Recent data suggests that *Rb* may repress transcription at least in part by remodeling chromatin. *Rb* interacts with a histone deacetylase (HDAC1), and, when associated with E2F, *Rb* recruits HDAC1 to DNA (Fig. 4). The interaction of HDAC1 with Rb requires an intact pocket domain in Rb, and naturally occurring *Rb* mutations reduce this interaction *(52,54).* A role for HDAC1 in Rb-mediated repression through E2F was demonstrated by experiments using specific inhibitor of HDAC1, trichostatin A (TSA).

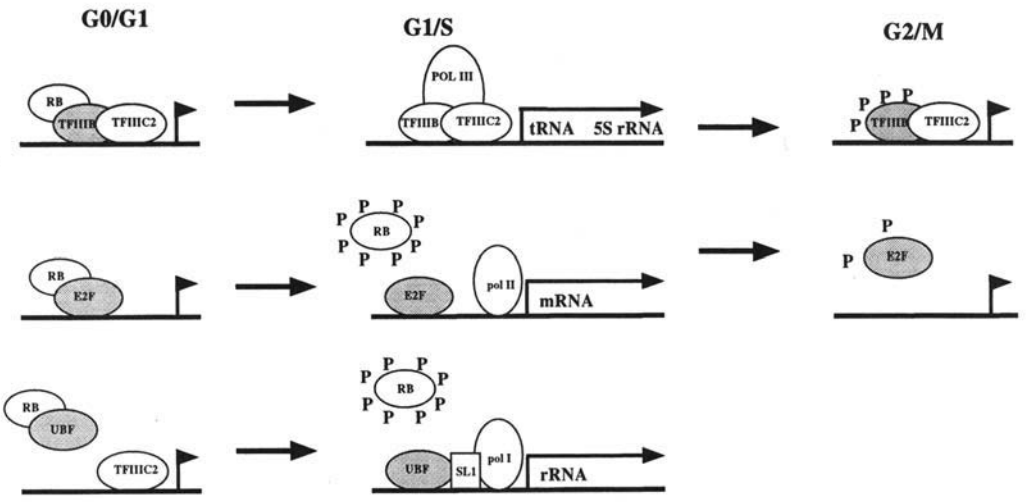


**Fig. 5.** Regulation of pol I, II, and III by Rb. *Rb* can regulate transcription by all three classes of polymerases. *Rb* represses pol I by preventing the general factor, UBF, from binding the complex. *Rb* represses pol II transcription through gene-specific TFs, such as E2F. *Rb* may repress pol III transcription through the general factor, TFIIB.

After treatment with TSA, *Rb*-mediated repression of an E2F-regulated promoter in a chromosomal context was abrogated *(52).* Transected DNA templates, whose chromatin structure may not precisely mimic chromosomal chromatin, are also responsive to the TSA treatment, raising a possibility that HDAC1 may target other nonhistone proteins important for transcriptional regulation *(52–54).* p107 and p130 were recently shown to interact in a similar manner with HDAC1 in repressing E2F4-mediated transcription *(126).* Moreover, recent studies *(84)* indicate that E1A not only inactivates Rb-mediated recruitment of histone deacetylase, but also binds and activates CBP, increasing its histone acetyltransferase activity. Cumulatively, these two processes would be expected to significantly open chromatin structure, promoting gene expression *(128–132).*

## 6. INVOLVEMENT OF RAS PATHWAY IN CDK ACTIVATION: LINKING INACTIVATION OF *RB* WITH EXTRACELLULAR SIGNALS

During the G1 phase of the cell cycle, cells integrate the various growth-stimulatory and -inhibitory signals in their environment, and make the decision to enter the division cycle or to exit the cell cycle. *Rb* appears to function both as an inhibitory barrier to S phase, which must be overcome through *Rb* inactivation to allow for S phase, and as a positive regulator of events which commit cells to a terminal differentiation pathway. A primary determinant of whether a cell will enter S phase appears to be the activity status of CDKs. These enzymes, composed of a catalytic CDK subunit and a regulatory cyclin subunit, control cell cycle transitions, and are the ultimate recipients of information intended to control proliferation. Two major classes of cyclins, D-type and E-type cyclins, have been implicated in the control of the G1–S transition, and in *Rb* inactivation *(133–141).* D-type cyclins (D1, D2, and D3) serve as specific activators of CDK4

and CDK6; cyclin E activates CDK2. Because of the critical roles played by these two kinases in bringing about S phase, elaborate and diverse regulatory pathways have evolved to control the extent and timing of their activity. Ultimately, the activity status of CDKs is controled primarily by two parameters: the level of relevant cyclins and the levels of CDK inhibitors. Two classes of CDK inhibitors are used to control the G1–S transition. The $p16^{INK4}$ class (p16, p15, p18, p19) specifically inhibits kinases activated by D-type cyclins; the $p21^{CIP1}$ class (p21, p27, and p57) inhibit D-, E-, and A-type cyclin–CDK complexes. During the past few years, some inroads have been made in elucidating the hardware linking receptor tyrosine kinases with both the transcriptional program controlling cyclin expression and mechanisms that control the levels of CDK inhibitors. Activation of receptor tyrosine kinases lead to activation of the Ras/Raf(MEK1) mitogen-activated protein kinase pathway, which in turn leads to induction of cyclin D1 transcription *(142,143).* Assembly of cyclin D1 with CDK4 is also regulated by mitogens in fibroblasts.

As described later in more detail, CDK4–cyclin D complexes are intimately involved in phosphorylation and inactivation of the negative growth control function of *Rb.* In keeping with this idea, microinjection of anti-Ras antibodies, or expression of dominant interfering alleles of Ras, block cyclin D1 accumulation, and cause cell cycle arrest in *Rb*-positive, but not *Rb*-negative, cells *(144,145).* Moreover, expression of a constitutively active form of MEK1 induces cyclin D1 expression and assembly of active CDK4–cyclin D1 complexes *(146,147).* Accumulation of cyclin D1–CDK4 complexes during G1 may have multiple consequences, depending on the status of CDK inhibitors, including *Rb* phosphorylation and titration of p27 to release active cyclin E–CDK2 complexes. In mitogen-deprived fibroblasts, p27 accumulates, and is thought to serve as a barrier to S-phase entry by blocking CDK-mediated *Rb* phosphorylation *(148–152),* and one of the functions of mitogens is to facilitate destruction of p27 through a proteasome-dependent mechanism *(153).* Cells engineered to overexpress cyclin D and CDK4 do not allow for S-phase entry in the absence of mitogens, apparently because of failure to assemble active kinase complexes. However, when these cells also express a constitutively active MEK1, still in the absence of mitogens, active cyclin D–CDK4 complexes are assembled and sequester p27, thereby releasing active CDK2/cyclin E complexes that further promote S-phase entry *(146,147).* Biochemical studies suggest that, although p27 binds tightly to both cyclin D and cyclin E-dependent kinases, it is an effective inhibitor of only CDK2. Thus, accumulation of cyclin D–CDK4 complexes may play two roles in transit through G1 in fibroblast: *Rb* phosphorylation, and titration of p27 to activate cyclin E–CDK2. How pervasive this mechanism is in various cell types, and whether sequestration of p27 plays a role in progression from mitosis to S phase, a setting where p27 levels are generally low, remains to be determined. In addition to MEK1, Raf-1 has also been shown to overcome *Rb*-mediated growth arrest. Whether this represents a direct interaction of Raf-1 with *Rb,* as suggested by this paper *(57),* or downstream effects of activation of cyclin D1 transcription and cyclin D–CDK4 assembly, remains to be determined.

## 7. *RB* PHOSPHORYLATION AND RELIEF OF GROWTH SUPPRESSION

*Rb* contains 16 possible CDK phosphorylation sites (Fig. 2), and undergoes cell-cycle-dependent phosphorylation. It is clear that cyclin D–CDK4 complexes are cen-

tral to *Rb* inactivation *(35).* There are several pieces of data that contribute to this idea, including the fact that overexpression of p16 can block *Rb*-positive, but not *Rb*-negative, cells in G1 *(137,139,154–157).* Because p16 is a specific inhibitor of CDK4/6 kinases, this data indicates that their ability to phosphorylate *Rb* is critical for cell cycle progression. In addition, cyclin D–CDK4 can efficiently phosphorylate *Rb* in vitro, and this phosphorylated *Rb* is unable to arrest *Rb*-negative cells in G1, upon microinjection *(96).* The ability of *Rb* to be inactivated by cyclin D–CDK4 in vitro requires phosphorylation of S795, a residue that is preferentially phosphorylated by CDK4–cyclin D in vitro. It is very likely that cyclin D–CDK4 is not sufficient to inactivate *Rb,* and, indeed, several studies suggest that cyclin E–CDK2 functions in combination with cyclin D–CDK4 to regulate *Rb (158–160).* It has also been suggested that there is a temporal dependence in *Rb* phosphorylation, although this may reflect the timing of action of the two kinases, rather than a strict biochemical requirement. CDK2 and CDK4 display overlapping but distinct specificities toward *Rb,* and it is possible that different phosphorylation events are cumulatively responsible for *Rb* inactivation *(167–169).* Recent studies indicate that some of the specificity in pocket protein phosphorylation results from interactions of cyclin–CDK complexes with substrate recognition motifs in pocket proteins. p107 and p130 associate tightly with cyclin A–CDK2 complexes through an RXL motif *(85–88),* a short sequence that is also found in the CIP/KIP family of CDK inhibitors. Tight association makes p107 and p130 obligate CDK substrates, and is required for efficient phosphorylation. In *Rb,* there are several RXL motifs located in the C-terminus of the protein *(165),* and one of these contributes significantly to the ability of cyclin A–CDK2 and cyclin D–CDK4 to phosphorylate *Rb* in the C-terminal region containing a cluster of phosphorylation sites. In contrast with p107, however, cyclins A and E do not bind tightly to Rb. In addition, cyclin D may interact with *Rb* through an N-terminal LXCXE motif in cyclin D *(137,138).* However, this sequence is not required for *Rb* phosphorylation in vitro, or for cyclin D to overcome the growth suppressive effects of *Rb* in tissue culture cells.

Although cyclin E–CDK2 complexes probably contribute to *Rb* inactivation, they also have substrates other than *Rb.* This is based on the fact that cyclin E expression can bypass growth suppression by a nonphosphorylatable Rb protein, and can do so without activating E2F *(166–169).* This activity probably reflects the ability of cyclin E–CDK2 to phosphorylate proteins whose activity is rate-limiting for S-phase entry. Although cyclin E can activate S-phase entry in this way, existing data indicate that this ectopic S phase is incomplete, and cells do not enter mitosis.

## 8. POCKET PROTEIN FUNCTION REVEALED THROUGH KNOCKOUT MICE

### *8.1. Rb Deficiency*

Knockout mice and fibroblast cell strains for all three known members of the family have been used extensively to analyze pocket protein function in vivo and in vitro. Mice lacking *Rb* die by embryonic day 16, and display multiple defects *(170–172).* These defects are seen, starting at d 11.5, as swelling near the fourth ventricle, and poor blood distribution. Extensive p53-dependent and -independent cell death occurs throughout the central nervous system (CNS), but no defects were observed in peripheral nervous system development. The likely cause of apoptosis in *Rb–/–* embryos is

inappropriate cell divisions in the CNS *(173).* In mutant mice, numerous dividing cells were observed in the nervous system, ocular lens, and liver, where wild-type (wt) embryos contain mostly differentiating and migrating cells. In the CNS of *Rb–/–* embryos, increased levels of cyclin E and free E2F were observed. Red blood cells (RBCs) of mutant embryos often have aberrant morphology, and peripheral red blood cells retain their nuclei, but peripheral blood of the wt embryos contain mostly enucleated erythrocytes *(170–172).* Experiments with chimeric mice pinpointed the defect in RBC maturation in *Rb–/–* embryos to a defect in hepatic stromal cells, and not in erythrocytes themselves *(174–176).* Because *Rb–/–* mice are inviable, analysis of transformation because of *Rb* loss has been analyzed in *Rb+/–* mice. *Rb* loss is associated with development of RB in humans, and, because of this, it was anticipated that mice deficient in *Rb* would also show retinal phenotypes. However, *Rb+/–* mice developed pituitary (PIT) tumors, and not RBs, indicating that mice and humans have distinct dependencies for particular tumor suppressor genes in specific tissues. Pituitary transformation was associated with loss of the wt *Rb* allele *(170–172,177–178).*

A major question has been to what extent the functions of pocket proteins overlap. This has been investigated in vivo by crossing *Rb–/–* mice with mice deficient for p107 *(179).* Where *Rb–/–* embryos die at 13.5–15.5 dpc, p107*–/–Rb–/–* embryos die at 10.5–12.5 dpc, and death is accompanied by increased rates of apoptosis in the CNS and liver. Thus, in the absence of *Rb,* p107 is required for some aspect of prenatal development. The elimination of p107 in Rb+/– mice leads to severe growth retardation and increased mortality, but these animals did not show any additional tumor phenotypes. However, these mice did develop focal lesions of the retina, with retinal displasia and elimination of the photoreceptor layer *(179,180).* The stochastic appearance of these lesions suggests that additional somatic events, possibly including loss of the existing wt *Rb* allele, may be responsible for this weak phenotype. The enhanced phenotype observed in *Rb*/p107 knockouts may reflect shared functions in certain tissues, or functional compensation by p107 in the absence of the Rb function.

### *8.2. p107 and p130 Deficiency*

p107 knockout mice have been created in two genetic backgrounds with different outcomes. p107 knockout mice, constructed on an 129/Sv genetic background, are fertile and viable, and do not show increased predisposition to tumor development. p107–/– embryos display subtle thickening of the long bones, compared with the wt embryos, implicating p107 in bone development. However, fibroblasts derived from p107–/– embryos did not reveal any significant defect in cell cycle regulation. These observations indicate the absence of a unique obligatory function for p107 in the embryonic development and cell cycle regulation in the embryonic fibroblasts *(179),* at least in this strain background.

In the alternative p107 knockout mice model, strain-specific effects were observed *(181).* Mice deficient for p107 on the Balb/cJ background were viable and fertile as adults, and embryos as well as newborn pups were indistinguishable from the wt and heterozygous littermates. In contrast with the situation in the 129/Sv background, pups in the Balb/cJ background display a severe postnatal growth deficiency, in which animals were approx 50% normal size at 3 wk of age. Postnatal development was not delayed, and animals could be weaned, and reached sexual maturity at normal times. As such, the basis of the growth rate decrease remains undefined. The immune

response of mutant mice was compromised, with frequent development of a diathetic myeloproliferative disorder *(181)*.

Embryonic fibroblasts derived from these p107–/– Balb/cJ mice displayed a markedly increased growth rate, with the doubling time of mutant fibroblasts about one-half that of wt. The rate of progression through all cell cycle phases seemed to be enhanced to similar extents, since there was no obvious change in cell cycle parameters in these strains. The increased rate of cell cycle progression may reflect in part the fact that cyclin E is derepressed in these p107 mutant strains, in contrast to the situation found with the 129/Sv background. The basis of strain-specific effects are still not understood, but it appears that there are modifier genes that can compensate for p107 loss in particular background *(179,181)*.

p130–/– mice constructed on 129/Sv genetic background are viable, and are without overt phenotypes *(182)*. Which was surprising, given that p130 is a major component of the E2F complex in G0 *(79,99,107)*. Analysis of the E2F complexes in serum-starved p130–/– cells revealed that, although the population of the free E2F increased, the majority of E2F was now complexed with the p107, instead of p130. Thus, in this instance, p107 can functionally compensate for p130 deficiency. When p130 deficiency was generated on a Balb/cJ background, homozygous-null animals were inviable *(183)*. Embryonic death was observed at 11–13 dpc, and animals at this stage were 75% smaller than heterozygous littermates. Mutant hearts displayed an abnormal morphology caused by dilation, and resembled hearts of earlier-stage normal embryos with two chambers. At 10.5 dpc, mutant embryos fail to form and extend hind limb buds. Histological examination of mutant mice revealed defects in neurogenesis and myogenesis, a poorly formed notochord, and abnormal cardiac morphology. The levels of apoptosis were greatly increased in the CNS in the absence of p130. Moreover, tissue-specific effects on proliferation were observed *(183)*.

The absence of dramatic effects in p107- or p130-deficient mice in the 129/Sv background potentially reflects the ability of pocket proteins to compensate for one another, a view that is supported by the shared structural and functional similarities of pocket proteins. To address this question, mice lacking both p107 and p130 were constructed *(182)*. After crossing p107+/– and p130+/– animals, only seven possible genotypes were observed soon after birth. Double-knockout mice survive until birth, but die shortly thereafter. At 18.5 dpc, double-knockout embryos were somewhat smaller than littermates, and had shortened limbs. Skeletal preparations revealed significant abnormalities in bone structure and in the timing of the bone deposition in double knockouts. Effects were observed in both the developing ribs and long bones, which are formed through the process of endochondral ossification. In contrast, bones formed through intramembranous ossification, such as most of the cranial bones, were normal. These data indicate that p107 and p130 are required for endochondral, but not intramembranous, bone formation *(182)*. Limb bones were also shortened, thickened and abnormally shaped. The shortening and thickening of the bones can be attributed solely to p107 loss, because p107–/–p130+/– embryos had the same defect. The defects in bone formation were traced to alterations in chondrocyte proliferation. In the absence of the p130 and p107 function, chondrocytes in epiphyseal centers proliferate at an increased rate, and were delayed in cell cycle withdrawal and differentiation *(182)*.

Analysis of p130–/– mice has also demonstrated functional compensation by *Rb* family members in peripheral T-lymphocytes *(184)*. T-lymphocytes form a uniform

population of quiescent cells, which rapidly proliferate and differentiate after an appropriate mitogenic stimulus *(99,106,185).* Lymphocytes maintain the quiescent resting state, with high levels of p130–E2F-4 complexes, which are promptly disrupted upon entering S phase. The postulated role of p130 in G0, in maintaining the quiescent state, predicts that p130–/– mice will have some disturbance in this phase of the cell cycle, but analysis of quiescent T-lymphocytes failed to reveal a difference in either the ability to enter quiescence or the rate of cell cycle entry, between p130–/– and p130+/– mice. The level of free and E2F-complexed p107 was significantly higher in p130–/– cells, suggesting that it compensating for p130 loss, and compensation appears to be essentially complete. T-lymphocytes lacking both p130 and p107 have elevated levels of free E2F, and also contain a Rb–E2F-4 complex, providing an additional means for compensation. But, in this case, the compensation does not appear as complete; these T-lymphocytes are hypersensitive to mitogens, and have derepressed a subset of E2F-regulated genes *(184).* Similar derepression was observed previously in embryonic fibroblasts, and in tissues of mice lacking both p107 and p130 *(108–182).* p130–/–p107–/– cells contain a new E2F complex lacking *Rb,* suggesting the possibility that another family member exists. It is not known at the moment if this hypothetical *Rb* homolog is important for compensation of p130 and p107 functions, or plays a role in control of cell cycle progression *(108).* Clearly, there may be functional overlap between pocket proteins in certain cell types, but whether this reflects a normal shared function or a gain of function, upon loss of another family member, is difficult to discern.

Analysis of pocket protein mutations in multiple-strain backgrounds has revealed major effects on phenotypes, and points to the importance of variable interacting genes that control the severity and penetrance of phenotypes. It is possible that the same modifiers are involved in the strain-specific phenotypes of both of these genes. Both p107- and p130-mutant mice do not display abnormal phenotypes on the 129/Sv or C57BL/6J genetic backgrounds, but their deletions on the Balb/cJ background lead to specific effects. Such differences could be explained by recessive loss-of-function mutations in the 129/Sv and C57BL/6J genetic background, or by dominant gain-of-function mutations in the BALB/cJ background. The mixture of both possibilities also cannot be excluded. The identification of these modifiers could provide insight into the specific and shared roles of pocket proteins in development and cell cycle control.

## 9. SUPPRESSION OF *RB* DELETION PHENOTYPES BY MUTATION OF E2F-1

Although heterozygosity for the *Rb* mutation leads to PIT and thyroid tumors in mice, homozygosity leads to midgestation lethality, because of defective erythroid and neuronal differentiation and apoptosis, and multiple cell cycle defects. The absence of Rb function leads to increased level of free, transcriptionally active E2F, and derepression of E2F target genes, such as cyclin E, which is important for cell cycle entry *(173).* To test if the proliferative and apoptotic disorders in *Rb*-mutant mice are related to derepression or transactivation of a subset of E2F-regulated genes, mice mutant for both *Rb* and E2F were created. E2F–/– mice by themselves have a mild lymphoproliferative disorder, decreased level of apoptosis in thymocytes and a wide array of tumors at advanced age *(186,187).* Loss of even one copy of E2F was enough to extend the life

of *Rb*+/– mice by several months, and reduced the incidence of thyroid tumors. Deletion of both copies of E2F leads to an even more extended life-span for *Rb*+/– mice, and to a decrease PIT tumor incidence *(188,189,190). Rb*–/– embryos die at E13–14, but *Rb*–/–E2F-1–/– embryos have a slightly prolonged life-span, dying at E17 with defects in blood, muscle, and lung development. Deletion of E2F-1 on an *Rb*–/– background leads to decreased levels of apoptosis in the lens, PNS, and CNS, and decreased incidence of abnormal S-phase entry that is characteristic of *Rb*–/– embryos. These observations indicate that the major consequence of *Rb* deletion is deregulation of E2F-1 function, although not all the effects of *Rb* deletion can be corrected by deletion of E2F-1 *(189).*

## 10. *RB* AND DIFFERENTIATION

Differentiation is known to be tightly coupled to cell cycle arrest in G1, but how this occurs is still not well understood. Two model systems, muscle cell (MC) differentiation and lens differentiation, have been extensively used to examine the role of *Rb* in the differentiation process. MC differentiation is controlled by a set of four myogenic basic helix-loop-helix transcription factors: MyoD, myogenin, MRF4/Myf-6/herculin and Myf-5. Each of these factors can activate the program of MC differentiation, when expressed in non-MCs. MyoD, Myf5, myogenin, and MRF4 have overlapping but distinct expression patterns during development of the skeletal muscles. Analysis of knockout mice lacking one or a combination of these TFs, has revealed their roles in muscle differentiation. MyoD and Myf5 act redundantly early in embryogenesis to establish the muscle lineage. Myoblasts, established with the help of MyoD and Myf5, continue to proliferate until receiving an unspecified differentiation signal, upon which myoblasts exit the cell cycle and form myotubes, a process controlled by myogenin. MRF4 is involved later in the process of myotube maturation. Mice lacking myogenin or a combination of MyoD and MRF4 have severe defects in skeletal muscle development, because of a failure of myoblasts to differentiate *(191–193).* Myoblasts are completely absent in mice lacking both MyoD and Myf5 *(194).* Because inactivation of both MyoD and MRF4 is required to inhibit differentiation, and of both MyoD and Myf5 to disrupt myoblast specification, it is clear that these sets of proteins have redundant roles, and can be compensated by another member of the group *(193,195).*

In order to terminally differentiate, cells must exit the cell cycle. In experiments involving ectopic expression of MyoD, differentiation was abolished if cyclin D, A, or E were co-expressed with MyoD *(196–198).* The cyclins act with CDK4 or CDK2 to phosphorylate and inactivate Rb. It is possible that, by inactivating Rb, CDKs interfere with differentiation. This notion is strengthened by the observation that viral proteins, which associate with and inactivate Rb, also interfere with MC differentiation *(199,200).* Cells lacking Rb fail to execute proper differentiation in vitro *(201,202),* also indicating that *Rb* plays a role in differentiation. The analysis of Rb's role in differentiation in vivo is complicated by the fact that *Rb*–/– mice die prenatally, before executing a program for secondary muscle differentiation *(170–172).* Myoblasts lacking Rb can differentiate into multinucleated myotubes in vitro, following serum withdrawal, in apparent contrast with observations in vivo. But these myotubes remain inappropriately sensitive to external signals, and can enter S phase and replicate their DNA in response to stimulation by serum growth factors *(201,203),* resembling results

of low level Rb expression in vivo *(204).* Rb–/– fibroblasts retain an ability to activate an aberrant muscle differentiation program after ectopic expression of MyoD, probably through compensation by other family members that are upregulated *(202).* This suggests potential functional compensation by p107 or p130, which can both regulate (HBP1)-mediated transcriptional inhibition of MyoD family members, and p107 is substantially upregulated in *Rb–/–* myoblasts in vitro *(201,203,40,205).* HBP-1 is a transcriptional repressor and a cell cycle inhibitor induced during MC differentiation. The relative ratio of *Rb* to HBP1 appears to be an important factor in determining whether cell cycle exit or full differentiation will occur. When the Rb:HBP1 ratio is low, cell cycle exit occurs, but tissue-specific gene expression is not observed. With a high ratio, full MC differentiation occurs. Therefore, the relative ratio of Rb to HBP1 may be a signal for activation of MyoD, implicating Rb function in controlling more than one step in the MC differentiation *(40).*

It is not clear which components of the myogenic regulatory pathway require Rb for function. It is known that Rb interacts with MyoD in vitro *(201),* although MyoD activity seems unaffected by *Rb* loss. p21 is a potential MyoD target; it is induced by MyoD expression, and could therefore couple the differentiation program to cell cycle arrest in G1 by blocking CDK-mediated *Rb* phosphorylation. The absence of muscle differentiation defects in p21–/– mice raised a possibility of functional redundancy of p21 in MC differentiation *(206).* Indeed, it was found that skeletal muscle expresses the p21 homolog $p57^{KIP2}$, and simultaneous deletion of both $p21^{Cip1}$ and $p57^{Kip2}$ leads to severe defects in skeletal muscle development, indicating that these two inhibitors cooperate to control cell cycle exit and muscle differentiation *(207).* MCs lacking both p21 and p57 display increased proliferation and apoptosis, and a phenotype shared by MCs lacking myogenin, or expressing inadequate levels of Rb, indicate that p21 and p57 function upstream of *Rb,* to facilitate MC differentiation. Additional links are suggested by the fact that Rb can physically associate with MyoD and myogenin *(201),* and that p21 may be a target of the myogenin, although its expression is not altered in myogenin-deficient cells *(208,209).*

The lens has also been an important experimental system for studying the role of *Rb* in differentiation, primarily because of its simplicity. The lens contains an anterior layer of proliferating epithelial cells that migrate to the equatorial zone, where they differentiate into lens fiber cells, and elongate. In *Rb*-mutant mice, lens epithelial cells fail to appropriately exit the cell cycle, and many cells undergo inappropriate apoptosis. In a situation parallel to that found in muscle, loss of two CDK inhibitors ($p57^{KIP2}$ and $p27^{KIP1}$) leads to a very similar phenotype, inappropriate S-phase entry, and apoptosis. One interpretation is that, in the absence of these inhibitors, *Rb* is inactivated by CDK-dependent phosphorylation; thus, the phenotype of the double-knockout mimics *Rb* loss, as with MCs. Additional studies are needed to understand whether *Rb* is required simply for cell cycle arrest, or is also directly involved in promoting the action of transcriptional pathways that promote lens epithelial cell differentiation.

## 11. OTHER ROLES FOR RB

### *11.1. Apoptosis*

Cell proliferation, differentiation, and apoptosis are major pathways controling cell fate in the developing organism. The role of Rb in cell cycle control and differentiation

well established. In the past few years, a role for Rb in apoptosis has been suggested, based on the fact that *Rb* deficiency leads to increased cell death in the developing lens and nervous system *(170–172).* Moreover, Rb–/– fibroblasts show an increased tendency to undergo apoptosis in response to growth factor withdrawal and DNA damage *(210).* p130-deficiency in certain genetic backgrounds also produces similar apoptotic effects in muscle, neural structures, and the developing eye *(183).* These data indicate that both Rb and p130 may have a role in protecting cells against apoptosis.

Rb's apoptosis protection function may result from its regulation of E2F-1. E2F-1, when overexpressed, can induce apoptosis in certain cell types *(123,211–214),* and, in the absence of *Rb,* E2F-1 may inappropriately activate apoptotic signals. That this is the case is suggested by reduced apoptosis when *Rb* deficiency is crossed onto an E2F-1-deficient background *(188,189).* The ability of E2F-1 to promote apoptosis may reflect its ability to activate ARF *(215,216).* The ARF protein is able to bind and inhibit the Mdm2 protein, which normally controls the levels of p53 through ubiquitin-mediated proteolysis *(217–220).* Blocking p53 destruction may activate p53-dependent apoptosis in this context. In addition to possibly being required to suppress E2F-1-mediated apoptosis, *Rb* is also a target of caspases involved in cleaving proteins during apoptosis *(221,222,223,224).* This area of research has recently been reviewed *(225).* Whether cleaved *Rb* is required for apoptosis is still an open question.

### *11.2. Tumorigenesis and DNA Damage Arrest*

The ability of a cell to arrest in response to DNA damage is essential for accurate transmission of genetic information. The failure of a cell to arrest in such circumstances can lead to propagation and accumulation of harmful mutations that facilitate transformation. The ability to confer cell cycle arrest in response to γ- or UV irradiation, or after treatment with chemotherapeutic agents, may be an important characteristic of certain classes of tumor suppressors. p53 is a tumor suppressor that is stabilized and activated upon DNA damage *(101,217–221).* Activated p53 has increased transcriptional activity, and is required for both the G1–S DNA damage checkpoint and the G2–M spindle checkpoint. $p21^{CIP1}$ is a major target of p53-mediated transcriptional activation following DNA damage, and is important for G1–S arrest *(212,226–230).*

Rb was recently identified as a mediator of p53-dependent arrest imposed by a diverse range of DNA-damaging agents *(231). Rb–/–* embryonic fibroblasts failed to respond to DNA damage by arresting the cell cycle, and continue to replicate damaged DNA *(170–172);* fibroblasts with mutant p130, p107, or both p107 and p130, had a response similar to that of wt cells *(179–183).* The rate of induction of p53 and p21 in response to damage was unchanged in Rb–/– and *Rb*+/– cells, indicating that Rb loss affects DNA damage pathways downstream of p21 *(170–172).* These data are consistent with the idea that p21 blocks CDKs from inactivating *Rb*'s growth-suppressive function. The presence of functional Rb correlates with the ability of p21 to arrest cells *(230),* although p21 can block *Rb*-negative cells when expressed at sufficient levels. Alternatively, the role for Rb in the G1 damage checkpoint may reflect an indirect role of Rb loss in gene regulation. The level of cyclin E, an important target of E2F–*Rb* regulation, is increased in *Rb–/–* cells *(108,232).* Increased cyclin E levels may require higher levels of p21 for complete inhibition than are achievable through p53-dependent induction. A specific role for Rb in the G1 checkpoint may underlie the fact that, of the three pocket proteins, only Rb is a tumor suppressor. The exact biochemical mecha-

nism of Rb-dependent cell cycle arrest remains unclear, and it would be important, for practical applications, to determine if the same pathway is at work in vivo.

## 12. THERAPEUTIC APPLICATIONS

Because the disruption of the Rb pathway (Fig. 1) is a common feature of tumors, the restoration of Rb function initially seemed to be an attractive way for correcting the deficiency. Indeed, introduction of wt *Rb* into transformed cells can suppress inappropriate growth. Despite the theoretical attractiveness of such an approach, there has been little progress in bringing Rb-based therapies to the clinic. Several problems of a conceptual and practical nature have yet to be solved. First, a highly efficient means of targeted drug delivery should be employed, because even rare cells with unrestored Rb function will be selected for growth, and any such cell can potentially give rise to tumors. Second, most human cancers are associated with multiple mutations, and restoration of just one mutated function may not lead to a fully functional restoration of the normal phenotype. Recently, it was shown that Rb is able to prevent replication of damaged DNA *(231),* with *Rb–/–* cells being able to replicate in the presence of the damage, despite the presence of functional p53. So, Rb loss may mediate propagation of mutations. This observation, made using Rb–/– mouse embryonic fibroblasts, may prove to be crucial to ideas on cancer treatment. Chemotherapy is a commonly prescribed procedure, but chemotherapeutic treatment of tumors with *Rb* mutations may actually propagate mutations, and so have deleterious effects. In addition, it is now clear that cyclin E overexpression can bypass the *Rb*–E2F pathway to initiate S phase *(166,167).* Thus, the use of nonphosphorylatable *Rb* as a growth inhibitor may not provide the expected outcome. As such, significant advances will need to be made before the *Rb* pathway becomes a viable target for cancer therapeutics.

## 13. CONCLUSION

The past 10 years has seen remarkable progress in the understanding of *Rb* function. However, there are several outstanding questions. Although a large number of *Rb*-interacting proteins have been identified, for most of these, very little is known about their roles in the *Rb* pathway or even whether they are important mediates of *Rb* function. In addition, although *Rb* is clearly required for differentiation of several cell types, it is unclear precisely what its role is in this process. The identification of *Rb* homologs in genetically tractable organisms will probably allow a better appreciation of these and other questions related to pocket protein function.

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# 7 p53 Tumor Suppressor Protein

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***Contents***



## 1. INTRODUCTION

The p53 protein was originally identified during the late 1970s, by several independent groups, as a novel cellular protein that was tightly associated with the large T antigen in cells transformed by simian virus-40 (SV40) *(1–3).* Although originally thought to function as an oncogene, isolation of the wild-type (wt) gene encoding p53 led to the discovery that p53 functioned as a potent tumor suppressor *(4–8).* A role for p53 in preventing malignant progression was subsequently demonstrated by the observations that transfection of p53 into cultured cells inhibited transformation by a number of oncogenes *(9),* and that mice lacking the *p53* gene rapidly developed tumors with high incidence *(10–12).* It is now known that *p53* is one of the most frequently mutated genes in human cancer, in which loss of function mutations contribute to the development of many major human malignancies. Approximately 50% of all human tumors carry a *p53* mutation, and at least 52 different types of tumor have *p53* mutations *(13–15).* During the past decade, p53 has been brought to the forefront of cancer research, and intensive investigation has provided insight into how it mediates its tumor suppressor activities, and how these activities are regulated. Elucidation of the mechanisms that activate and regulate p53, and the identification of upstream and downstream effectors and targets involved in p53 function, should contribute to understanding how cancers arise, and to the development of new therapeutic tools for their treatment.

## 2. STRUCTURAL FEATURES OF THE p53 PROTEIN

The human *p53* gene encodes a protein of 393 amino acids, which can be divided into several well-characterized structural and functional domains, based chiefly on the

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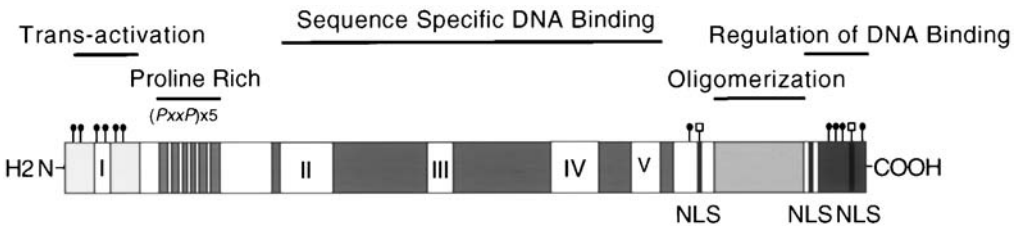


**Fig. 1.** Schematic representation of the human p53 protein. The important functional domains (transactivation, proline-rich, sequence-specific DNA-binding, oligomerization, regulation of DNA binding), conserved box regions I–V, sites of phosphorylation within the N- and C-terminal regions (filled circles), and sites of acetylation within the C-terminus (open squares), are indicated.

ability of p53 to function as a transcription factor transcription factors (Fig. 1). The *p53* gene has been identified in several species, from human to squid. Sequence comparison of the p53 protein from different species shows five highly conserved regions (Fig. 1). Four of these conserved regions lie within the central core domain of p53, which comprises a sequence-specific DNA-binding region. The N-terminal region of p53 is rich in acidic residues, and contains a transcriptional transactivation domain, which has been shown to form a direct contact with several basal transcription factors, such as the TATA-binding protein (TBP) *(16–20),* TBP-associated factors in the TFIID complex *(21–23),* and the transcriptional co-activators, p300 and CBP *(24,25).* This region also contains the site for binding to Mdm2, the product of a p53-inducible gene that plays an important role in negatively regulating p53 function *(26,27).* A proline-rich region links the transcriptional activation domain with the central core, and has been shown to be important for the growth-suppressor function of p53 *(28–31).* The C-terminal region of p53 is rich in basic residues, and shows nonspecific DNA/RNA-binding, which can regulate the DNA-binding activity of the central core *(32,33).* Modification of the extreme C-terminus of p53 by deletion, phosphorylation, or binding of a specific antibody activates the sequence-specific DNA-binding activity. The native p53 protein is a tetramer in solution *(34),* and residues 324–355 within the C-terminal region are essential for oligomerization *(34–37).* The C-terminal region also contains three nuclear localization sequences *(38–40)* and a nuclear export signal that regulates the subcellular localization of the p53 protein *(41).* p53 is posttranslationally modified by phosphorylation at specific serine (Ser) residues within the N-terminus and C-terminus of the protein, and by acetylation at specific lysine residues within the C-terminal region *(42).* Two common polymorphic variants of p53 exist, arising from a single base-pair substitution at codon 72, encoding either a proline or an arginine residue *(43,44).* Although both polymorphic forms share similar growth-suppressive activities, recent studies suggesting subtle differences in their regulation and potency may be reflected in increased cancer susceptibility in some individuals *(45,46).*

In recent years, two genes showing structural similarities to p53 have been identified *(47–53).* The *p73* gene encodes at least four distinct isoforms; the full-length version, which gives rise to the protein called p73α, and three splice variants, which encode proteins referred to as p73β, γ, and δ. p73 has been shown to mediate at least some functions in common with p53, including apoptosis, transcriptional transactivation of $p21^{WAF1/CIP1}$,

a known target of p53, and suppression of cell growth. The *p73* gene has been mapped to chromosome 1p36.3, a locus that is deleted in neuroblastoma and some other human cancers *(49,54).* Studies to date indicate, however, that, in contrast to p53, p73 tumor-derived mutations appear infrequently *(54–56),* although this may be a reflection of the observation that p73 is imprinted. Another p53 family member, referred to as *p40, p51, p63, p73L,* or the rat homolog, *KET,* encodes at least six isoforms that are expressed in a tissue-specific manner, and have different transactivation potentials *(53).* A contribution of p63 to tumor suppression has not yet been established, although mice deleted of the *p63* gene suffer severe developmental defects *(57,58).*

## 3. CELLULAR FUNCTIONS OF THE p53 PROTEIN

Although mice deleted of the *p53* gene show a high incidence of cancer, their viability and relatively normal development indicated that p53 function is not essential for cell growth and differentiation. In contrast, analysis of transgenic mice expressing a p53-responsive *lacZ* reporter gene demonstrated that tissue-specific overexpression of p53, following irradiation, is detrimental to normal embryonic development (59–61), indicating the importance of the tight negative regulation of p53 function during normal cell growth. It is now clear that p53 plays a major role in the cellular response to stress, and that activation of p53 following various insults prevents the growth of damaged and abnormal cells, and in some cases contributes to the ability of the cell to repair the damage.

Under normal circumstances, endogenous p53 protein is rapidly degraded and maintained at very low levels within the cell. However, a variety of stress signals, including DNA damage *(62,63),* hypoxia *(64),* oncogene activation *(65–68),* heat shock, metabolic changes, viral infection, or cytokine treatment *(69),* or withdrawal *(70)* lead to the rapid elevation of p53 levels, principally through stabilization of the protein. In most cell types, the activation of p53 results in either cell cycle arrest or cell death through apoptosis, thereby preventing the propagation of potentially abnormal cells. p53 can also contribute to cellular senescence *(71–73)* and differentiation *(74–77),* and, in mouse cells, loss of p53 results in rapid loss of the mechanisms that normally limit the proliferative capacity of cells in culture. Further functions of p53 include regulation of adhesion, metastasis, and angiogenesis *(78,79).* p53 activity is also important for normal centrosome duplication *(80),* and a role for p53 in DNA repair, replication, and recombination has also been described *(81–87).*

### *3.1. Transcriptional Activation by p53*

As mentioned earlier, one of the most important functions of p53 in tumor suppression is its ability to inhibit cell growth by inducing cell cycle arrest or apoptosis. Although these are independent activities of p53, the ability of p53 to function as a transcription factor contributes to both responses *(88).* Many cellular genes have been shown to be transcriptional targets of p53 *(89),* although only a few of these have been verified as direct mediators of the p53 response. These genes can be broadly divided into those likely to contribute to activation of a cell cycle arrest, and those that are more likely to play a role in mediating the apoptotic response *(88).*

Activation of p53 in many cell types leads to an arrest at G1 and G2–M phases of the cell cycle, which under some circumstances, is reversible *(90).* One of the principal

mediators of both G1 and G2/M arrests is the *p21$^{WAF1/CIP1}$* gene, a direct target of p53 transcriptional activation *(91).* p21$^{WAF1/CIP1}$ is a broad inhibitor of cyclin-dependent kinase (CDK) complexes, which are essential for cell cycle progression *(92,93).* p21$^{WAF1/CIP1}$ also binds to proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase, and directly inhibits DNA replication *(94).* Overexpression of p21$^{WAF1/CIP1}$ in a number of cellular backgrounds leads to G1 and G2 arrests indistinguishable from those mediated by p53 *(95).* Deletion of *p21$^{WAF1/CIP1}$* from human cells dramatically reduces the ability of the cell to undergo cell cycle arrest in response to p53 activation *(96),* although the retention of some activity in p21$^{WAF1/CIP1}$-null mouse cells suggests that other p53-inducible genes also contribute to this response *(97,98).* Mice deficient in the *p21$^{WAF1/CIP1}$* gene develop normally, but do not show the high incidence of tumor development seen in p53-deficient animals, suggesting that this response is not the primary mechanism by which p53 functions as a tumor suppressor. A number of other p53-responsive target genes have been described that may contribute to the cell cycle arrest. The *GADD45* gene is a member of a group of growth arrest and DNA damage-inducible genes (GADD) *(99),* and is induced by ionizing radiation in many cell types containing wt p53 *(100).* Expression of GADD45 has been associated with the activation of both the G1 and G2/M arrest *(101,102).* GADD45 binds PCNA, to stimulate excision repair of damaged DNA *(103),* and has recently been shown to modify DNA accessibility on damaged chromatin *(104).* p53-dependent activation of *GADD45* can be mediated via direct p53/DNA interactions, as described above, but also through interaction of p53 with the product of the Wilm's tumor gene, *WT1 (105),* indicating that further p53-responsive genes, which lack p53-binding sites within their promoter region, may remain to be identified. Recently, *PA26,* another novel p53 target gene that belongs to the GADD family, was identified *(106).* Although no functional clues have been revealed by its sequence, *PA26* appears to be negatively regulated by serum factors, and so, like *GADD45,* may also play a role in growth regulation *(106).* Another p53-target gene, which could play a role in regulating proliferation, is *IGFBP-3,* which encodes a secreted inhibitor of insulin-like growth factors *(107).* The human homolog of the *Drosophila sina* gene, *SIAH-1,* has also been shown to play a role in p53-dependent cell-cycle arrest *(108,109)* and the 14-3-3 σ protein has been shown to be a potent p53-mediated regulator of G2–M progression *(110).* 14-3-3 proteins have also been shown to specifically bind to a DNA-damage-inducible 14-3-3 consensus binding site within the C-terminal region of p53, which enhances sequence-specific DNA-binding function in vitro *(111).* However, the precise physiological role for this interaction, in terms of p53 transcriptional regulation, remains unclear.

Activation of transcription also plays a role in the ability of p53 to activate apoptotic cell death *(112).* The apoptotic targets of p53 appear to be independent of the cell cycle arrest targets, and activation of genes, such as *p21$^{WAF1/CIP1}$*, play no role in the induction of cell death *(112).* Apoptosis is a form of programmed cell death, and is characterized by morphological changes that include cell shrinkage, plasma membrane blebbing, nuclear condensation, and DNA fragmentation *(113).* These cellular changes have been shown to involve a family of cysteine proteases called caspases (ICE/CED-3 proteases) *(114),* which can be activated through two main pathways, one involving the activation of death receptors, such as Fas/APO1 and DR5, at the cell surface *(115),* and the other involving cytochrome-*c* dependent activation of the adaptor protein, Apaf-1 *(116).* Although the mechanisms by which p53 initiates apoptosis are not fully under-

stood, both the death-receptor-associated pathways and the Apaf-1-dependent apoptotic pathway have been shown to be involved in mediating p53-dependent cell death *(117).* Several transcriptional targets of p53 have been identified, which directly link p53 to caspase activation, including the proapoptotic Bcl-2 family member, Bax *(118),* which functions by releasing cytochrome-*c (119),* and the cell surface death receptors, DR5 and Fas *(120,121).* None of these appear to represent the only component of the p53-mediated death mechanism, however, since mice deficient in *bax* or *Fas* are still able to mediate apoptosis in response to activation of p53 in some cell types *(122–125).*

Other potential apoptotic transcriptional targets of p53 include IGFBP-3 *(107),* which inhibits IGF survival function; *PAG608,* which encodes a nuclear zinc finger protein with apoptotic activity *(126);* and the gene that encodes the cathepsin-D protease, which contributes to cytokine-mediated apoptosis *(127).* Reactive oxygen species participate in cell death mechanisms mediated by many different stimuli, and it has been proposed that p53 may transcriptionally regulate genes *(128)* or cooperate with cellular targets that are sensitive to reactive oxygen species *(129).* Several further novel p53-induced genes, which are highly expressed in the colorectal cancer line, DLD-1, before the onset of apoptosis, have been identified using sequence analysis gene expression *(128),* although their contribution to the p53 response has not yet been fully characterized.

Finally, p53 has also been shown to activate the expression of a number of metastasis- or angiogenesis-related genes, including epidermal growth factor, matrix metalloprotease (MMP-2, also called human type IV collagenase), cathepsin-D, thrombospondin-1 *(130)* and BAI1, an inhibitor of angiogenesis *(131).* Drugs specifically targeting some of these genes (MMP inhibitors), have been used, in combination with radiotherapy, to combat the induction of metastases in the treatment of some tumors that express wt p53 *(130).*

### *3.2. Transcriptional Repression by p53*

In addition to activating genes with p53-binding sites, p53 can also repress promoters that lack the p53-binding element, and this may be of considerable biological importance in mediating the apoptotic response of p53 *(112,132).* Indeed, a number of genes, including interleukin-6 *(133),* nuclear factor-κB RelA (NF κB) *(134,135),* cyclin A *(136,137),* PCNA *(138),* and a number of metastasis-related genes *(130),* have been shown to be transcriptionally repressed by p53 in this way. Additionally, both RNA polymerase II and III transcription can be repressed by p53 *(139,140).* Despite genetic evidence showing a strong correlation between the ability of p53 to induce apoptosis and the retention of transcriptional repression, identifying the important targets that are transcriptionally repressed by p53 has been difficult. Relatively few of the potential target genes for repression by p53 have been verified by showing downregulation of endogenous gene expression following p53 expression, and the mechanisms of function of those that have been authenticated in this way, such as mitogen-activated protein 4 *(141,142)* remain unclear. Furthermore, the observation that transcriptional repression of some genes may be the consequence, rather than the cause, of apoptosis may further complicate the identification of *bona fide* targets of p53 *(143).* The difficulty in examining this activity of p53 is clearly demonstrated by the c-*fos* gene promoter, which had for many years represented the archetypal target for transcriptional

repression by p53 *(144),* until it was recently shown that the endogenous gene is transcriptionally activated by p53 *(145).*

### 3.3. Transcriptionally Independent Activities of p53

Despite the clear contribution of transcriptional activation to p53-mediated apoptosis in many systems, considerable evidence has accumulated to support the existance of a transcriptionally independent function of p53 in the activation of cell death. p53 can mediate cell death when RNA and protein synthesis are blocked *(146),* and mutational studies have shown that, although loss of transcriptional activity invariably results in loss of the ability to induce cell cycle arrest, the ability to induce apoptosis is not necessarily impaired *(147).* Conversely, C-terminal mutants of p53 that retain transcriptional activity show defects in apoptotic activity *(148,149),* despite retaining cell cycle arrest functions. The proline-rich region of p53 has also been shown to be important for apoptosis in several systems *(28–31),* although this region may in fact play a role in differential regulation of p53 transcriptional function *(150).* The observation that the requirements for p53-induced apoptosis are dependent on the cell type under examination has further complicated the identification of these activities, although several functions of p53 that are not dependent on transcription have been described. One activity of p53 with clear implications for the induction of apoptosis is the ability to traffic the death receptor, Fas, to the cell surface, thereby sensitizing cells to Fas-mediated apoptosis *(151).* Whether p53 shows a general ability to redistribute death receptors to the cell surface remains unknown. The direct interaction of the extreme C-terminal region of p53 with the XPB and XPD DNA helicases has also been shown to play a role in p53-mediated apoptosis *(152,153),* although this activity is clearly not required under all circumstances *(154).* The N-terminal proline-rich domain within p53 shows some similarities to an SH3-binding domain, and binds to the SH3 domain of c-Ab1 tyrosine kinase. This interaction has recently been shown to enhance p53 expression by neutralizing the inhibitory effects of Mdm2 *(155),* and so far it is unclear whether the interaction of c-Ab1 with p53 via the proline-rich domain is important for p53-mediated apoptosis *(156,157).*

### 3.4. Cell Cycle Arrest or Apoptosis

The identification of distinct p53 target genes and mechanisms that are involved in p53's ability to mediate cell cycle arrest or apoptosis indicates that these are separable and independent physiological functions of p53, and p53 mutants, which retain one, but not the other, function, have been described in several studies *(28,30,31,143,147,158).* The cellular response to p53 in undergoing cell cycle arrest or apoptosis represents a critical choice between a potentially transient delay in cell growth and the clearly irreversible process of cell death. Although the choice of response to p53 is strongly influenced by the cell type, the cellular environment (including presence of survival factors) and other genetic abnormalities sustained by the cell, it is clear that, to some extent, the choice can be mediated by p53 itself. Overall p53 levels can determine the choice of response *(149),* and it has been suggested that this in part determines the response to DNA damage. In this model, low levels of damage, which may be repairable, induce low levels of p53, and lead to a cell cycle arrest; extensive, irrepairable damage, which induces high levels of p53, leads to apoptosis.

Because distinct transcriptional targets of p53 mediate the cell cycle arrest and apoptotic responses, it is possible that activation of the two sets of target genes may be dissociated under some circumstances. The observation that apoptotic cell death in response to p53 obscures an underlying cell cycle arrest *(159,160)* implies that, in these cells, the choice of response is not either cell cycle arrest or apoptosis, but rather cell cycle arrest, or cell cycle arrest with apoptosis. These observations suggest that the transcriptional targets that mediate cell cycle arrest are more sensitive to p53 than the apoptotic targets, with some evidence that the promoters of cell-cycle-arrest genes contain p53-binding sites with higher affinities for p53 than the apoptotic target gene promoters *(161,162).* Several mutants of p53, which contain amino acid substitutions in the DNA binding domain, show a temperature-sensitive loss of the ability to activate apoptotic targets, while retaining activation of cell-cycle-arrest target genes *(143).* This may reflect a reduction in DNA binding activity of these mutants, so that they fall below a threshold necessary to activate the apoptotic targets. Similarly, mutation within the proline-rich domain of p53, which regulates sequence-specific DNA binding *(30,163),* also results in loss of the ability to activate a specific subset of p53-responsive genes.

## 4. REGULATION OF p53 FUNCTION

### *4.1. Regulation of p53 Protein Stability*

The principal mechanism by which p53 function is regulated is through the stability of the protein, although transcriptional and translational control of p53 expression has also been reported *(164–166).* Endogenous p53 protein is maintained at very low levels within the cell, because of its rapid degradation by ubiquitin-dependent proteolysis. It is now clear that a direct transcriptional target of p53, Mdm2, is an essential component of this process *(26,27).* Mdm2, unlike the other transcriptional targets described above, has not been shown to directly contribute to cell cycle arrest or apoptotic functions of p53 *(167,168).* However, Mdm2 directly binds to residues within the N-terminal transactivation domain of p53, repressing transcriptional activation *(169)* and targeting p53 for degradation *(26,27,170).* In this way, *Mdm2* negatively regulates p53 stability and transcriptional function. The importance of this regulation was clearly illustrated by the development of mice deficient in Mdm2, which resulted in lethality at the implantation stage of development *(171,172).* This lethality was rescued by breeding onto a *p53*-null background, demonstrating that the ability of Mdm2 to regulate p53 protein levels is essential during development.

The abundance of cellular Mdm2 protein depends mostly on its transcriptional activation by p53, and, in normal cells, p53 and Mdm2 exist in a tightly regulated feedback loop, through the direct binding of Mdm2 to p53. This mutual regulation of p53 and Mdm2 is lost in cells expressing mutant forms of p53, which have lost the ability to activate transcription, such as those expressed in many cancers. These p53 mutants are unable to activate the expression of Mdm2, and are therefore usually stable and expressed at high levels *(170,173,174).* The observation that mutant p53 is usually expressed at high levels has been used as a prognostic indicator in some tumor types *(175).*

Two activities of Mdm2 have recently been described that are likely to contribute to the regulation of p53 stability: the ability to function as a ubiquitin ligase, and the reg-

ulation of subcellular localization. Mdm2 has specific ubiquitin ligase E3 activity, and will readily ubiquitinate p53, upon association *(176),* thus targeting p53 for degradation *(177).* This degradation depends on the direct interaction between the two proteins, although regions outside the binding sites on both p53 and Mdm2 are also essential *(170,174,178).* For example, deletion of the last 30 amino acids from the C-terminus of p53 renders the protein partially resistant to Mdm2-mediated degradation *(174,179),* and deletion of the p53-conserved box II sequences in the DNA-binding region also results in slight resistance to Mdm2-mediated degradation *(173,174).* It is possible that the latter observation reflects a contribution of binding between p53, Mdm2, and the transcriptional co-activator p300, in allowing efficient degradation of p53 *(180).* Sequences in Mdm2 apart from the p53-binding region are also necessary for degradation, with evidence that the RING finger domain at the C-terminus of Mdm2 is critical for ubiquitin ligase activity *(177).* Mdm2 also appears to regulate its own stability. Mutants of Mdm2 that fail to mediate ubiquitin ligase activity are themselves stable *(170,178),* and Mdm2 can target its own ubiqutination *(176,177).* Mdm2 can also target the degradation of other proteins to which it can bind *(181,182),* and the stability of other important components of the cell cycle machinery, such as the transcription factor E2F, may be regulated by Mdm2 *(183).*

In addition to acting as a ubiquitin ligase, Mdm2 also plays a role in regulating the subcellular localization of p53. The Mdm2 protein contains both nuclear import and export sequences, and the importance of the nuclear export function of Mdm2 in the degradation of p53 suggests that Mdm2 plays a role in relocating p53 to the cytoplasm, where degradation takes place *(184).* This interpretation is complicated by the identification of nuclear export sequences in p53, which also result in transport of p53 to the cytoplasm, without a requirement for Mdm2 *(41),* although this activity may depend on the oligomerization status of p53. It seems likely that the subcellular location of these proteins will be as important as their expression levels and activities in determining their ability to function.

Although Mdm2 is a major player in regulating p53 stability, other proteins also contribute to the degradation of p53. The jun-N-terminal kinase (JNK) plays a role in targeting p53 for ubiquitination and degradation, in an Mdm2-independent manner *(185,186),* although the mechanism is unknown, and does not depend on the kinase activity of JNK. Viral proteins, such as the papillomavirus E6 and E1B, and E4orf from adenovirus, can also target p53 for degradation *(187–189).* p53 stability may also be regulated by other proteases, such as calpain *(187,190),* although the pathways involved are not yet understood. The p53-related protein, p73, retains the ability to bind Mdm2, but is not degraded following this interaction *(191,192),* although p73 protein levels do appear to be regulated in a proteasome-dependent manner *(191).* It is possible that other Mdm2-related proteins, such as MdmX *(193–195),* play a role in regulating the stability of p53 family members, although there is at present no evidence for such a mechanism.

Since the Mdm2 protein plays a central role in negatively regulating p53 stability, it seems highly likely that inhibition of this regulatory mechanism would be essential for stabilization of the protein under conditions of cellular stress. It is becoming clear that many different pathways can lead to the inhibition of Mdm2-mediated degradation of p53, and different stress signals are likely to utilize different mechanisms to stabilize p53 (Fig. 2). One way to render p53 resistant to Mdm2 is to prevent interaction

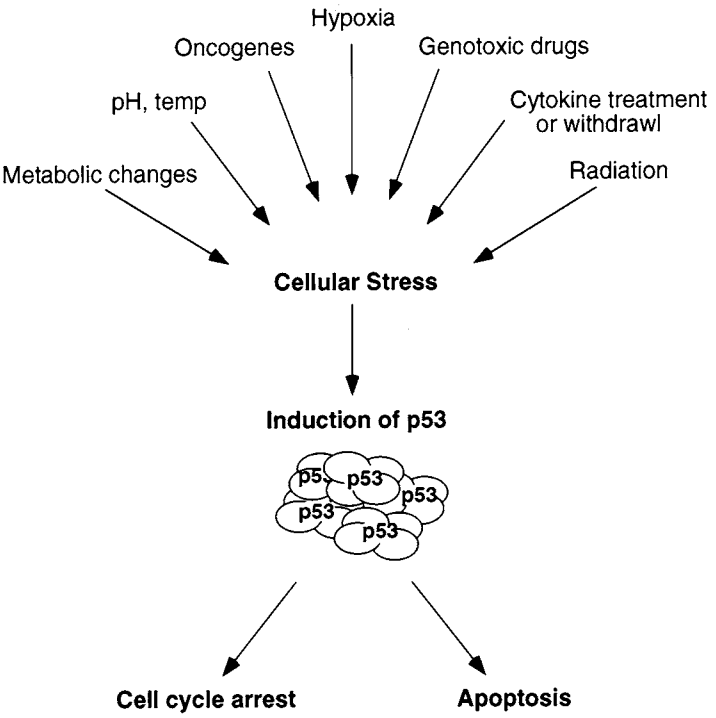


**Fig. 2.** Multiple pathways lead to the stabilization of p53.

between the two proteins, and phosphorylation of p53 or Mdm2 has been shown to reduce the p53–Mdm2 interaction *(196,197),* and to inhibit Mdm2-mediated ubiquitination in vitro *(177).*

Several kinases have been shown to phosphorylate the N-terminus of p53 in vitro, close to or within the Mdm2-binding region. One candidate is the ATM kinase, which is encoded by the gene mutated in the human genetic disorder, ataxia telangiectasia, and is activated in response to ionizing radiation, but not UV radiation *(198).* ATM can directly associate with p53, and phosphorylate it at Ser15 in vitro *(198–200),* and this residue is inducibly phosphorylated upon DNA damage, simultaneously with stabilization of the protein *(201,202).* Cells deficient for ATM show a delayed ability to stabilize p53 in response to ionizing radiation, although the UV response remains intact, supporting a role for ATM in governing p53 stability *(202).* The ATR kinase, a protein closely related to ATM, also phosphorylates Ser15 and Ser37 in vitro, and overexpression of an inactive ATR kinase prevents phosphorylation of Ser15 in response to UV radiation *(203).* Recently, phosphorylation of p53 at Ser20, within the Mdm2-binding site, has also been shown to occur in response to some forms of DNA damage *(204),* and has been proposed to play a role in regulating p53 stability *(205),* although the kinase responsible for this phosphorylation has not been identified.

DNA damage-induced phosphorylation of p53 provides an attractive mechanism for mediating the stabilization of the protein. However, analyses of p53 mutants that cannot be phosphorylated, either at individual sites or at multiple sites within N- or C-terminal regions of the protein, have shown that phosphorylation is not essential for

stabilization in response to several forms of DNA damage in vivo *(173,206).* Indeed, the p53 protein is stabilized in response to ionizing radiation, even under conditions when Ser15 phosphorylation has been abolished by the expression of an antisense ATM construct *(200).* Furthermore, some DNA-damaging agents, such as actinomycin D, induce rapid stabilization of p53 in the absence of detectable Ser15 phosphorylation *(207).* These studies indicate that phosphorylation is not essential for DNA-damage-induced stabilization of p53, and other mechanisms may also play a role. One mechanism employed by topoisomerase inhibitors seems to be the specific downregulation of Mdm2 expression at the transcriptional levels, thereby releasing p53 from degradation by reducing Mdm2 protein levels in the cell *(207,208).* Other proteins that bind Mdm2 or p53, such as the retinoblastoma tumor suppressor protein (Rb) *(209)* or c-Abl *(155),* have also been shown to regulate the degradation of p53.

In addition to DNA damage, several other forms of stress stabilize and activate p53. Many studies have implicated a role for p53 in responding to abnormal proliferative signals generated by oncogene activation, and stabilization of p53 in response to such oncogenic stress represents a powerful fail-safe mechanism to protect cells from malignant progression. Stabilization of p53 in response to signals generated by oncoproteins, such as E2F1, Myc, Ras, and adenovirus E1A, is mediated though the $p14^{ARF}$ (mouse, $p19^{ARF}$) protein *(65,66,210),* which binds directly to Mdm2 *(211–213),* and inhibits the ability of Mdm2 to function as a ubiquitin ligase *(177).* Expression of $p14^{ARF}$ therefore results in the stabilization of both p53 and Mdm2. $p14^{ARF}$ is a direct transcriptional target of E2F1; therefore, most forms of deregulated proliferation, which lead to E2F1 activation, are likely to result in $p14^{ARF}$ activation. Complex feedback mechanisms, through which p53 can inhibit $p14^{ARF}$ expression, exist *(68,212,214),* probably to prevent activation of a p53 response during each normal cell cycle.

Although $p14^{ARF}$ very efficiently stabilizes p53, it does not appear to be involved in the DNA damage response pathway, because cells deficient in $p14^{ARF}$ mediate p53 induction in response DNA damage *(215).* Conversely, ATM, which plays a role in the activation of p53 in response to some DNA-damaging agents, is dispensable for the activation of p53 by proliferative abnormalities *(216).* The observation that many tumor cell lines that retain wt p53 suffer loss of $p14^{ARF}$ suggests that it is loss of the $p14^{ARF}$ pathway that is of critical importance during tumor development. Therefore, the failure to respond to proliferative abnormalities following loss of either $p14^{ARF}$ or p53 is a key step for tumor development.

### *4.2. Regulation of p53 Activity*

Although activation of a p53 response is clearly dependent on stabilization of the protein, in many cases, other mechanisms that regulate p53 activity have also been described. Evidence from in vitro studies suggests that the conformation of the p53 protein is crucially important for its ability to bind DNA, and there is evidence that this activity can be regulated by the C-terminus of the p53 protein. One model suggests that this C-terminal region of p53 allosterically regulates activation of the latent non-DNA-binding form of p53 to the active wt conformation *(35,217).* Others have suggested that the C-terminus reciprocally regulates the ability of p53 to bind DNA via the sequence-specific DNA-binding core *(218,219).* Many different mechanisms, which modify the C-terminus of p53, have been shown to activate DNA binding. These include phosphorylation *(220,221),* acetylation *(25,222,223),* O-glycosylation *(224),* binding to the C-

terminal antibody, PAb421 *(33),* binding to short strands of DNA *(225),* C-terminal deletion, or point mutations *(226).* Dephosphorylation within this region, following IR, has also been shown to correlate with binding of the 14-3-3 proteins to the p53 C-terminus, and subsequent activation of transcription *(111).* A number of other proteins that bind to p53 have also been shown to regulate its function *(221).* The redox/repair protein, Ref-1, is a potent activator of p53 DNA-binding and transcriptional activation functions *(227),* and a recently identified activator of p53, HMG-1, has also been shown to enhance p53 DNA-binding function *(228).*

Despite the clear ability to activate p53 protein produced in vitro, the contribution of these regulatory modifications to controling p53 activity and stability is much less clear in a physiological context. Several studies have shown a dissociation between activation of p53 function and stabilization of p53 protein *(220,229),* and, conversely, stabilization of inactive p53 has been described in teratocarcinoma cells *(230).* These results suggest that regulation of latent and active conformational states could play an important role in controling p53 function in cells.

Regulation of protein binding to the N-terminus of p53 also contributes to the modulation of p53 activity. Phosphorylation at the N-terminus of p53 decreases its interaction with TFIID in virus-transformed cells *(231);* by contrast, phosphorylation of Ser15 within this region of p53 increases the recruitment of transcriptional co-activators CBP/p300 *(232).* Both CBP and p300 show histone acetyl transferase activity, and have also been shown to acetylate the C-terminus of p53 *(233,234).* This has led to the hypothesis that phosphorylation at the N-terminus of p53 mediates acetylation at the C-terminus, and thus enhances DNA-binding and transcriptional activation function *(223).* In an interesting elaboration of this system, p300 was recently shown to be more important for p53 activation of Mdm2 than other transcriptional targets that mediate the cell cycle arrest and apoptotic responses *(235).* Inhibition of p300 activity, following adenovirus E1A expression, for example, can therefore stabilize p53 by inhibiting Mdm2 expression *(235),* the growth-inhibitory activities of p53 may be regulated, under physiological conditions, by activation of other transcription factors, such as NF-κB, which compete with p53 for binding to p300 or CBP *(236).*

### *4.3. p53 Cellular Localization*

In addition to regulation of protein levels and protein activity, p53 function can also be modulated by control of subcellular localization. p53 is subject to both nuclear import and export mechanisms *(38);* the C-terminal region of p53 contains three nuclear localization sequences *(38–40)* and a nuclear export signal *(41).* Translocation to the nucleus is essential for p53 function *(237),* and inactive wt p53, which fails to localize to the nucleus has been described in some tumor cells *(238,239).* The anti-apoptotic protein, Bcl-2, has also been shown to regulate p53 nuclear import *(240).* As described earlier, degradation of p53 by Mdm2 protein has been shown to be dependent on export from the nucleus *(184,241),* although, in this case, nuclear export appears to depend on signals in Mdm2, rather than p53 itself.

## 5. p53 IN TUMOR DEVELOPMENT

Increased understanding of the regulation and functions of p53 has allowed a reassessment of the frequency of p53 functional loss during tumorigenesis. Mutations

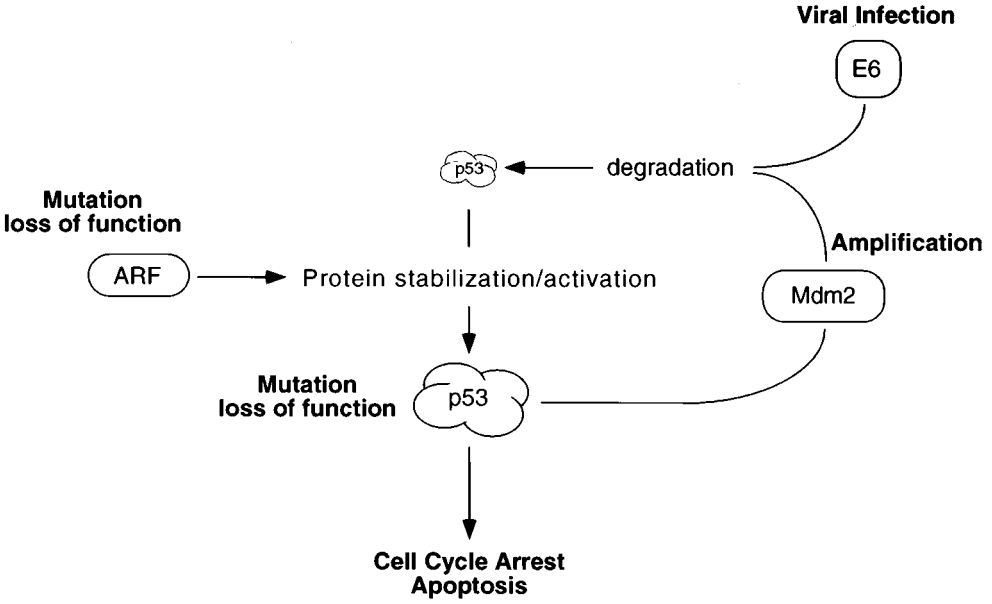


**Fig. 3.** Multiple mechanisms for loss of p53 function during tumor development.

in *p53* are common event in many major human malignancies, yet around one-half of all human cancers arise despite retention of wt p53 sequences. It is now apparent that many of these cancers have lost p53 function through other mechanisms, such as overexpression of Mdm2 or the human papillomavirus E6 protein, which target p53 for degradation, thus preventing stabilization in response to stress (Fig. 3). Another common defect that prevents p53 activation in response to abnormal proliferation is loss of the $p14^{ARF}$ protein, which normally inhibits Mdm2 degradation of p53 following oncogene activation. Other proteins that function like $p14^{ARF}$ may also play a role in mediating tumor-suppressor activities of p53. Tumors may also show loss of the downstream effectors of p53 function, and mutation of Bax, for example, may protect tumor cells from apoptosis following activation of p53 *(242).* Taken together, it seems likely that loss of the normal p53 response pathway, through one mechanism or another, is a common and possibly even obligatory step in cancer development.

Despite the identification of several mechanisms by which p53 activity can be lost, mutation within the *p53* gene itself is likely to be the most efficient mechanism to release the cell from the negative growth control mediated by p53. Mutant forms of p53 that are expressed in many cancer cells can show dominant-negative activity in inhibiting co-expressed wt p53 *(243),* and even show an oncogenic gain of function independent of the expression of wt p53 *(244–246).* Tumor cells in which evasion of p53-mediated tumor suppression is the result of loss of upstream activators of p53, such as $p14^{ARF}$, show only partial protection from p53 activation. The observation that $p14^{ARF}$-negative cells retain the ability to activate p53 in response to DNA damage suggests that tumors of this type may be more sensitive to DNA-damaging chemotherapeutic treatments than tumors with mutations in p53 itself. Furthermore, in addition to loss of function, mutant forms of p53 show dominant transforming activities that may

reflect their ability to oligomerize with, and inhibit the activity of, other p53 family members, such as p63 and p73 *(247).*

The observation that p53 responds to many signals appears to be mirrored in the ability of p53 to function at several stages during tumor development. The p53 response to DNA damaging events, such as UV radiation through excessive exposure to sunlight *(248,249),* could inhibit the accumulation of potentially oncogenic mutations and the initial stages of tumor development, and loss of p53 may also be necessary to tolerate the loss of DNA-repair-associated genes, such as *BRCA-1 (250,251).* Abnormal proliferation, a hallmark of cancer cells, also activates p53, and the ability of E2F-1, both to drive cell proliferation and to induce p53, embodies part of the complex protective mechanism that make cells exquisitely sensitive to abnormal growth. Finally, p53 may also be involved in suppression of later stages of tumor development, responding to hypoxic conditions that occur as tumors grow in size, and inhibiting angiogenesis necessary for progressive tumor growth.

The high frequency of loss of p53 function in a broad range of human tumors has encouraged many attempts to restore p53 pathways in tumor therapy. The prospects for these approaches have been further strengthened by indications that activation of p53 might preferentially kill tumor cells, sparing normal cells by inducing a reversible cell cycle arrest. The basis for this selectivity appears to reside in the deregulation of E2F-1 activity, which occurs in almost all tumors. In addition to stabilizing p53 through $p14^{ARF}$, E2F-1 also sensitizes cells to apoptotic signals in a p53-independent manner *(252),* and activation of E2F-1 and p53 potently induces cell death under circumstances in which activation of p53 alone would lead to only cell cycle arrest *(68).* The concept that tumor cells, with deregulated E2F-1, are more likely to undergo apoptosis in response to p53 than normal cells, has generated a great deal of enthusiasm for the use of p53 in tumor therapy, and several mechanisms for the activation of p53 function are under investigation. Depending on the situation, these could involve inhibition of Mdm2 activity, restoration of $p14^{ARF}$ expression, or, in cells expressing high levels of mutant p53, reactivation of the DNA-binding function of p53 itself *(253,254).* Whatever the mechanism, restoration of p53 function to selectively kill tumor cells in a broad range of major human cancers may be a distant, but attainable goal.

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# 8 p16 Tumor Suppressor

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***CONTENTS***



## 1. INTRODUCTION

p16, a protein named for its migration rate on denaturing gels, is a member of a class of functionally similar proteins called cyclin-dependent kinase inhibitors (CDKI). p16 and its relatives bind to CDKs and inhibit their kinase activity. Because cyclins and CDKs form the core of the cell cycle apparatus, p16 is positioned to directly regulate some of the most basic cell cycle decisions. In eukaryotic cells, the cell cycle is typically described in terms of four component phases: G1, S, G2, and M. Most regulation occurs at the G1–S and G2–M transitions. In higher eukaryotes, the G1 checkpoint is especially important. This is precisely where p16 acts, and its activity is biochemically and functionally linked to other regulators of the G1–S transition, including the well-known cell cycle regulator (and tumor suppressor protein), Rb.

Over the past 5 years, a variety of evidence has accumulated that ties p16 dysfunction to the development of cancer. As an inhibitor of CDKs, p16 is a compelling candidate for a tumor suppressor gene (TSG). Consistent with this view, the *p16* gene is a target for inactivation in many types of tumor, and germ line mutations in *p16* predispose to melanoma. In addition, as the complexities of the *p16* locus have been teased apart, it is becoming apparent that this locus sits astride a second pathway of growth control that involves the notorious tumor suppressor, p53.

Here is reviewed current knowledge about p16, giving special attention to its role in cancer. The chapter addresses the biochemical and physiological functions of p16, the extraordinary molecular biology of the locus, and some of the outstanding questions related to the *p16* gene and its neighboring sequences.

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## 2. P16'S INVOLVEMENT IN HUMAN CANCER

Genes involved in growth control and other aspects of biology relevant to cancer may be identified by mutations in sporadic tumor cells or in the germ line. Certain cancer-related genes are found to be altered only in sporadic tumors; other mutant cancer genes are only seen in the germ line. Most commonly, however, genes involved in cancer predisposition syndromes contain both germ line and somatic mutations. *p16* is a constituent of this latter class, with mutations that occur in sporadic tumors and in the germ line of cancer-prone individuals. Although p16 was initially identified biochemically, based on its interaction with CDK4 *(1),* its role in cancer was first demonstrated by analysis of lesions in tumor cells and cell lines *(2,3).*

### *2.1. p16 Somatic Mutations*

Loss of heterozygosity (LOH), deletions of chromosomal regions or entire homologs, can be found in nearly all tumors and cell lines. These karyotypic abnormalities are not only characteristic of cancer, but in certain instances can also be related causally to tumor progression *(4).* It has been presumed that regions frequently deleted in cancer cells harbor TSGs; inactivation of the resident genes by deletion and/or mutation results in a growth advantage conferred upon the evolving tumor cell *(5).* Often, one member of an allelic pair of genes is inactivated by a detectable deletion, providing the means to localize the underlying mutant gene.

LOH on the short arm of chromosome 9 (9p21) is one of the most common chromosomal aberrations observed in human cancer *(6).* Furthermore, homozygous deletions that remove tens, hundreds, or even thousands of kilobase pairs in 9p21 are relatively common, allowing an especially straightforward route to the presumptive TSG. At the center of these deletions lies the *p16* locus. In one study *(2),* this locus was shown to be deleted in nearly one-half of all cell lines derived from tumors (Table 1). An impressive range of tumor cell lines exhibits such homozygous *p16* deletions, including melanoma, glioma, breast, and bladder malignancies. However, not all types of tumor cell line display *p16* loss; for instance, cell lines from colon cancers and neuroblastomas rarely contain 9p21 homozygous deletions. *p16* deletions are also observed in a variety of primary tumors, although several studies have reported detection of homozygous deletions at lower rates than those observed in cognate cell lines *(8).* This disparity may relate to heterogeneity of primary tumors, growth advantages in tissue culture enjoyed by cells lacking p16, and/or the difficulty of detecting homozygous deletions in primary tumors that contain stromal tissue *(9).*

In contrast to other TSGs, inactivation by mutation is relatively rare in *p16,* compared to homozygous deletion. But, in a fraction of cells derived from tumors that do not harbor homozygous deletions of *p16,* mutant *p16* genes are found. Almost invariably, the mutations occur in tumors that exhibit 9p21 LOH. The smaller lesions include nonsense, missense, and frameshift mutations, and microdeletions. In many cases that have been analyzed, missense mutations have been shown to affect p16's ability to inhibit CDK4 enzymatic activity in vitro *(10–13).* Yet homozygous deletion of the locus is by far the most common mechanism of inactivation. For example, in a survey of melanoma cell lines, 60% contained homozygous deletions; only 15% had mutant *p16 (2).*

Methylation leading to decreased transcription may be yet another mechanism by which *p16* is rendered inactive in tumors. There have been numerous reports of methy-

Table 1
*p16* Mutation/Deletion Frequencies

| *Germ line* | *<1/10,000* |
|---|---|
| *Somatic Homozygous Deletions in Tumor Lines*[a] | |
| | *(%)* |
| Astrocytoma | 82 |
| Bladder | 33 |
| Breast | 60 |
| Colon | 0 |
| Glioma | 88 |
| Leukemia | 64 |
| Lung | 36 |
| Melanoma | 62 |
| Neuroblastoma | 0 |

[a] From ref. *2*.

lation-silenced *p16* alleles in cell lines and primary malignancies. For example, in cancers of the colon, bladder, and lung (nonsmall cell), methylation of C residues in presumptive gene control regions near *p16* has been proposed to interfere with p16 expression *(14–17).* The occurrence of methylated, nontranscribed *p16* genes in tumors is indisputable, but it has been difficult to link methylation causally to tumor progression. Nevertheless, *p16* methylation remains a tenable hypothesis for epigenetic alteration of growth control during tumor development.

### *2.2. p16 Germ-line Mutations*

Germ-line mutations in *p16* were identified through analysis of melanoma-prone kindreds *(7,18).* These kindreds were ascertained and studied because they display unusually high incidence of melanoma, as well as a preponderance of individuals with skin covered by moles, or nevi. Initially, the relationship to moles, thought to be precursors to melanoma, was strongly emphasized. A syndrome called "dysplastic nevus syndrome" (DNS) was defined, characterized by a large number of abnormal (dysplastic) moles and a tendency to develop melanoma *(19).* DNS has been a focus for controversy concerning its diagnosis, genetic basis, and relation to melanoma *(20).* The major melanoma-predisposing gene, *MLM,* was mapped by ignoring DNS in one kindred set, and concentrating on melanoma as a dominant trait *(21). MLM* maps within 9p21, with some kindreds showing high-confidence statistical linkage to markers in 9p21 logarithm of the odds of linkage (LOD scores > 3).

The identification of *p16* as the 9p21-linked target for deletion and mutation in tumor cell lines led to its analysis as a candidate for *MLM (7,18).* To date, a large number of melanoma-prone kindreds have been examined, and numerous germ-line mutations in *p16* (missense, nonsense, frameshift, microdeletion) have been documented *(22,61).* However, in some kindreds with high LOD scores, mutations in the *p16*-coding sequences have not been detected *(7).* This has fostered a belief that another cancer gene located in 9p21 plays a part in familial melanoma *(23).* However, there is no solid evidence for such a gene, and it remains possible that some kindreds may carry *p16*

genes with mutations that lie in noncoding sequences that have not been systematically screened. Indeed, at least one such regulatory mutation has been defined *(24)*.

The overall incidence of germ-line *p16* mutations in the population is probably very low. Estimates of the percentage of familial melanoma, as opposed to nonfamilial or sporadic melanoma, cluster between 5 and 10% *(21)*. Yet, in several studies in which there is some evidence of familiality, but in which linkage analysis is impossible, very few mutations in *p16* have been observed *(7)*. In a few other studies, however, high rates of *p16* mutation have been detected *(25)*. Persons afflicted by sporadic melanoma also rarely carry *p16* mutations. On balance, therefore, it appears that the majority of melanomas typically described as familial, as well as sporadic melanoma, cannot be explained by germ-line mutations in *p16*. Because melanoma afflicts roughly 1% of the U.S. population, the incidence of *p16* germ-line mutations in the population at large is probably very low, perhaps on the order of 1/10,000. Nevertheless, *p16* remains the most significant cancer gene discovered to date that controls melanoma susceptibility.

Germ-line *p16* mutations increase melanoma risk by roughly 50-fold in certain populations, depending on the level of sun exposure and skin pigmentation *(26)*. The penetrance of mutant *p16* alleles, i.e., the probability of incurring disease over a lifetime, is approx 50%. Thus, inheritance of a defective *p16* gene does not guarantee melanoma: It merely increases risk. Because risk can be significantly modified by sun exposure, it is possible to prescribe a lifestyle that, even in the presence of mutant *p16* alleles, should substantially reduce the likelihood of melanoma.

The relationship between *p16* and other malignancies is unclear. The prevalence of *p16* mutations and deletions in many cancer types suggests a role for *p16* germ-line mutations in cancers other than melanoma. Some studies have linked *p16* mutations to increased pancreatic cancer risk, but the effect appears to be substantially less than for melanoma risk *(27)*. Thus, despite *p16*'s involvement in numerous sporadic cancer types, germ-line mutations of *p16* predominantly, if not entirely, alter melanoma risk. This apparent paradox may be resolved by consideration of rate-limiting genetic changes in cancer progression. Perhaps only in melanoma development is p16 inactivation rate-limiting.

The connection between *p16* and DNS is also obscure. Some studies have found a correlation between *p16* mutations and mole number and size *(26)*. If heterozygous *p16* mutations influence moles, they must be dominant or co-dominant, or the nevi must display loss of the wild-type *p16* allele. In support of this view, some reports of 9p21 LOH in dysplastic nevi have appeared *(28)*.

At least one other familial melanoma gene has been discovered, probably affecting less than one-tenth as many people as *p16 (29)*. This second melanoma gene is CDK4, one target of p16's inhibitory action. A specific missense substitution in CDK4 produces a CDK4 protein that is insensitive to p16 inhibition *(30)*. Mutant CDK4 acts as an oncogene, stimulating cell growth even in the presence of wild-type components of the p16–Rb pathway. The elucidation of the role of mutant CDK4 in familial melanoma further underscores the importance of the p16 pathway in cancer (Table 2).

## 3. BIOCHEMICAL FUNCTION OF P16

p16, as a CDKI, binds to specific CDKs. CDKs comprise a variety of types, denoted CDK1–6 in mammals *(31)*. They and their ancillary factors are responsible for transi-

**Table 2**
**Role of p16 in Human Cancer**

| | |
|---|---|
| Germ-line mutation | Melanoma predisposition; pancreatic cancer? |
| Somatic mutation | Immortalization? |
| Somatic overexpression | G1 cell cycle arrest |
| Biochemical activity | CDK4/6 inhibitor |
| Physiological activity | Cell cycle arrest, life-span control |
| Other genes mutated in pathway | *CDK4, Rb1* |

tion through different control points in the cell cycle, the so-called "checkpoints". CDKs are convergence sites for signals emanating from the cell's environment, which affect decisions related to the cell cycle. Thus, CDKs form part of a cellular switch that controls the all-or-none commitments to replicate DNA and undergo cytokinesis.

Cyclins are protein co-factors that form complexes with CDKs. Like the CDKs, cyclins comprise a family of proteins, termed "cyclins A–E" *(31).* Certain cyclins preferentially associate with particular CDKs, and specific complexes regulate the G1–S or G2–M transitions. CDK4(6)–cyclin D and CDK2–cyclin E complexes influence the G1–S choice to proliferate or not. These complexes, when appropriately phosphorylated by the action of additional regulatory kinases and phosphatases, phosphorylate a variety of substrates. The fundamental features of this control mechanism are conserved in all eukaryotes studied, including fungi, plants, and animals. *(31).*

To date, several CDKIs have been identified, including CDKIs from single-cell eukaryotes, such as yeast *(32).* In mammals, these molecules fall into two general classes: the p16 family (p15, p16, p18, p19), which contains sequence motifs present in the cytoskeletal protein, ankyrin, and the p21 family (p21, p27, p57), which shares other homology elements. Although all CDKIs interfere with CDK activity, the mechanism of action of p16 family inhibitors is different from p21 family members *(32).* p16 and its homologs bind to free CDKs, and competitively inhibit binding of the activating cyclin co-factors. In contrast, p21-like inhibitors bind to the CDK–cyclin complex, and block its interaction with substrates. The end result is the same: All CDKIs prevent phosphorylation of CDK substrates.

Certain CDKIs, such as p21, bind to all CDK–cyclin complexes; p16 is more selective *(32):* It specifically binds to CDK4 and CDK6 in the free form (i.e., not in complex with cyclin D). The crystal structure of a complex between p16 and CDK6 has been solved *(33),* providing a detailed, atomic-resolution view of the biochemical interaction. p16 resides near the adenosine triphosphate-binding region of the catalytic site, opposite the cyclin-binding site. p16 binding appears to prevent cyclin interaction by altering the conformation of the cyclin-binding site.

p16 exerts its biochemical effects at the G1–S checkpoint (Fig. 1). CDK4 and CDK6, in association with D-type cyclins, promote transition through the checkpoint. A variety of proteins, including histones, HMG proteins, E2F proteins, and Rb, are substrates for CDKs *(34).* From the perspective of growth control, Rb and E2Fs are especially significant substrates. Rb in its hypophosphorylated form binds several proteins. Among these are members of the E2F family of transcription factors, which play important roles in downstream aspects of growth regulation. E2F1, for example, is

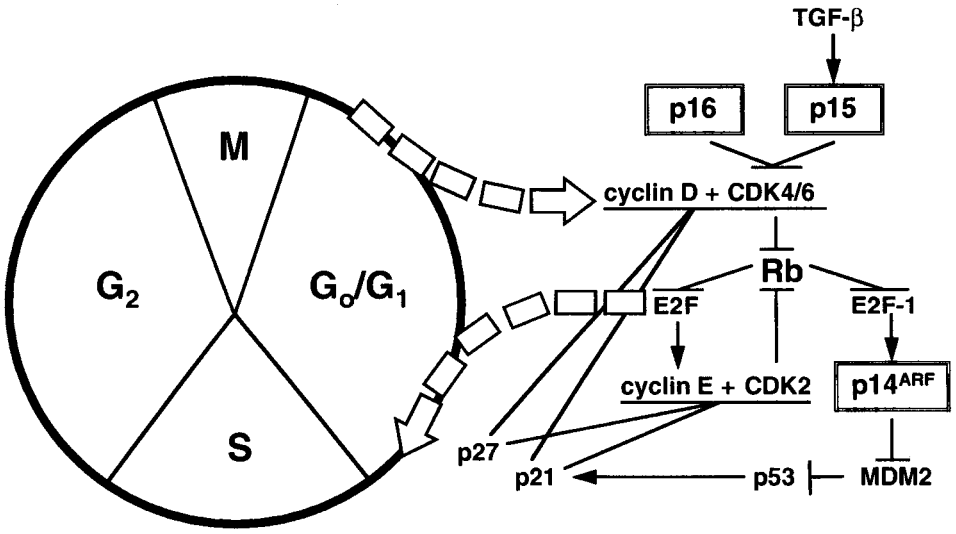


**Fig. 1.** Cell cycle and components of the p16, p15, and $p14^{ARF}$ pathways. Arrows indicate activation; blunt-ended lines indicate inhibition.

implicated in transcription of genes in early S phase, such as thymidylate synthase and dihydrofolate reductase, enzymes involved in synthesis of DNA precursors *(35)*. In addition, cyclin E expression is induced by E2Fs, thus propelling cells across the G1–S boundary. The entire set of biochemical interactions involving p16 and its downstream pathway components supports a model whereby p16 inhibits CDK4/6 by interfering with cyclin D binding; in the absence of phosphorylation by CDK4, Rb binds E2Fs; E2Fs sequestered by Rb are unable to activate transcription of cyclin E to enter S phase, thus trapping cells in G1.

## 4. PHYSIOLOGICAL FUNCTION OF P16

As described above, p16's biochemical function is to inhibit activators of cell division (i.e., specific CDKs). Thus, it has a principal physiological role in regulating cell growth. In this general sense, it differs little from other CDK inhibitors, such as Pho81 and FAR1 in yeast, or p21 and p27. All these molecules participate in control of cell growth. What distinguishes p16 from other CDK inhibitors is its physiological role.

Growth control is important in biology, and there are multiple layers of growth regulation in each cell, as well as a plethora of tissue-specific mechanisms for regulating cell division. Cell growth is obviously critical during development, but it is also important in tissue regeneration and maintenance. Apart from nonmitotic, terminally differentiated cells, all cells retain the ability to divide, and must do so in an appropriate context. In general, a go/no-go decision is made after receiving positive, growth-stimulatory signals from outside and/or a threshold-crossing decrease in growth-inhibitory stimuli. Normal cells not only integrate signals from their environment, but also assess their internal condition. Cellular machinery surveys the integrity of the genomic DNA, the availability of DNA biosynthetic precursors, and the state of the mitotic spindle. If problems are detected, cell cycle progression may be aborted at one of the checkpoints.

This may cause a delay in division, followed by resumption of the orderly cell cycle program, or it may result in apoptosis. The feedback loops which regulate the decisions to proceed, temporarily arrest, or commit suicide are increasingly apparent *(36).* Many components and several independent pathways participate. A number of these components are targets for mutation in cancer.

A second aspect of growth control involves genetically programmed limits on cellular life-span. With the exception of germ-line cells (i.e., embryonic stem cells), normal cells are not capable of indefinite division, and eventually senesce. The genetic determinants responsible for the timing and the mechanism of senescence are beginning to emerge *(37).* Again, some of these components play a role in tumorigenesis.

Cancers progress from relatively benign types, which differ little from normal tissue, to malignant, highly abnormal cell masses. As they evolve, cancer cells gradually lose their dependence on growth signals in their environment. Checkpoint controls go awry, genomes become unstable, and cells avoid senescence to become immortal. Many CDKIs are implicated in specific aspects of tumor progression. For example, p21 is related to the apoptosis decision, and p27 functions in transforming growth factor β (TGF-β)-mediated cell cycle arrest *(38,39).*

p16, however, is unique, because it is involved intimately with tumor progression. p16, in contrast to other CDKIs, is a principal target for mutation during tumor development. Overexpression of p16 (like some other CDKIs) causes G1-phase cell cycle arrest in normal cells, and in many tumor cells. However, in Rb cells that have lost *Rb1* gene function, p16 overexpression does not cause arrest *(40,41).* This result suggests that p16 functions in the same pathway upstream of Rb, an observation consistent with the biochemical roles of the proteins. In addition, *p16* mutations and *Rb1* mutations, though commonly found in many tumor types, are seldom found in the same tumors, suggesting that inactivation of one of the components is sufficient to compromise the pathway *(42).*

A role of p16 in cellular senescence has been proposed *(43,44).* Evidence supporting this proposition comes from studies of p16 levels in aging cells. As cells approach the crisis point at which cellular life-span controls come into play, p16 mRNA and protein levels rise. In cells that traverse this point, p16 levels fall dramatically. This senescent state can be mimicked in younger cells by overexpression of the *ras* oncogene, causing G1 arrest and buildup of p16 and p53 *(43).* Inactivation of p16 permits escape from this condition, at least in certain cells. These studies support the view that p16 participates in the physiology of cellular life-span.

One observation that does not fit neatly with the senescence model for p16's physiological role involves the timing of p16 loss in tumor development. In certain tumor types that have been studied carefully, *p16* gene inactivation appears to occur early during progression *(6,62).* Because immortalization is generally considered to be a late-stage tumor phenotype, these findings imply that p16 has a different activity regarding tumor formation. It is possible that p16 has multiple physiological roles, at least one of which is relevant in early phases of tumor growth, and another in the later stage of immortalization.

Genetic analysis of p16 mutations in mice prove the hypothesis that p16 functions as a tumor suppressor in vivo. Gene knockouts (i.e., homozygous, loss-of-function mutations) are revealing with respect to p16's part in physiology. Homozygous-null p16 mice are viable, demonstrating that p16 is neither essential for the survival of normal

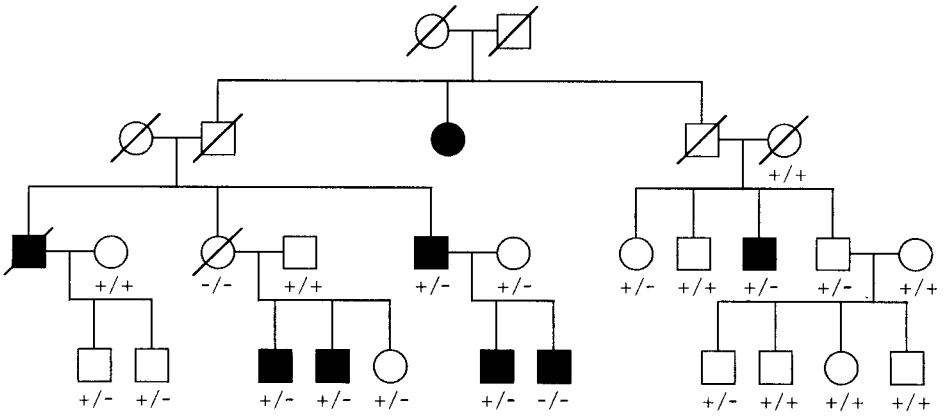


**Fig. 2.** Pedigree of Dutch melanoma-prone family segregating a *p16* microdeletion. Filled circles/squares indicate individuals affected by melanoma. The *p16* genotype is shown below each family member. Note the presence of two *p16* homozygous nulls (–/–) in the pedigree, one affected, one unaffected *(46)*.

cells, nor for the proper development of the mouse *(45)*. Homozygous-null mutants develop tumors more readily than control animals, confirming a cancer-predisposing role for p16 in mammals. Furthermore, fibroblasts isolated from the homozygous-null mice are demonstrably different from normal mouse fibroblasts. These fibroblasts are exceptionally sensitive to transformation by introduced *ras* oncogenes, suggesting that they have sustained at least one hit in their growth-control pathways. Heterozygous *p16*-mutant mice are not cancer-prone, however, unless they are stressed with carcinogens *(45)*.

Homozygous p16 human knockouts also exist *(46)*. Two individuals have been identified who inherited the same defective *p16* allele from each parent (Fig. 2). Both individuals were normal, although one developed two primary melanomas by age 15 yr. The other individual, however, was melanoma-free, dying at age 55 yr of an adenocarcinoma. These observations confirm that, as in mice, *p16* is not an essential gene. Furthermore, they illustrate that the same cancer-predisposing mutation can produce different consequences depending on genetic background, environmental factors, or random biological processes.

## 5. MOLECULAR BIOLOGY OF *P16* LOCUS

The *p16* gene and its neighboring sequences comprise a locus of exceptional interest and complexity. Two genes other than *p16* contribute to this complexity: *p15* and $p14^{ARF}$ (*p16E1*β). Together, they endow *p16* with some of the most fascinating and surprising molecular biology of any human locus.

*p16* consists of three exons: E1α, E2, and E3. E3 contains only three codons, with the remainder of the coding sequence roughly split between the first two exons *(48);* (Fig. 3). All the exons are remarkably GC-rich, averaging 70% G or C residues. The p16 mRNA gives rise to the 156-amino-acid-long p16 protein *(1,48)*.

Located about 12 kilobase pairs upstream from E1α is another exon, E1β, which is spliced to E2 and E3, forming a second transcript type, the β transcript, originating

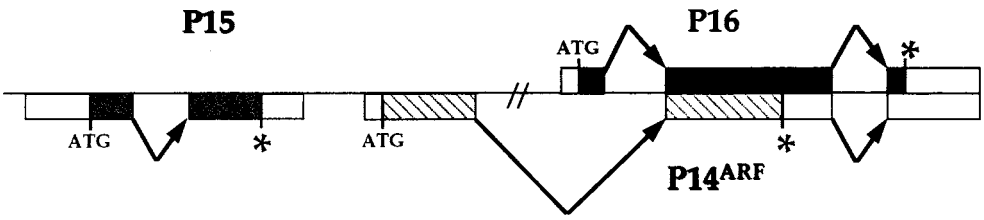


**Fig. 3.** *p16* locus. Asterisks indicate translation termination sites; ATG indicates translational initiation. Distances between genes and exons not drawn to scale.

from the *p16* locus. This transcript is not alternatively spliced from the p16-encoding transcript; rather, it is generated from an independent promotor upstream from E1β *(48).* If translated in the p16 reading frame, the β transcript could initiate translation at a methionine codon located in E1β, eight codons from the E1β–E2 junction. This hypothetical protein would be approx 10 kDa. A second alternative open reading frame exists, which could produce a 14 kDa protein (19 kDa in mouse), if translated. This protein has been detected in vivo, and shown to produce cell cycle arrest when expressed at high levels in cultured cells *(49).* Moreover, knockout constructs that specifically target E1β give rise to mice that resemble *p16*-null mice in phenotype *(50).* These findings have produced a flurry of inquiries directed at $p14^{ARF}$.

Despite intensive efforts to find mutations that specifically affect the $p14^{ARF}$ protein, none has been identified. To date, all *p16* germ-line and somatic mutations that have been described affect the p16 protein, though some also alter $p19^{ARF}$ (Table 3). The only lesions that affect the β transcript, leaving *p16*α intact, are deletions. Several examples of homozygous deletions that disrupt the β transcript, but leave the *p16* mRNA intact, have been defined *(47,63).*

Further investigation of the $p14^{ARF}$ protein has uncovered several other features of special relevance to its role in cancer. $p14^{ARF}$ appears to activate p53 by blocking its degradation *(51–55).* Moreover, one of Rb's targets, E2F1, directly induces $p14^{ARF}$ transcription. Thus, when Rb levels fall, E2F levels rise, leading to increased $p14^{ARF}$ expression. This in turn elevates p53 levels, potentially providing a feedback circuit that compensates for failure of the Rb pathway. It has also been observed that activated Ras protein induces $p14^{ARF}$ expression, causing apoptosis via the p53 pathway *(56).* Again, the implication is that the *p16* locus and p53 are functionally intertwined, connected through $p14^{ARF}$. $p14^{ARF}$'s involvement with the p53 pathway may have deep biological significance, bridging the p16–Rb pathway and the p53 pathway.

Immediately upstream of E1β is a third gene, *p15,* that encodes a protein with substantial similarity to p16. *p15* comprises two exons that are, like *p16* exons, extremely GC-rich. The *p15* and *p16* genes are similar over their entire length, but especially so near their 3′ ends. Comparative studies of these genes in humans and mice indicates that they were involved in a gene conversion event that occurred about the time of the establishment of the ape lineage *(57).* The p15 protein is essentially indistinguishable from p16 in its binding and inhibition of CDK4 and 6, but, despite this similarity, *p15* does not appear to be a solitary target for mutation in cancer (Table 3). No germ-line mutations in *p15* have been found, although the gene has been subjected to intense scrutiny, particularly in 9p21-linked melanoma kindreds for which *p16* mutations have

Table 3
Genetics of *p16* Locus

| | *p16* | *p15* | $p14^{ARF}$ |
|---|---|---|---|
| **Somatic (human and mouse)** | | | |
| Homozygous deletions | Yes | Yes | Yes*[a] |
| Microlesions | Yes | No | No |
| Methylation | Yes | Yes | – |
| **Germ line (human)** | | | |
| Heterozygous phenotype | Yes | – | – |
| Homozygous phenotype | Yes (viable) | – | – |
| **Germ line (mouse)** | | | |
| Heterozygous phenotype | No | – | No |
| Homozygous phenotype (knockout) | Yes (viable) | – | Yes |

[a] All homozygous deletions that affect $p14^{ARF}$, but not *p16,* also affect *p15.*

not been detected. In addition, virtually no somatic mutations have been seen in *p15* in melanomas or other tumor types *(47).* The majority of homozygous deletions that remove *p16* also delete *p15.* However, apart from the few that also affect E1β, no deletions remove *p15* alone, leaving *p16* intact. Thus, there is no evidence that *p15* is involved in cancer as a mutation target.

All three genes of the *p16* locus are regulated independently: *p15* is upregulated in response to TGF-β in certain cell lines; *p16* E1α and E1β are not *(58).* There is no appreciable cell cycle regulation of E1α transcription in lymphocytes; E1β levels oscillate, depending on cell cycle phase *(47).* E1α expression is substantially higher in Rb cells, suggesting a role in a feedback loop with Rb; the expression of the other transcripts is unchanged.

## 6. OUTSTANDING QUESTIONS

Since the discovery of p16 in 1993, progress has been made in understanding its role in normal tissues and in tumorigenesis. However, several aspects of p16 biology remain to be clarified. The central question pertains to the difference between p16 and other CDKIs. Why is the *p16* gene an attractive target for mutation, but other CDKI genes are never, or infrequently, mutated? This question applies especially to *p15* and $p14^{ARF}$, which do not suffer mutations in the manner of *p16.* It is of course possible that the prevalence of *p16* homozygous deletions reflects the presence of neighboring genes, *p15* and $p14^{ARF}$, which can be inactivated jointly by a single deletional event. However, the puzzle remains as to why p16 has point mutations but other genes such as p15 do not.

Although several components of the p16 pathway have been defined, the upstream molecules that regulate *p16* expression have not been elucidated. These factors are especially important, because it is likely that they are the key to p16 physiology. The biochemical similarity between p15 and p16 implies that their different physiological roles may result from idiosyncrasies of gene regulation. If p16 plays a major role in controling cellular life-span, it will be interesting to dissect the circuitry that links growth limits to p16 expression.

A further intriguing aspect of p16 biology involves the peculiar structure of the *p16* locus. A teleological explanation for *p16* and $p14^{ARF}$ is not obvious. What, if anything, is the value of such densely packed genetic information? In the case of certain bacteriophages, it is possible to rationalize overlapping genes, based on maximizing protein-coding sequence in the face of DNA packaging constraints. But, in light of the profligate use of DNA among mammals, it is not clear what evolutionary pressures underpin the compressed features of the *p16* locus.

Even more uncertain is the potential of p16 as a genetic diagnostic or prognostic indicator of disease. In the realm of risk analysis, it seems likely that germ-line screening for *p16* mutations will be a useful strategy for preventing melanoma. Sun exposure is a great risk factor for melanoma that can be conveniently reduced. Thus, genetic information could be used to encourage lifestyle modification. But the low frequency of *p16* germ-line mutations in the population may produce economic barriers to testing that must be overcome by, e.g., technical advances in screening. Somatic *p16* mutations may also ultimately prove useful in directing cancer therapy *(59).* However, clinical correlation studies must be completed before this type of molecular pathology has a significant impact on therapy and outcome.

As always, therapeutic implications of a cancer gene, such as *p16,* are most difficult to predict. Several companies are presently developing and testing drugs that mimic CDKIs. The obvious shortcoming of this approach is the likelihood of side effects. p16 overexpression in normal cells causes growth arrest. Thus, dividing cells, including hematopoietic stem cells and intestinal epithelial precursors, would probably also experience arrest. Gene therapy, with functional *p16* sequences to complement loss in tumors, remains an appealing prospect. However, tumor targeting and escape are formidable problems. An alternative strategy is to use *p16* to arrest and protect normal cells prior to exposure to therapeutic doses of cancer drugs *(60).* Alesson learned from the study of p16 is that even when a cancer gene is relatively well understood, such as *p16,* the inherent complexities of physiology provide daunting obstacles to therapy that must be overcome.

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# 9 DNA Mismatch Repair in Tumor Suppression

*Guo-Min Li, PhD, Scott McCulloch, PhD, and Liya Gu, PhD*

***Contents***



## 1. INTRODUCTION

It is believed that cancer is caused by mutations *(1)*. In addition to the mutations induced by DNA damage, mutations can arise from mismatched base pairs (bps) generated during DNA replication and recombination. However, to avoid mutagenesis, cells possess mutation avoidance systems, one of which is the DNA mismatch repair (MMR) pathway. MMR is the primary cellular pathway that is responsible for correcting mispairs that arise during normal DNA metabolism. In bacteria, the importance of MMR in maintaining genomic stability was demonstrated with the observation, made more than 25 years ago, that defects in this pathway lead to elevated spontaneous mutability *(2,3)*. Inactivation of MMR in eukaryotic cells results in a mutator phenotype, and thus MMR is also crucial in maintaining genomic stability in eukaryotes. A dramatic example of this fact is the direct association of MMR deficiency with human hereditary and sporadic cancer.

There are multiple MMR pathways in both bacteria and eukaryotic cells. In the past several years, a rapid increase in knowledge of MMR in yeast and humans has led to the current understanding of the molecular mechanism(s) of MMR in eukaryotic cells. The focus of this chapter is human MMR and its role in cancer avoidance. Because the human MMR pathway and understanding of it are based on its homology to the *Escherichia coli* methyl-directed and MutS/MutL-dependent pathway, the *E. coli* pathway is discussed briefly. Readers interested in other MMR pathways and MMR in other organisms are referred to a number of excellent reviews *(4–13)*.

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## 2. *E. COLI* METHYL-DIRECTED MMR

The best-characterized MMR pathway is the *E. coli* methyl-directed and MutHLS-dependent repair system, which has been reconstituted using 10 purified proteins *(14).* Among these proteins, MutS, MutL, and MutH are the three key components specifically required for this system. The other proteins involved in the process are UvrD (helicase II), single-stranded DNA-binding protein (SSB), three exonucleases (ExoI, RecJ, and ExoVII), DNA polymerase III holoenzyme, and DNA ligase. The MutS protein (97 kDa) is the mismatch recognition protein that binds to both base–base mismatches *(15)* and small nucleotide (1–3) insertion/deletion (ID) mispairs *(16).* The function of MutL is not clear, although it interacts with MutS, and is required for activation of MutH *(17,18).* Recently, MutL has been shown to load UvrD to the repair initiation site *(19),* and to possess an adenosine triphosphatase (ATPase) activity *(20).* Since the interaction between MutS, MutL, and MutH is an ATP-dependent reaction *(17,18),* the ATPase activity of MutL may drive this process. MutH is a latent endonuclease, which nicks hemimethylated d(GATC) sequences on the unmethylated strand, when in its activated form *(21).* This strand-specific nicking activity of MutH overcomes the dilemma that exists when both bases in a mismatch are the normal components of a Watson-Crick bp. Normally, DNA in *E. coli* is methylated at the $N^6$ position of adenine residues in d(GATC) sequences. But newly replicated DNA is transiently unmethylated in these sequences, which provides a signal for MutH to identify and target repair to the newly synthesized daughter strand. The requirement for d(GATC) hemimethylation and MutH is bypassed, if mismatched DNA contains a pre-existing strand break *(14).*

A model illustrating current understanding of the *E. coli* methyl-directed MMR pathway is shown in Fig. 1. Mismatch correction is initiated by the binding of a MutS homodimer to the mismatch. The MutS–DNA complex recruits MutL and MutH proteins to the repair complex, in a reaction dependent on ATP. The repair complex then bidirectionally draws flanking DNA through itself, to form an α-loop structure *(22).* When the protein complex encounters a hemimethylated d(GATC) site, activated MutH binds to the sequence, and cleaves the unmethylated strand in the DNA substrate. With the help of MutL, UvrD loads at the nick *(19,23).* UvrD unwinds the duplex from the nick toward the mismatch, revealing a ssDNA region of the unnicked strand to which SSB binds, preventing attack on it by nucleases. Depending on the position of the strand break relative to the mismatch, exonuclease I (3′–5′ exonuclease) or exonuclease VII or the RecJ exonuclease (both 5′ and 3′ exonucleases), degrades the nicked strand from the nicked site up to and slightly past the mismatch. The resulting single-stranded region is filled in by DNA polymerase III holoenzyme, and the nick is sealed by DNA ligase.

The *E. coli* methyl-directed and MutHLS-dependent MMR pathway possesses the following features: First, it is strand-specific, i.e., the system can recognize which strand is correct and only replace the incorrect base; second, the repair is bidirectional, being able to process mispairs (base–base and ID) that lie either 5′ or 3′ to the strand discrimination signal *(24,25);* finally, the system has a defined, broad specificity as to what type of substrates it can process. These characteristics of *E. coli* MMR depend on MutS, MutL, and MutH proteins: For example, the bidirectional capability of the system, which is achieved through the action of exonucleases, requires functional MutS

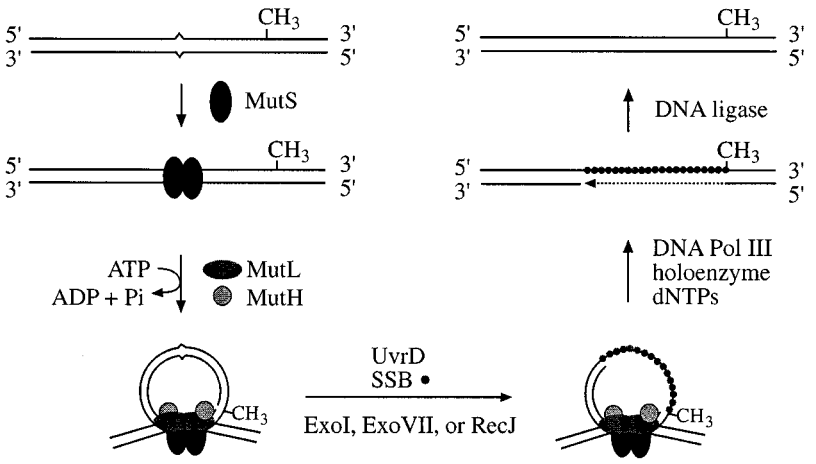


**Fig. 1.** Mechanism of methyl-directed MMR in *E. coli.*

and MutL. Although *E. coli* methyl-directed MMR is the best-studied MMR system, it is far from being perfectly understood. Further studies are required to address many unanswered questions, such as characterizing the interaction between MutS, MutL, and MutH, and precisely defining the function of MutL.

## 3. MMR IN HUMAN CELLS

Human cells possess a MMR pathway homologous to the *E. coli* methyl-directed and MutHLS-dependent system *(26,27).* As shown in Table 1, these two pathways share many similarities. Like the *E. coli* pathway, the human system efficiently repairs both base–base mismatches and small ID mispairs *(26–30).* However, human cells have a broader substrate specificity than *E. coli,* as evidenced by their ability to efficiently repair C–C mismatches and ID mispairs larger than four nucleotides *(26,28–33)* which cannot be corrected by the *E. coli* system. The strand discrimination signal in human cells does not appear to involve methylation *(26),* but it does involve recognition of and targeting to a strand containing a pre-existing strand break *(31).* This suggests that, like the *E. coli* reaction, the strand-specific repair in human cells is also nick-directed. As in *E. coli,* the human MMR reaction is capable of bidirectional processing of a mispaired base. Regardless of the polarity (5′ or 3′) of the strand break, relative to the mismatch, mismatch-provoked excision always occurs between the nick and the mismatch *(31).*

A search has been undertaken to find related protein components that are involved in the *E. coli* and human MMR systems. This approach has provided convincing evidence that the human strand-specific MMR reaction is a homolog of the *E. coli* methyl-directed and MutHLS-dependent MMR reaction. Human homologs of *E. coli* MutS and MutL, two of the three known MMR-specific *E. coli* functions, have been identified and shown to be required in the human MMR pathway (*see* Subheading 3.2.).

The DNA resynthesis step in human nuclear extracts is sensitive to aphidicolin, an inhibitor for eukaryotic DNA polymerases α, δ, and ε, suggesting that MMR in human cells may require replicative DNA polymerases *(26,27,31).* The requirement for prolif-

Table 1
Similarities Between MMR in *E. coli* and Human Cells

| | *E. coli* | *Human* |
|---|---|---|
| Substrate | Base–base and ID mispairs | Base–base and ID mispairs |
| Strand discrimination signal | Methyl group-directed strand break | Single-strand nick |
| Bidirectionality | Yes | Yes |
| Protein components | | |
| MMR-specific | MutS homodimer | hMutSα, hMutSβ |
| | MutL homodimer | hMutLα, hMutLβ |
| | MutH | ? |
| Helicase | UvrD | ? |
| Exonuclease | ExoI, RecJ, ExoVII | ExoI? |
| Replicative polymerase | PolIII holoenzyme | Polδ, PCNA |
| ssDNA binding | SSB | RPA |
| Ligase | DNA ligase | ? |

erating cellular nuclear antigen (PCNA), an accessory factor for DNA polymerases δ and ε during MMR-associated DNA resynthesis, supports the involvement of one or both polymerases in MMR *(34)*. The identification of DNA polymerase δ in the human strand-specific MMR reaction *(35)* has assigned a role for the polymerase in MMR, although it remains possible that the other two aphidicolin-sensitive polymerases are also involved in MMR in human cells.

Activities involved in the unwinding and excision steps of the human reaction have not been specified. A human homolog of yeast EXO1 *(36)* has been proposed to participate in human MMR, based on the interaction of the yeast protein with MSH2 *(37)*. However, given the participation of EXO1 in a number of DNA transactions, its actual role in MMR awaits further characterization. Recently, human ssDNA-binding protein RPA has also been implicated in human MMR *(38,39)*. The discovery of similarities in substrate specificity, component activities, and repair mechanism(s) in prokaryotes and eukaryotes has greatly advanced current understanding of the human MMR pathway.

### *3.1. Function of Human MutS Homologs*

The first identified human gene homologous to *E. coli MutS* was *hMSH3,* initially called *DUG-1 (40)*. In fact, the *hMSH3* gene was identified fortuitously during study of the *DHFR* gene promoter, which is a divergent promoter that also regulates the expression of *hMSH3*. Even though the gene was identified 10 yr ago, the actual function of *hMSH3* was only determined recently. By virtue of their homology to bacterial MutS, additional human MutS homologs were identified, including *hMSH2 (41,42), hMSH4 (43),* and *hMSH5 (44,45),* thanks to the pioneering work in yeast by Kolodner et al. *(46,47)*. *hMSH6* was cloned based on partial protein sequence information obtained from peptides of the 160 kDa G–T mismatch-binding protein, also called GTBP *(48–50)*. All MutS homologs identified to date share a highly conserved carboxy-terminal region, which includes the consensus ATP-binding site. Among these human MutS

homologs, only hMSH2, hMSH3, and hMSH6 are known to participate in strand-specific MMR in human nuclear extracts.

The human MSH2 protein was initially thought to be the human mismatch recognition protein, because it binds with high specificity to both base–base mismatches and ID mispairs *(51,52).* However, later experiments revealed that functional human mismatch recognition activity involves an hMSH2-containing heterodimer. This was illustrated by co-purification of the 105-kDa hMSH2 with a 160-kDa polypeptide *(30).* This heterodimer, designated as hMutSα *(30),* was subsequently reconstituted using recombinant hMSH2 and hMSH6 proteins *(53,54).* Although the identification of hMutSα as a mismatch recognition complex was a step forward, the existence of this complex is not sufficient to adequately explain the distinct mutator phenotypes of *hMSH2*- and *hMSH6*-mutant cells. Tumor cells with mutations in *hMSH2* are defective in repair of both base–base mismatches and small ID mispairs *(29,30,55).* Accordingly, these cells exhibit an elevated number of mutations in both the *HPRT* gene and in simple repeated sequences *(56). hMSH6* mutant cells are deficient in repair of base–base and single-nucleotide ID mispairs, but partially proficient in the processing of ID mispairs that are two nucleotides or larger *(30,57).* This observation is consistent with the fact that *hMSH6*-deficient cells have no detectable instability in di- and trinucleotide repeated sequences *(58),* while displaying hypermutability in single-base ID at the *HPRT* locus *(57,58).* As expected, purified hMutSα restores full MMR activity to both types of mutants *(30).* These findings strongly suggest that base–base mismatches and ID mispairs are recognized by different human MutS homologs, with the former recognized by hMutSα and the latter recognized by an activity involving hMSH2, but not hMSH6 *(30).*

This hypothesis has been confirmed by experiments in both yeast and humans. Marsischky et al. *(59)* demonstrated that yeast *MSH3* mutants show a low but increased rate of frameshift mutations, but essentially no increase in the rate of base substitutions. However, the *MSH3* and *MSH6* double mutants display the same phenotype as *MSH2* single mutants. It was concluded that there are two mismatch recognition heterodimers: one that consists of MSH2 and MSH6, and the other that consists of MSH2 and MSH3. Indeed, there is evidence for physical interaction between MSH2 and MSH3 *(59,60)* and a MSH2–MSH3 heterodimer, which is referred to as MutSβ *(33,61),* has been isolated from human and yeast cells in native form *(33)* and recombinant form *(53,61,62).* Recently, protein interaction domains mediating the interface between MSH2 and MSH6, and between MSH2 and MSH3, have been identified *(63,64).* MSH2 interacts with MSH3 and MSH6 through the same domain *(64).*

The function of MutS-like proteins is to recognize mispairs. The mismatch binding capability of hMutSα and hMutSβ heterodimers has been demonstrated, indicating that these protein complexes are indeed mismatch recognition proteins. hMutSα is capable of binding to both base–base mismatches and ID mispairs *(30,53,54),* but hMutSβ only recognizes ID mispairs, which is consistent with the in vitro repair data *(30,33).* This result substantiates the earlier proposal by Marsischky et al. *(59)* regarding the substrate specificity of these two MutS heterodimers.

In addition to mismatch recognition, MutS-like proteins also possess a weak intrinsic ATPase activity *(65–67),* which has been associated with hMutSα *(54,68).* A clear function of the ATPase activity has not yet been established, but it is likely to be important, because a single mutation in the MutS consensus ATP-binding domain leads to a

mutator phenotype *(65,67).* ATP affects several key steps of MMR process: ATP negatively regulates the binding of MutS homologs to mismatches *(30,54,68);* ATP is required for interactions between MutS and MutL homologs *(34,69);* and it drives translocation of MMR proteins along the DNA helix *(22,33)* or the molecular switch model proposed by Fishel et al. *(68,70).* Thus, ATPase may play an important role as a motor for MutS homologs in mediating these transactions.

### *3.2. Function of Human MutL Homologs*

Three human MutL homologs (hMLH1, hPMS1, and hPMS2) have been identified *(71–73),* based on their homology to the *E. coli* and yeast MutL proteins. Early genetic studies on *Saccharomyces cerevisiae* mutants with increased postmeiotic segregation (PMS) identified the yeast MutL homolog, *PMS1 (74).* The homology between yPMS1 and bacterial MutL protein has led to identification of *yMLH1 (75)* and *yMLH3 (11,76),* and the three mammalian *MutL* homologs *(71–73,77,78).*

Similar to the eukaryotic MutS homologs, functional human MutL homologs are also heterodimers. In fact, the concept of Mut homologs as heterodimers originated from the studies of yeast MutL homologs. Based on the indistinguishable mutator phenotypes of *yMLH1–yPMS1* double mutants and either *yMLH1* or *yPMS1* single mutants, Prolla et al. *(75)* proposed that these two MutL homologs exist as a heterodimer that functions in the MMR pathway. This idea is supported by the demonstration that yMLH1 and yPMS2 physically interact with each other *(75,79,80).* However, more direct evidence supporting the heterodimeric form of the MutL homolog was the co-purification of hMLH1 and hPMS2 (human homolog of yPMS1) from HeLa nuclear extracts, which was designated hMutLα *(81).* Although efforts to determine the biochemical function of hMutLα have not yet succeeded *(81),* it was established that individual components of hMutLα are absolutely required for strand-specific MMR. Cells defective in either *hMLH1* or *hPMS2* are hypermutable, and are deficient in the repair of both base–base mismatches and ID mispairs *(28,82,83).* hMutLα is capable of fully restoring MMR to mutants of *hMLH1 (81)* and *hPMS2* (Li, unpublished results).

The MMR pathway in both *E. coli* and humans possesses a bidirectional processing capability *(25,31),* i.e., it is capable of removing mismatches either from a 5′ to 3′ orientation or from a 3′ to 5′ orientation, depending on the relative position of the strand break and the mismatch. MMR components that have a polar activity are helicases and exonucleases. The authors have found an *hMLH1*-deficient cell line that is only defective for repair in one orientation, suggesting that human MutL homologs are involved in selecting MMR orientation. This cell line possesses a wild-type (wt) *hMLH1* gene, but the expression of the gene is inhibited by hypermethylation of its promoter region. As a result, this cell line is defective in the repair of heteroduplexes with a strand break 3′ to the mismatch, but proficient in the processing of heteroduplexes with a strand break 5′ to the heterology (Li and Modrich P, unpublished results). A similar result has also been found in a cisplatin-resistant cell line with defective *hMLH1 (83).* Also, though cells defective in *hMLH1* fail to carry out repair in a 3′ to 5′ orientation, a cell line defective in *hPMS2* has been shown to be defective in repair in the 5′ to 3′ orientation *(84).* It was recently observed that *E. coli* MutL plays a role in loading UvrD at the site of the strand break *(19,23).* Thus, the association of human MutL homologs with

repair in a specific orientation suggests that hMLH1 may be involved in delivering a helicase or exonuclease to the 3′ nick accordingly hPMS2 may be responsible for directing these enzymes to the 5′ nick. Further biochemical studies are required to address and resolve these issues.

The role of hPMS1 in MMR has not been determined. However, a recent study in yeast has demonstrated that deficiency in *MLH3,* a yeast homolog of *hPMS1,* causes a small but significant increase in the frameshift mutation rate. This phenotype is similar to the phenotype of *yMSH3* single and *yMSH3–yMLH3* double mutants, suggesting that MSH3 and MLH3 are involved in the same ID mispair correction pathway *(76).* In addition, this study showed that yMLH3 interacts with yMLH1 in a two-hybrid system, implying the existence of a second heterodimeric MutL complex consisting of yMLH1 and yMLH3 *(76).* Given the close homology between MMR systems in all species, it would not be surprising if a second human MutL heterodimer of hMLH1–hPMS1 were identified. Indeed, Jiricny et al. have recently found that hMLH1 and hPMS1 proteins co-immunoprecipitate with each other, both in HeLa nuclear extracts and in baculovirus recombinant proteins, although the biochemical function of this complex has not been determined *(84a).*

The bacterial MutL protein has been described as a molecular matchmaker *(85)* or a molecular chaperon *(12)* that facilitates the interactions between MutS and MutH. Although direct evidence for human MutL homologs as molecular matchmakers or chaperons is lacking, hMutLα has been shown to be crucial for formation of the initiation complex. Gu et al. *(34)* have demonstrated that hMSH2, hMLH1, hPMS2, and PCNA can be co-immunoprecipitated in HeLa nuclear extracts, but this co-precipitation is not observed in the extracts derived from *hMLH1*-deficient cells. In addition to enhancing protein–protein interactions, MutL homologs have been shown to enhance the binding of MutS homologs to mismatches in DNA *(79,86).*

Taken together, a model can be proposed for the role of MutS and MutL homologs in human MMR (Fig. 2). Mismatch recognition involves two heterodimers of human MutS homologs, hMutSα and hMutSβ: hMutSα recognizes both base–base and ID mispairs; hMutSβ binds only to ID mispairs. The substrate specificity of these two complexes implies that human cells may use hMutSα in routine repair processes, and hMutSβ as a partial backup system, although both complexes function redundantly on ID mispairs. A number of lines of evidence support this view. First, cells with genetic deficiency in *MSH6* display a much more severe mutator phenotype than *MSH3*-defective cells *(87,88),* because the former cells are defective in base–base mismatch correction, but retain partial repair activity on ID mispairs *(30).* Second, the overexpression of MSH3 (which competes with MSH6 for MSH2) leads to a strong mutator phenotype, despite the fact that these cells are proficient in ID mismatch correction *(89,90).* Third, under normal circumstances, human cells possess much more hMutSα than hMutSβ *(33,89).* The two hMutL heterodimers, hMutLα and hMutLβ (hMLH1 and hPMS1), may have a similar relationship. hMutLα seems to interact with both hMutSα and hMutSβ; hMutLβ only interacts with hMutSβ. The proposed roles for hMutL homologs are consistent with genetic studies in yeast *(76),* mice *(91),* and human tumors, in which only a limited number of germ-line mutations are documented in *hPMS1* and *hPMS2;* a relatively large number are reported in *hMLH1* (see Table 2).

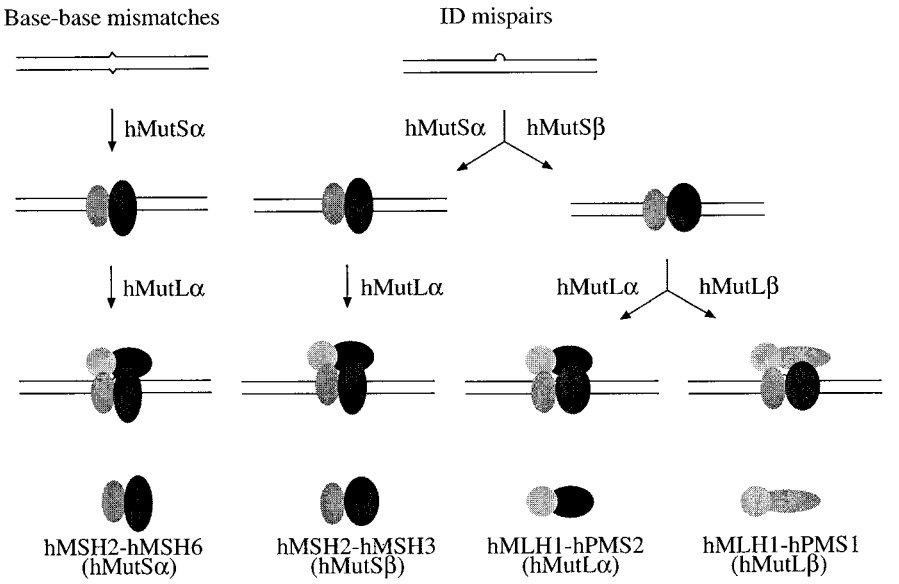


**Fig. 2.** Proposed function for MutS and MutL homologs in human MMR.

## 4. MMR DEFICIENCY AND COLORECTAL CANCER

MMR has long been postulated to be an important cellular mechanism to prevent genomic instability. However, it was not implicated as a cause of cancer susceptibility until 1993, when MMR defects were associated with hereditary nonpolyposis colorectal cancer (HNPCC) and a subset of sporadic colon cancers (CCs). HNPCC is a heritable autosomal dominant disease that is defined by the presence of at least three family members with CC in two successive generations, with one affected member having been diagnosed at less than 50 yr of age *(92).* HNPCC accounts for 4–13% of all CC *(93),* one of the most common forms of neoplasia in Western populations *(94).* In addition to CC, patients with HNPCC are at increased risk to develop cancers of the endometrium, ovary, stomach, urinary tract, brain, and other epithelial organs *(95,96).* Although the HNPCC syndrome was suspected to be a heritable disease more than 85 yr ago *(97),* the molecular pathogenesis of this disease was not established until 1993.

### *4.1. Microsatellite Instability in HNPCC and Sporadic CC*

The discovery of the molecular basis of HNPCC started with the observation of frequent alterations in simple repeated sequences (also called microsatellite instability [MSI]) in HNPCC tumors. Tumor susceptibility genes are often subject to loss of heterozygosity in tumors. To explore the hypothesis that the genetic basis of HNPCC involved a tumor suppressor gene, Aaltonen et al. *(98)* employed microsatellite markers to determine allelic losses in two large HNPCC kindreds in chromosome (chr) 2p15–16, an HNPCC locus identified previously by genetic linkage analysis *(99).* However, allelic losses were not found in this HNPCC locus; instead, they observed insertion or deletion mutations at these repetitive sequences in 11/14 tumors examined *(98).* These unexpected mutations were evident in each di- and trinucleotide repeat

tested, and were referred to as replication error positive *(98)*. In addition, those authors also found a subset of sporadic CCs with a similar phenotype, but occurring at a much lower incidence (6/46) *(98)*.

At the same time, two other groups, Ionov et al. *(100)* and Thibodeau et al. *(101),* independently reported microsatellite alterations in 12–15% of sporadic CCs. These observations were made using random microsatellite markers throughout the genome, rather than the markers within the 2p15–16 region, used by the first group. Taken together, these findings suggest that MSI in HNPCC, and a subset of sporadic CCs, is a genome-wide phenomenon, and may be caused by a common mechanism. Although the genetic basis of HNPCC remained unidentified at that time, these studies provided an important clue as to the mechanism of its action.

## *4.2. MMR Deficiency Is the Genetic Basis of HNPCC*

### 4.2.1. Breakthrough

The identification of MSI in CCs received a great deal of attention from cancer investigators, as well as from geneticists and biochemists working on DNA MMR, because the mutational fingerprint is similar in HNPCC and MMR-deficient cells. At that time, the following points had been established: Loss of MMR function leads to genome-wide base–base substitutions, as well as to frameshift mutations *(4,57);* MMR proteins recognize and process ID mispairs *(16);* and repetitive dinucleotide sequences undergo frequent contractions or expansions in MMR-deficient bacterial cells *(102,103)*. Therefore, the hypothesis was made that the genetic defects in HNPCC involve the loss of MMR function.

Several groups tested this hypothesis using different approaches. First, Strand et al. *(104)* examined the stability of poly(GT) tracts in yeast strains with either a single or double knockout of *MSH2, MLH1,* or *PMS1*. All mutants (both single and double mutants) exhibited 100–700-fold elevated levels of tract instability involving insertion or deletion of 2–4 repeated units *(104)*. That study strongly supports an association of MMR defects with the HNPCC syndrome.

Second, two teams, Fishel et al. *(41)* and Leach et al. *(105),* independently searched for human MMR homolog genes, and determined their association with HNPCC kindreds. Both teams reported in succession the cloning of the *hMSH2* gene, using polymerase chain reaction products of degenerate primers derived from two highly conserved regions of the known bacterial MutS and yeast MSH proteins, and located the gene on chr 2p (there was a subtle conflict on the exact position of the gene on the chr) *(41,105)*. Germ-line mutations of *hMSH2* were indeed identified in HNPCC families *(105)*.

Third, two laboratories *(28,29)* took a biochemical approach, and examined the MMR proficiency of tumor cells derived from HNPCC and sporadic CCs with MSI. Both laboratories demonstrated that cell extracts of these tumor cells are completely defective in repair of base–base and ID mispairs *(28,29)*. These in vitro biochemical studies provided definitive evidence supporting the hypothesis that MMR defects are the genetic basis of HNPCC.

### 4.2.2. Genetic Evidence

Immediately after the first HNPCC-linked locus was mapped to chr 2p15–16 *(99),* Lindblom et al. *(106)* identified a second locus linked to HNPCC predisposition at chr

3p21–23. Since the MMR pathway involves multiple components (*see* Subheading 3.) and characterization of the first HNPCC-associated locus led to the cloning of *hMSH2 (41,105),* investigators looked for additional HNPCC-associated human Mut homologs. In a remarkably short period of time, three human *MutL* homolog genes *(hMLH1, hPMS1,* and *hPMS2)* were cloned *(71–73).* Bronner et al. *(71)* identified the *hMLH1* gene, using an approach similar to that used to identify *hMSH2 (41,105).* The gene was mapped to chr 3p, and a missense germ-line mutation was characterized in a family with a history of HNPCC *(71).* At the same time, Papadopoulos et al. *(73)* searched a large database of human cDNAs and also identified the *hMLH1* gene. In addition, they reported two more human *MutL* homologs, *hPMS1* and *hPMS2,* located on chr 2q and 7p, respectively *(72).* Germ-line mutations of each human *MutL* homolog were found in HNPCC kindreds, with defects in *hMLH1* present in the majority of the HNPCC kindreds *(72,73).*

Since the initial identification of HNPCC-linked genes, HNPCC kindreds have been extensively screened for mutations in each of these genes *(107–119).* It is now clear that mutations of *hMSH2* and *hMLH1* are very common, and are found in more than 90% of HNPCC kindreds, but mutations of *hMPS1* and *hMPS2* are rare (*see* Table 2). Less is known about germ-line mutations in *hPMS1* and *hPMS2,* two MMR genes not identified through HNPCC linkage analysis. There is one report of a germ-line mutation in *hMSH6 (120),* and no reports of *hMSH3* mutations in HNPCC. However, somatic mutations in each of the MMR genes in sporadic cancer and cell lines with MSI, as well as in HNPCC, have been documented *(87,121–130),* although mutations of *hMSH2* and *hMLH1* are also predominant. The protein products of these two genes play a central role in MMR, through their essential functions in hMutS and hMutL heterodimers. In contrast, overlapping roles exist for the hMSH3 and hMSH6 proteins, and for the hPMS1 and hPMS2 proteins (*see* above). Thus, the observed distribution of mutations in these genes, in HNPCC and sporadic cancer, is consistent with the relative importance of their functional roles in MMR.

Mice with a knockout mutation in each *MMR* gene are available *(77,78,91,131–134b).* Although most of them display a typical hypermutable phenotype and a predisposition to develop cancer, it is surprising that none of these MMR-deficient mice develop CC as in HNPCC (Table 3). Mice deficient in *MSH2* and *MLH1* demonstrate a similar pattern of cancer development. Both of these mice genotypes usually develop lymphomas *(78,91,131,132),* intestinal adenomas and adenocarcinomas, and, to a lesser extent, skin neoplasms and sarcomas *(78,91,132).* However, the *MLH1*-deficient mice (both male and female) are infertile *(78,133).* In contrast to *MSH2*- or *MLH1*-deficient mice, *PMS2*-deficient animals do not develop intestinal adenomas and adenocarcinomas, although they do appear prone to sarcomas and lymphomas *(77,91).* In *PMS2* knockouts, only male mice are infertile *(77). MSH6*-deficient animals develop a spectrum of tumors, but the most predominant are gastrointestinal tumors and lymphomas *(134),* suggesting that germ-line mutations of *MSH6* may be associated with a cancer predisposition. Unlike all the MMR-deficient mice described above, mice deficient in *PMS1* gene show no instability in repeat sequences, except for a small mutation rate in mononucleotide repeats *(91).* The *PMS1*-knockout mice do not develop any tumors *(91).* The same seems to be true for the *MSH3*-knockout mice (134a, 134b), although MMR-deficient mice do not develop CC, these studies certainly

**Table 2**
**MMR Genes in HNPCC**

| *Gene* | *Chromosomal location* | *Mutations in HNPCC* |
|---|---|---|
| MutS Homologs | | |
| *MSH2* | 2p | 45%[a] |
| *MSH3* | 5q11–13 | None |
| *MSH6* | 2p16 | One family |
| MutL homologs | | |
| *MLH1* | 3p21–23 | 49%[a] |
| *PMS1* | 2q31–33 | One family |
| *PMS2* | 7p22 | 6%[a] |

[a]From ref. *120.*
[b]From ref. *72.*

**Table 3**
**Characteristics of MMR-deficient Knockout Mice**

| *Gene* | *Tumor* | *MSI* | *Other* |
|---|---|---|---|
| *MSH2* | Lymphoma<br>Intestinal adenoma | Yes | |
| *MSH3* | None | Not determined | |
| *MSH6* | Lymphoma<br>Gastrointestinal | No instability in dinucleotide repeats | |
| *MLH1* | Lymphoma<br>Intestinal adenoma<br>Skin tumor and sarcoma | Yes | Male and female infertility |
| *PMS1* | None | Mononucleotide repeats only | |
| *PMS2* | Lymphoma Sarcoma | Yes | Male infertility |

support the view that MMR defects lead to genomic instability, and eventually to cancer, as originally proposed, based on studies of the HNPCC syndrome.

Strong evidence supporting the concept that MMR genes are crucial to genome stability was provided by chr or gene-transfer experiments *(135–139).* Koi et al. *(135)* reported that the transfer of human chr 3, carrying wt *hMLH1* gene to an *hMLH1*-deficient colorectal tumor cell line, restores MMR to the cell line *(135).* Similarly, human chr 2, containing both the *hMSH2* and *hMSH6* genes, can complement MMR defects in *hMSH2*- and *hMSH6*-deficient tumor cell lines *(136,137,140).* Recently, restoration of MMR repair to a *PMS2*-deficient human cell line and a *MLH1*-knockout mouse cell line has been achieved by introduction of *hPMS2* or *hMLH1* genes into these lines, respectively *(138,139).* These studies further confirm that the MMR system plays an essential role in the maintenance of genomic stability, and provides molecular insights with potential for application in HNPCC gene therapy.

### 4.2.3. Biochemical Evidence

The most convincing evidence that the HNPCC syndrome is caused by MMR defects may be the biochemical studies of this disease. Functional biochemical assays of extracts prepared from a number of cell lines, which were derived from HNPCC and sporadic tumors with MSI, have clearly demonstrated that these cells are deficient in nick-directed MMR *(28–30,55,82,128,141–144).* Further characterization of these cell lines has defined at least two in vitro complementation groups *(29,30,55),* which led to the isolation of hMutLα and hMutSα *(30,81).* Using an in vitro complementation analysis, Li and Modrich *(81)* purified a heterodimer from HeLa nuclear extracts that restores strand-specific *MMR* to an *hMLH1*-deficient colorectal tumor cell line. This heterodimer has two components, 85 kDa hMLH1 and 110 kDa hPMS2, and is called hMutLα *(81).* Using a similar approach, Drummond et al. *(30)* isolated hMutSα (105 kDa hMSH2 and 160 kDa hMSH6), based on its ability to restore MMR to tumor cells defective in either *hMSH2* or *hMSH6.*

### 4.2.4. Epigenetic Evidence

As discussed previously, mutations in *MMR* genes, which account for the hypermutable phenotype, are associated with the HNPCC syndrome and a subset of sporadic CCs with MSI. However, in a significant fraction of sporadic MSI colon tumors, no mutations have been identified in *MMR* genes *(124,125,145),* suggesting that a novel mechanism may be involved in causing MSI in these cases. The search for the novel mechanism has linked these tumors again to MMR defects. This time, it is an epigenetic factor, methylation, responsible for suppressing the expression of *MMR* genes *(121,123,143,146–149).*

Kane et al. *(123)* demonstrated that hypermethylation of the *hMLH1* promoter is correlated with a lack of *hMLH1* expression in several sporadic colon tumors and cell lines that are free of mutations in the *hMLH1* gene. Those authors suggested that hypermethylation is probably a common mode of *MMR* gene inactivation in sporadic cancer *(123).* Since then, hypermethylation of *MMR* genes, especially *hMSH2* and *hMLH1,* has been extensively studied *(143,146–149).* According to the Bethesda guidelines *(150),* sporadic tumors can be classified into three types, based on their MSI status: microsatellite stable, low-frequency MSI (MSI-L), and high-frequency MSI (MSI-H). It has been reported that more than 95% of MSI-H tumors are caused by loss of expression of *hMLH1 (151).* Almost all MSI-H tumors that do not have a detectable mutation within the *hMLH1* gene demonstrate hypermethylation in the *hMLH1* promoter *(148,149).* In contrast, hypermethylation of the *hMSH2* gene is not observed in sporadic tumors *(148).*

To determine the nature of the hypermethylation of the *hMLH1* promoter in these MSI tumors, two independent research groups, Veigl et al. *(143)* and Herman et al. *(149),* treated several tumor cell lines deficient in *hMLH1* expression because of hypermethylation in the *hMLH1* promoter with the demethylating agent 5-aza-deoxycytidine. This treatment successfully restores hMLH1 protein expression in all tumor cells that lack *hMLH1* expression because of a methylated *hMLH1* promoter. The expression of hMLH1 is associated with the presence of unmethylated *hMLH1* alleles *(143,147).* More importantly, extracts derived from the drug-treated cells are capable of performing strand-specific MMR *(147).* These experiments indicate that both genetic defects and epigenetic modification in MMR genes can result in a mutator phenotype.

## 5. MMR DEFICIENCY AND NON-CC

### 5.1. Microsatellite Instability in Non-CC

The identification of MSI in HNPCC in 1993 led to the dramatic elucidation of the molecular pathogenesis of this disease. Since then, a great body of work has been published demonstrating that MSI is also associated with a wide variety of non-HNPCC and noncolonic tumors (for detailed reviews, *see* refs. *150,152,* and *153).* These tumors include endometrial *(154–166),* ovarian *(167–173),* gastric *(174–190),* cervical *(163,191–195),* breast *(196–208),* skin *(209–220),* lung *(221–226),* glioma *(227–229),* prostate *(230–238),* bladder *(239–242),* and lymphoma *(243,244)* (Table 4). These studies were carried out using various numbers of different microsatellite markers, and employing different numbers of samples; thus, it is not surprising that the observed mutation rates vary from study to study, and in some cases are not in agreement with one another *(195,210,245–256).* Therefore, it is difficult to draw a firm conclusion based on the MSI work published to date. To address this problem, an international workshop was recently held in Bethesda, Md, to develop international criteria for MSI studies, referred to as the "Bethesda guidelines" *(150).* The workshop resulted in the suggestion that at least five loci should be used in future MSI studies, with instability in one of five loci scored as MSI-L, and instability in two or more loci scored as MSI-H *(150).*

Table 4 shows results of some of the MSI studies in non-HNPCC and noncolonic tumors that can be judged by the Bethesda guidelines *(150).* As observed in sporadic CCs, noncolorectal tumors exhibit the MSI-H and MSI-L phenotypes. Most of the sporadic endometrial and gastric tumors, lung cancers, and lymphomas display a high level of MSI in many markers (Table 4). Some tumors demonstrate greater instability in one marker than another. Tumors with MSI can be divided into two groups: one that displays elevated instability at mono- and dinucleotide markers and, to a lesser degree, at larger-repeat markers, and a second group that displays elevated instability only at specific larger-repeat markers, such as tri- and tetranucleotide repeats. Endometrial and gastric tumors usually belong to the first group; bladder, lung, head, and neck cancers belong to the second group *(150,257).*

The presence of MSI in these sporadic noncolonic tumors stimulated a search for somatic mutations in the *hMSH2* and *hMLH1* genes in these tumors. Initially, no mutations of these two HNPCC genes were detected *(183,258–261).* However, several laboratories have recently demonstrated *(149,162,262)* that the *hMLH1* promoter is hypermethylated in sporadic endometrial and gastric tumors. This result suggests that hypermethylation of *hMLH1,* and its subsequent transcriptional silencing, may be a major cause of sporadic noncolonic tumors with MSI. In addition, somatic frameshift mutations in *hMSH2, hMSH3,* and *hMSH6* have been reported in gastric cancers *(130,263,264).* Biochemical studies have demonstrated that cell lines derived from sporadic endometrial, ovarian, and prostate cancers are defective in strand-specific MMR *(29,55,128).* These findings suggest that MMR defects are a likely cause of noncolonic sporadic cancer with MSI, although other mechanisms may also be involved in causing the MSI mutator phenotype.

### 5.2. MMR Deficiency and Inactivation of Genes Critical for Cellular Growth

The work described above conclusively demonstrates that loss of function in MMR genes can cause HNPCC. This result implies that MMR genes are tumor suppressor

**Table 4**
**MSI in Noncolonic Tumors[a]**

| *Tumor* | *Loci* | *MSI* | *Frequency no. (%)* | *Ref.* |
|---|---|---|---|---|
| Endometrial | Dinucleotide | ≥2/8 loci | 10/109 (9) | *(159)* |
| Endometrial | *D1S126* | ≥2/5 loci | 18/77 (22) | *(259)* |
| | *D2S393* | | | |
| | *D3S1067* | | | |
| | *D5S644* | | | |
| | *TP53* | | | |
| Ovarian | | ≥2/5 loci | 2/68 (3) | |
| Endometrial | Dinucleotide | ≥2/8 loci | 12/68 (18.5) | *(156)* |
| Gastric | *D2S123* | ≥2/5 loci | 15/24 (62.5) | *(333)* |
| | *D2S126* | | | |
| | *D3S1067* | | | |
| | *D11S922* | | | |
| | *TP53* | | | |
| Gastric | *AP(δ)3* $(A)_{18}$ | ≥2/5 loci | 25/167 (15) | *(130)* |
| | *hMSH3* $(A)_{26}$ | | | |
| | *D1S158* | | | |
| | *D5S421* | | | |
| | *D8S199* | | | |
| | *BAX* $(G)_8$ | | 16/25 (64) | |
| | *hMSH3* $(A)_8$ | | 16/25 (64) | |
| | *hMSH6* $(G)_8$ | | 13/25 (52) | |
| Gastric | Dinucleotide | ≥2/6 loci | 5/98 (2) | *(177)* |
| Cervix | Dinucleotide | ≥2/30 loci | 5/98 (3.3) | *(244)* |
| | Trinucleotide | | | |
| | Tetranucleotide | | | |
| Breast | Dinucleotide | ≥2/12 loci | 2/100 (2) | *(207)* |
| Skin cancers | 47 loci[b] | ≥2 of loci tested | | *(213)* |
| Basal | | | 1/47 (2) | |
| Squamous | | | 1/49 (2) | |
| Melanoma | | | 0/41 (0) | |
| Lymphoma | | | | |
| MALT[c] | *D3S1262* | ≥2/5 loci | 21/40 (52.5) | *(334)* |
| | *D3S1265* | | | |
| | *D3S11* | | | |
| | *D3S1261* | | | |
| | *D6S262* | | | |
| HIV-positive | 9 loci | ≥2/5 loci | 4/6 (66) | *(335)* |
| Lung | Dinucleotide | ≥2/8 loci | 12/35 (34) | *(336)* |
| Gliomas | | ≥1/2 loci | | *(228)* |
| Glioblastoma | *D2S123* | ≥1/2 loci | 5/24 (21) | |
| | *D3S1067* | | | |
| Astrocytoma | *D2S123* | ≥1/2 loci | 2/16 (12.5) | |
| | *D3S1067* | | | |
| Glioblastoma | *TGFβ RII* $(A)_{10}$ | | 4/24 (17) | |
| Astrocytoma | *TGFβ RII* $(A)_{10}$ | | 2/16 (12.5) | |
| Prostate | 23 loci | >2/23 loci | 4/47 (8.5) | *(237)* |

[a]Used with permission from ref. *(150)*

[b]Not all tumors investigated at all loci.

[c]MALT, mucosa-associated lymphoid tissue.

genes, at least with respect to certain cancer phenotypes. It has also established that the MMR pathway is a mutation avoidance system or a caretaker system *(265).* Therefore, loss of MMR function will have a great impact on genome stability, especially on the stability of genes critical for cellular growth, such as other tumor suppressor genes and oncogenes. Because of technical limitations, it is impossible to assess the impact of MMR-deficiency on a genome-wide basis, and to identify all mutations that accumulate as a result of MMR deficiency. However, using MSI analysis, it is possible to readily detect frameshift mutations in genes that contain simple repeat sequences within their coding regions, which in most cases lead to truncated proteins. Therefore, repeat tracts within coding regions have been studied in MMR-deficient cells, to determine if they are frequent mutational targets.

Markowitz et al. *(266)* reported that mutations in the type II transforming growth factor-β receptor *(TGFβ RII)* gene is associated with sporadic CC cells defective in MMR. These mutations are all frameshift mutations, and occur either in a 6-bp GTGTGT repeat or in an $(A)_{10}$ mononucleotide repeat *(266).* In each case, the frameshift mutation results in a mutant protein form of TGFβ RII. Subsequent studies *(267–273)* have demonstrated that frameshift mutations of simple repeat tracts in the *TGFβ RII* gene are common in colorectal tumors with MSI. Similar mutations of *TGFβ RII* have also been observed in MSI gastric cancer *(274–277),* glioma *(228),* uterine cervical cancer *(278),* squamous carcinoma of the head and neck *(279),* ulcerative colitis-associated neoplasm *(280),* and sporadic cecum cancer *(281).* It is known that *TGFβ RII* is required for transduction of the TGFβ growth inhibitory signal to suppress epithelial cell growth. The loss of TGFβ RII function in tumors with MSI represents a crucial mechanism for escape from growth control. The targeted mutations in simple repeated sequences in *TGFβ RII* are characteristic of what is expected from a MMR defective system.

In addition to *TGFβ RII,* somatic frameshift mutations of mononucleotide runs have been documented in several genes critical for cellular growth in tumors with MSI. These genes include the apoptosis gene *bax (160,282–288),* insulin-like growth factor 2 receptor *IGF2R (285,289–291),* transcription factor *E2F-4 (276,292),* MMR genes *hMSH3* and *hMSH6 (129,130,263,264),* and tumor suppressor genes *APC (293–295)* and *PTEN (162,289,296–298).* All of these genes are crucial for cellular growth control, and the inactivation of any of these genes would be a key mechanism by which tumors with MSI become neoplastic. Therefore, the potential impact of loss of the tumor suppressor function of MMR is not only relevant to HNPCC, but to virtually all types of cancer.

It is worth noting that not all genes that contain simple repeat sequences undergo frameshift mutations in MSI tumors. For example, this type of mutation is not associated with the *p53* gene *(275,299–301).* It appears that there is a large variation in the propensity for mutation in repeat sequences at different sites in the genome in MMR-deficient cells, which is not yet completely understood. It is also important to point out that defects in MMR cause base–base substitutions and frameshift mutations in nonrepeat sequences, and these mutations can also inactivate essential genes. This kind of mutation has not been extensively examined in MMR-deficient cells, because it can be tedious and difficult to detect mutations in nonrepeat sequences. Nevertheless, it has been documented that MSI tumors and tumor cell lines that are defective in MMR exhibit an elevated rate of base–base substitutions and frameshift mutations in the

*HPRT* locus *(56,57,302).* Therefore, further studies are required to determine the impact of MMR defects on mutagenesis and cancer.

### *6. MMR as a Sensor of Genomic Damage*

As discussed in subheading 5.2., MMR acts as a tumor suppressor, through its ability to maintain genomic stability by correcting base–base and ID mispairs. However, recent studies *(303,304)* suggest that MMR participates in other cellular functions, such as transcription-coupled nucleotide excision repair. DNA recombination *(305,306),* and chr synapsis *(77,78).* In addition, human MMR plays an important role in cellular response to DNA damage induced by many physical and chemical agents, a concept supported by the fact that MMR-deficient cells are much more resistant to killing by these agents than MMR-proficient cells.

The first evidence for this concept came from studies of *E. coli dam*$^-$ mutants *(307–309).* These cells lack the methylase activity required for strand discrimination during MMR, and are sensitive to killing by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a methylating agent that modifies guanine residues in DNA to produce $O^6$-methylguanine ($O^6$-meG). However, double mutants that are also defective in *mutS* or *mutL* are highly resistant to killing by MNNG. The mutagenic capability of the drug is similar regardless of the genotype (wt, *dam*$^-$, *dam*$^-$/*mutS*$^-$, *dam*$^-$/*mutL*$^-$), leading to the conclusion that the wt MMR system is involved in the MNNG-induced cytotoxicity of *dam*$^-$ cells. A similar phenotype was also observed in MMR-deficient human cells.

The best-characterized MNNG-resistant human cell line is MT1. MT1 was derived from a lymphoblastoid cell line by selection with a high dose of MNNG *(310).* Compared with the parental TK6 cells, MT1 cells not only exhibit more than a 500-fold increased resistance to killing by MNNG, but also display an elevated mutability at the *HPRT* locus in the absence of the drug *(57,310).* The MT1 cell line was found to harbor a mutation in *hMSH6 (58),* and to be defective in strand-specific MMR *(57).* While MMR deficiency can be acquired following treatment with the alkylating agent MNNG, human tumor cells also demonstrate a correlation between MMR-deficiency and resistance to alkylating agents. For example, an *hMLH1*-defective colorectal tumor cell line is resistant to killing by MNNG; however, when it receives a wt copy of *hMLH1* by chr 3 transfer, it becomes sensitive to MNNG-induced cytotoxicity and killing *(135,311).* Similarly, cellular deficiency in other MMR genes also confers resistance to alkylating agents *(82,131,312–315).*

Tumor cell lines resistant to other chemotherapeutic drugs, including cisplatin, procarbazine, and temozolomide, are also known to be deficient in MMR. Cisplatin damages DNA by forming inter- and intrastrand crosslinks between two guanine residues *(316,317).* It has been reported that several ovarian tumor cell lines lose MMR function and gain resistance to cisplatin by a single-step selection process *(318,319).* Accordingly, MMR-deficient cells are resistant to the cytotoxic effects of cisplatin *(320).* Like MNNG, temozolomide and procarbazine are also DNA-methylating agents, and exert their cytotoxic effects primarily through methylation at the $O^6$ position of guanine. Similar results have been obtained with cisplatin and these two drugs. Serial in vivo exposure of a glioblastoma multiforme xenograft in nude mice, with procarbazine, led to a resistant tumor that lost expression of *hMSH2 (321).* It is also documented that MMR-deficient cells are resistant to temozolomide *(315,321–323).* Recently, the list of

agents to which MMR-deficient cells gain resistance has been extended to include ionizing radiation *(324,325)* and environmental chemical carcinogens, such as polycyclic aromatic hydrocarbons and aromatic amines, which react covalently with DNA (preferentially at guanine residues) to form bulky DNA adducts. These findings suggest that MMR components may have a function in sensing DNA damage induced by drugs or carcinogens.

Although the mechanism for drug resistance in MMR-deficient cells is unclear, several models have been proposed. The first model suggests that the death of wt MMR cells, in response to drug or chemical treatments, results from an attempt by the MMR pathway to correct a lesion in the template strand, by repairing the newly synthesized strand, which leads to a reiterative cycle of excision and resynthesis of the daughter strand, and leaves the adducts in the template strand unaffected. Such a futile repair cycle may lead to cell death by an uncharacterized pathway (for reviews, *see* refs. *326* and *327*). Alternatively, cell death may result from the binding of MMR proteins to DNA adducts, which may block DNA transactions, such as replication, transcription, and proper damage repair. A third model proposes that a carcinogen-induced dsDNA break acts as the signal for cell death *(12)*. In this model, DNA adducts may dissociate the DNA replication machinery at the adduct site, and terminate chain elongation. DNA synthesis can be reinitiated behind the lesion by a newly formed replication complex, which can lead to a single-strand gap opposite the DNA adduct. A double-stranded break is formed during the subsequent cycle of DNA replication, when the gapped strand is used as a template. This model includes no proposed role for MMR components.

Strong support for the first two models has recently been provided by both in vitro and in vivo experiments. First, gel shift analyses demonstrated that human MutS homologs specifically recognize DNA adducts induced by MNNG *(328),* cisplatin *(328–331),* aromatic amines *(329),* and polycyclic aromatic hydrocarbons *(325a)*. Second, D'Atri et al. *(323)* have shown that cell death induced by temozolomide is associated with increased expression of p53. The carcinogen-induced p53-dependent apoptosis was also shown to be dependent on functional hMutS and hMutL homologs *(325a)*. Finally, $O^6$-msG can provoke strand-specific MMR in vitro *(329a)*. These observations strongly support the first hypothesis.

Taken together, the mechanism by which MMR triggers carcinogen-induced apoptosis can be proposed as follows (Fig. 3). When a DNA replication fork encounters a carcinogen–DNA adduct in the template strand, DNA polymerase incorporates a base opposite the adduct, which can be recognized by hMutS homologs as a mismatch. The binding of hMutS homologs to the damaged bp initiates a strand-specific MMR reaction. Because repair is restricted to the daughter DNA strand, the adduct persists in the template strand. Thus, repair resynthesis actually restores the original adduct-containing bp, which will retrigger a strand-specific MMR process. This futile repair cycle is a signal to induce p53 expression, which leads to programmed cell death. Recently, a p73-dependent apoptotic response has also been proposed *(329b)*.

The recognition and processing of carcinogen–DNA adducts by MMR may play an important role in ensuring genomic integrity. Normally, DNA damage induced by physical and chemical carcinogens is repaired by other repair systems, e.g., $O^6$-meG by $O^6$-meG methyltransferase, and bulky DNA adducts by base and/or nucleotide excision repair. However, when these systems are not available and genomic integrity is in dan-

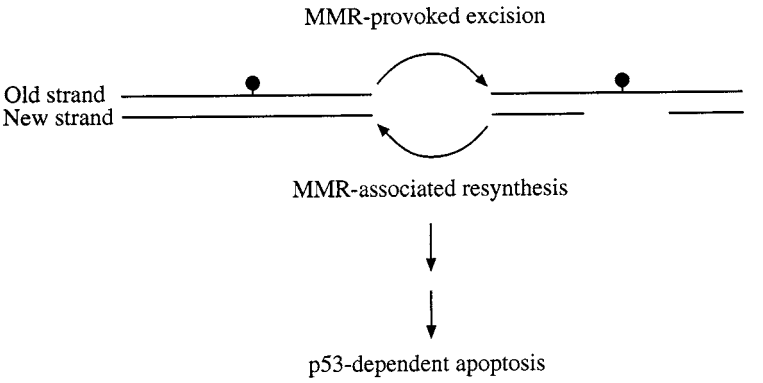


**Fig. 3.** Proposed mechanism for MMR triggering apoptosis. The filled circle represents the carcinogen adduct.

ger, cells will rely on MMR to commit suicide. Therefore, the authors propose that the MMR pathway possesses at least two functions that preserve genomic stability: MMR corrects mismatches, and it also provides a signal for cell death when DNA damage cannot be repaired.

Although MMR maintains genomic stability in two different ways, the differential response of MMR-deficient and MMR-proficient cells to a number of chemotherapeutic drugs may provide a significant basis for understanding the effects of cancer chemotherapy. First, certain widely used clinical drugs e.g., temozolomide, procarbazine, and cisplatin, are expected to be harmful for patients with tumors caused by MMR defects, because these drugs actually kill the patient's normal tissue, rather than the tissue of the tumor. Second, since some tumor cells can acquire MMR deficiency upon exposure to certain drugs *(301,318,319,321),* the use of these drugs in clinical practice may lead to a secondary cancer characterized by MMR defects.

The rapid advances in the field of eukaryotic MMR, described in this review, demonstrate important new connections between DNA repair and the fields of carcinogenesis and toxicology. The implications of these findings are only beginning to be fully appreciated. Areas of importance for future exploration in this field may include screening, diagnosis, and gene therapy for cancer with MMR defects.

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# 10 PTEN

## *Regulator of Phosphoinositide 3-Kinase Signal Transduction*

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***CONTENTS***



### 1. INTRODUCTION

The chromosome (chr) region spanning 10q22–10q25 exhibits loss of heterozygosity (LOH) in multiple tumor types, including carcinoma of the prostate, endometrium, breast, kidney, and thyroid, as well as glioblastoma, melanoma, and meningioma *(1–14).* In addition, the gene for Cowden disease (CD), an autosomal dominant familial cancer syndrome that is characterized by multiple hamartomas of the skin, breast, thyroid, and intestines, and an increased risk of breast and thyroid malignancies, has been localized to chr bands 10q22–23 *(15)* by linkage analysis. Microcell-mediated transfer of chr 10q into glioblastoma cells inhibits soft-agar colony formation and tumor formation in nude mice *(16).* Likewise, chr transfers of 10q into rat prostate cancer (PC) cells suppresses the metastatic ability of the highly metastatic parental cells *(17).* Taken together, these findings suggest the presence of one or more tumor suppressor genes (TSGs) in the chr 10q22–25 region. In 1997, the TSG phosphate and tensin homolog deleted on chr 10 *(PTEN)*/mutated in multiple advanced cancers 1 *(MMAC1)*/transforming growth factor Fβ-regulated and epithelial cell-enriched phosphatase *(TEP1),* located on chr subband 10q23.3, was identified by three independent groups *(18–20).* Mutational analysis of the nine coding exons of *PTEN/MMAC1/TEP1* (hereafter referred to as *PTEN*) demonstrated germ-line mutations in the related familial hamartoma syndromes, CD and Bannayan-Zonana syndrome (BZS), and LOH accompanied by somatic mutation of the remaining allele in multiple tumor types.

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The protein product of the *PTEN* (PTEN) encodes a dual-specificity phosphatase. Recent evidence has demonstrated that PTEN functions, not only as a protein phosphatase, but as a lipid phosphatase as well. This latter activity is associated with the ability of PTEN to regulate signaling through the phosphoinositide 3-kinase (PI3K) pathway, and appears to be required for PTEN function as a tumor suppressor. This chapter reviews the biochemical, functional, and genetic data that have led to current understanding of PTEN tumor-suppressor function.

## 2. *PTEN,* THE GENE

### 2.1. Germ-line Mutations

CD is an autosomal dominantly inherited hamartoma syndrome that is characterized by hamartomas in multiple organ systems, as well as by an increased risk of breast and thyroid malignancies *(21).* Hallmarks of CD include benign tumors of the hair follicle infundibulum, known as trichilemmomas and mucocutaneous papillomatosis. A subset of patients with CD are found to have Lherhmitte Duclos disease (LDD), characterized by an altered gait, epilepsy, and megencephaly secondary to dysplastic gangliocytoma of the cerebellum *(15).* The clinical phenotype of BZS partially overlaps CD, and includes the presence of hamartomatous polyps of the intestine, macrocephaly, and lipomas. However, families afflicted with BZS are not found to have an increased risk of malignancy. Additional characteristics of BZS may include vascular malformations, Hashimoto's thyroiditis, speckled penis, mental retardation, and intracranial tumors *(21).*

Germ-line mutations of *PTEN* have been found in 81% of families with CD, including those with LDD, and in 57% of BZS families *(15,21,22).* Thus, mutations of *PTEN* appear to be associated with multiple phenotypes. This has led to speculation as to whether or not these syndromes represent a spectrum of a single entity that is modified by the developmental timing of the mutations or other epigenetic and/or genetic events.

Alternatively, certain PTEN mutations could give rise to specific clinical phenotypes; that is, there may be genotype–phenotype correlates. However, this latter possibility seems unlikely, because most *PTEN* mutations cluster within the minimal PTEN phosphatase domain (*see* Subheading 2.2.), and would therefore be predicted to disrupt its phosphatase activity. In addition, one mutation, R233X, has been found in the germ line of a family with BZS, and in two unrelated CD families *(21).* These data would also argue against the possibility that differences in mechanisms of mutational inactivation of PTEN underlie the differences in the observed clinical spectra.

### 2.2. Somatic Mutations

Somatic mutations of *PTEN* have been identified in high-grade gliomas, advanced-stage prostate carcinomas, endometrial carcinomas, and malignant melanomas, and less commonly in small cell lung, thyroid, bladder, renal, and sporadic breast carcinomas and lymphoid and hematological malignancies *(23–49).*

LOH on chr 10q has been observed in 69–82% of glioblastomas *(24,45,46,50).* Intragenic mutations in the *PTEN* gene have been identified in 60% of primary glioblastoma cell lines *(18,20)* and in approx 20–44% of glioblastoma multiforme, the most aggressive subtype of astrocytic tumors *(18,20,24,39,45,51–53).* In contrast, in low-grade gliomas (such as anaplastic astrocytomas) and meningiomas, although they

also harbor chr 10q loss, *PTEN* alterations are comparatively rare *(24,39). PTEN* mutations therefore appear to be correlated with the more advanced tumors.

Although two studies have found a low frequency of *PTEN* mutations in PC *(27,38),* a number of additional studies have shown that *PTEN* alterations are more frequent in higher-grade tumors, and in metastatic tumors *(54–56).* LOH on chr 10q is observed in about 30–60% of tumors, and is associated with tumor progression and metastasis *(57).* In one series, 10/23 (43%) tumors with LOH of chr band 10q23 had a second inactivation event at *PTEN (55).* Seven of the 10 tumors with *PTEN* alterations in this series had pelvic lymph node metastases. Similarly, homozygous deletions of *PTEN* were detected in 10–15% of stage B prostate carcinomas, which are reported to have between 0–40% chr 10q loss *(55).* Study of PTEN expression, using immunohistochemistry, demonstrated that loss of PTEN expression correlated significantly with Gleason scores (a measure of the tumor grade) of 7 or greater *(56).* Taken together, these data suggest that, as in glioblastoma, *PTEN* inactivation is a late event in prostate tumorigenesis, and thus might be associated with the progression from a primary to a metastatic tumor.

PTEN mutations can be found in 30–40% of malignant melanomas *(29,44),* correlating with a LOH frequency of 30–50% on chr 10q *(1,58).* In addition, in specific instances, PTEN mutations were found in metastatic foci, but not in the corresponding primary tumors, again suggesting that *PTEN* is involved in tumor progression *(44).*

In contrast to findings in glioblastoma, prostate carcinoma, and melanoma, *PTEN* alterations are detected in 30–50% of endometrial carcinomas of the endometrioid type *(36,42,47,48.* Consistent with this data, chr 10q LOH can be found in approx 40% of endometrial cancers (Jones, 1994 #907; Nagase, 1996 #956; Peiffer, 1995 #967). In addition, *PTEN* mutations are found in approx 20% of endometrial hyperplasias, which are thought to be premalignant precursor lesions of invasive endometrial carcinomas *(59),* suggesting that here, *PTEN* mutations are not associated with advanced staging.

These findings suggest that *PTEN* may exhibit differential roles in specific tumor types. For example, in brain and prostate carcinomas, *PTEN* mutations probably occur as late events in tumorigenesis, and have been hypothesized to be associated with the metastatic phenotype. In contrast, in endometrial carcinomas, *PTEN* mutations may instead be initiating events. The latter role of *PTEN* as an initiator of transformation is also supported by the predisposition to breast and thyroid cancers conferred to certain individuals bearing a germ-line *PTEN* mutation. This role of *PTEN* as an initiator or as a progression factor is not a unique finding among TSGs. Loss of the retinoblastoma gene *(Rb)* predisposes to retinoblastoma and sarcoma, but somatic *Rb* mutations are found as late events in a number of tumors *(60).* Likewise, *p53* mutations can be initiating events, as in Li-Fraumeni syndrome, or can occur late in the evolution of certain sporadic cancers: Therefore, it is not necessary to ascribe different biological roles of the protein product to the action of a tumor suppressor in initiation or progression.

### *2.3. Evidence for Second TSG Near PTEN?*

In certain tumors, the frequency of chr 10q23 LOH exceeds the frequency of intragenic *PTEN* mutations. The most striking example is found in primary breast tumors. Although the frequency of chr 10q LOH in primary breast tumors is as high as 50%, most series report only rare *PTEN* mutations *(37,43,61,62).* This was particularly sur-

prising, because CD patients, who have germ-line mutations in *PTEN,* have an increased risk of breast tumors.

Other noted examples of this discordance include lung cancer, in which LOH on chr 10q near *PTEN* reaches 91% in small cell and 41% in nonsmall cell lung carcinomas *(6).* Again, *PTEN* mutations or homozygous deletions are significantly less, at approx 10% *(31).* Similarly, in thyroid tumors, LOH on chr bands 10q22–23 occurs at about 25% frequency, but *PTEN* mutations are comparatively rare *(13,35,49).*

A number of possible explanations for the discordance between 10q23 LOH and PTEN mutation can be envisioned. First, there may be a systematic underestimation of PTEN inactivation. For example, mutations in noncoding regions, which have not yet been characterized, may play a role in transcript or protein stability. Alternatively, other epigenetic mechanisms, such as promoter methylation, may inactivate *PTEN.* Second, monoallelic inactivation or haploinsufficiency of *PTEN* may be sufficient in some tumors to promote growth and/or progression. Third, other TSGs may reside within this locus.

Currently, only indirect evidence exists for the role of methylation in transcriptional repression. For example, in advanced prostate tumor xenografts, loss of PTEN protein and mRNA was found in the absence of PTEN mutation. Treatment of these xenografts with the demethylating agent, 4-azadeoxycytidine, restored PTEN protein expression, suggesting the presence of inactivating methylation events *(63).* On the other hand, one study *(55)* found no evidence of methylation in six primary prostate tumor samples. Whether transcriptional or posttranscriptional mechanisms for *PTEN* inactivation exist remains an open question.

Evidence for the possibility of a significant role for *PTEN* haploinsufficiency in tumor formation is supported by studies in *PTEN*+/– mice. Here, as will be discussed later, loss of one *PTEN* allele leads to diffuse proliferative abnormalities in the prostate, colon, skin, and lymph nodes *(64).* The diffuse nature of these lesions makes it unlikely that each and every cell has sustained a second inactivating mutation. Therefore, it is likely that this proliferative abnormality arises solely as a consequence of the loss of one *PTEN* allele. Furthermore, heterozygous embryonic stem cells show partial activation of Akt, compared to their wild-type (wt) and homozygous-null counterparts *(65).* Finally, heterozygous lymphocytes and embryonic fibroblasts are protected from Fas-induced apoptosis, and have partially deregulated Akt activity *(66).* These data suggest that loss of one allele of PTEN can lead to a significant alteration in cell behavior.

Two candidate TSGs have been identified distal to *PTEN: MXI1* max interactor 1 *(MXI1)* and deleted in malignant brain tumors *(DMBT1) (67,68).* Although MXI1 is capable of suppressing the growth of glioblastoma cells in culture lines *(68),* most studies of glioblastomas and prostate and lung carcinomas have found few to no mutations or homozygous deletions in *MXI1 (11,69–75).* Homozygous deletions of *DMBT1* appear to occur frequently in gliomas, and less frequently in lung and gastrointestinal carcinomas *(51,67,76–78).* An analysis of the prognostic significance of *PTEN* and *DMBT1* alterations in gliomas suggested that, although *PTEN* alterations were associated with progression of disease, *DMBT1* may be involved in the early stages of tumorigenesis *(77).*

In addition, *FAS,* the cell-surface receptor involved in death signaling, is located on chr subband 10q24.1, just distal to *PTEN.* However, alterations of *FAS* are rare in non-

lymphoid malignancies *(79–83).* An interesting possibility with respect to Fas is that combined haploinsufficiency of *FAS* and *PTEN* could combine to promote cell survival (discussed below). Most recently, another inositol phosphatase, multiple inositol polyphosphate phosphatase *(MINPP1),* was identified and mapped to chr band 10q23 *(84),* probably in close proximity to *PTEN.* Given its function and location, mutational analyses of *MINPP1* in tumorigenesis will be of interest.

### *2.4. PTEN Is a Tumor Suppressor*

Reconstitution of wt PTEN in PTEN-deficient glioblastoma cells, either through adenoviral, retroviral, or transfection-based methods, results in suppression of colony formation in culture, soft-agar growth, and tumor formation in nude mice *(85,86).* Likewise, reconstitution of PTEN in PTEN–/– PC cells suppresses the outgrowth of colonies in vitro *(87,88).* Chr transfers of wt chr 10 into either glioblastoma or malignant melanoma cell lines found that the microcell hybrids that escaped the chr-mediated suppression selectively lost portions of the chr band where *PTEN* is located *(50,89).* Furthermore, as described below, mice rendered heterozygous for PTEN, through gene targeting, develop a variety of tumors that are associated with loss of the wt *PTEN* allele *(90,91).* These data provide functional evidence that *PTEN* is in fact a TSG.

## 3. PTEN, THE PROTEIN

### *3.1. Introduction*

PTEN contains 403 amino acid residues, and is a member of the VHR family of dual specificity phosphatases, containing the signature motif common to protein tyrosine (Tyr) phosphatases: HCXXGXXRS/T. Although PTEN bears homology to the cytoskeletal protein, tensin, and to the secretory vesicle protein, auxilin *(19,20),* their region of homology encompasses the phosphatase homology region. Tensin and auxilin were observed to have homology to phosphatases prior to the cloning of *PTEN (92).* However, in each case, certain critical residues, typically required for phosphatase activity, are absent. This observation led to speculations that, for these proteins, this domain may serve as a novel phosphate-binding motif *(92).* It is therefore likely that the homology between PTEN and auxilin and tensin is more a reflection of the presence of phosphatase-like domains in the latter, rather than of a parallel function.

PTEN has phosphatase activity toward serine (Ser), threonine (Thr), and Tyr phosphorylated substrates *(93).* PTEN, however, does not dephosphorylate a number of typical phosphatase substrates, including myelin basic protein and RCML *(93);* rather, the phosphatase activity appears to be restricted to acidic substrates, such as $polyGlu_4$ $Tyr_1$ This preference for acidic substrates prompted Maehama and Dixon *(94)* to ask whether PTEN could act as a lipid phosphatase.

PTEN specifically dephosphorylates the D3 position on the inositol ring of phosphatidylinositol and inositol phosphates *(94).* It exhibits the highest activity against phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-$P_3$) and phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-$P_2$); and, in decreasing order, against phosphatidylinositol-3-phosphate (PtdIns3P) and inositol-1,3,4,5-tetrakisphosphate (Ins-1,3,4,5-$P_4$) *(87,94).* The specificity for the 3 position led those authors to predict that PTEN may function as an antagonist of PI3K signaling.

## *3.2. PTEN as a Lipid Phosphatase*

### 3.2.1. Overview of PI3K/Akt Pathway

Phosphoinositide kinases are a family of lipid kinases involved in intracellular signaling. There are three classes of PI3Ks. Class I kinases are typified by their association with regulatory subunits, and by the ability to utilize PtdIns-3,4-$P_2$ and PtdIns-3,4,5-$P_3$ as substrates *(95)*. For the purposes of this chapter, PI3K will refer to PI3Kα, the first-identified and cloned member of its class. PI3K is a class I kinase, and exists as a heterodimer with an 85-kDa regulatory subunit (p85) and an 110-kDa catalytic subunit (p110). Activation of PI3K by receptor and nonreceptor tyrosine kinases (TK), growth factors, and activated Ras initiates an intracellular signaling cascade that impacts cell proliferation and survival *(96–99)*. In addition, this pathway has also been implicated in vesicle trafficking, exocytosis, and actin assembly *(99)*. PI3K catalyzes the formation of PtdIns-3,4-$P_2$ and PtdIns-3,4,5-$P_3$, both of which can be dephosphorylated in the presence of PTEN *(87,94)*. In quiescent cells, PtdIns-3,4,-$P_2$ and, particularly PtdIns-3,4,5-$P_3$ are normally absent. However, in response to certain growth stimuli, such as platelet-derived growth factor (PDGF), insulin-like growth factor 1, and nerve growth factor, this signaling cascade is initiated. Typically, receptor TKs undergo an auto- or transphosphorylation reaction, leading to the creation of docking sites for the p85 subunit. Docking of PI3K to the receptor serves two purposes: first, PI3K is brought into proximity with the membrane-localized lipid substrates, and, second, it is likely that this docking activates PI3K. These steps lead to the production of PtdIns-3,4-$P_2$ and PtdIns-3,4,5-$P_3$ *(97)*. These phosphoinositides in turn recruit a variety of cytosolic proteins, including Grp-1, Akt, Bruton's TK, adapter protein-2, and synaptotagimen, to the membrane *(99)*. This interaction between the membrane-bound phospholipid and specific proteins is mostly mediated by binding of the phospholipid to either pleckstrin homology (PH) domains or other lipid-binding domains, such as Src-homology-2 domains *(99)*.

One cytosolic protein that has stimulated great interest is the Ser/Thr kinase, Akt. Binding of Akt to PtdIns-3,4-$P_2$ and/or PtdIns-3,4,5-$P_3$ is mediated by its amino-terminal PH domain *(97,100)*. Binding of the lipid to the Akt PH domain may result in a conformational change in Akt. More importantly, the PH domain binding translocates Akt to the plasma membrane, where Akt can then be activated by phosphorylation *(97,101)*. This activation requires phosphorylation at two sites, Thr308 and Ser473. These phosphorylations are mediated by the PtdIns-3,4,5-$P_3$-dependent protein kinase-1 (PDK-1) and a second unknown kinase, designated PDK2, respectively *(100,102)*. PDK2 may in fact be a form of PDK1 that has altered substrate specificity *(103)*.

### 3.2.2. Akt and its Downstream Targets

*Akt/RAC/PKB* was first identified as a retroviral oncogene isolated from rat T-cell lymphomas, as well as by homology to the related PKA and PKC *(104–106)*. The retroviral fusion protein, Gag-Akt, creates a constitutively active form of Akt that is targeted to the membrane independent of PtdIns-3,4-$P_2$. Likewise, a constitutively active form of Akt, created by insertion of a myristylation signal that targets Akt to the membrane, is capable of transforming chicken embryo fibroblasts in vitro, and producing hemangiosarcomas in chickens in vivo *(107)*. Intense interest has centered on the identification of downstream targets of Akt that may be essential for cell growth, survival, and/or transformation.

**Table 1**
**Downstream Targets of Akt**

| *Target* | *Action* | *Ref(s).* |
|---|---|---|
| Bad | Prevents binding to Bcl-$X_L$ | Datta et al., 1997 |
| Caspase-9 | Inhibits protease activity | Cardone et al., 1998 |
| GSK3 | Inhibits kinase activity | Cross et al., 1995<br>Dudek et al., 1997<br>Vanhesebroeck et al., 1997 |
| PhasI/4E-BP1 | Inhibits binding to eIF4E | Sonnenberg et al., 1997, 1999 |
| FKHRL1 | Prevents nuclear localization | Brunet et al., 1999 |
| AFX | | Kops et al., 1999 |
| FKHR | | Tang et al., 1999<br>Nakase et al., 1999<br>Guo et al., 1999 |
| CREB | Stimulates transcriptional activity | Keyong et al., 1998 |
| eNOS | Enhances NO production | Dimmeler et al., 1999<br>Fulton et al., 1999<br>Michell et al., 1999 |
| IKKα | Prevents binding to NF-κB | Ozes et al., 1999<br>Romashkova and Makarov, 1999<br>Kane et al., 1999 |

A number of Akt substrates have been identified (Table 1; Fig. 1), including proapoptotic factors, such as Bad, caspase-9, IKKα, and members of the forkhead transcription factor family, as well as other substrates, such as glycogen synthase kinase-3 (GSK3), $p70^{S6K}$, eIF4E-binding protein-1 (4E-BP1), and endothelial nitrogen oxide synthase (eNOS) *(100,108–121).* Of note, it is unclear whether the interaction between Akt and $p70^{S6K}$ is a direct one *(100).* A general, but not universal finding, is that Akt phosphorylations render the target substrate inactive. For example, Akt phosphorylation of Bad creates 14-3-3 binding sites. Once bound to 14-3-3, Bad is unable to interact with Bcl-$X_L$. Bcl-$X_L$ is an antiapoptotic Bcl-2 family member, and thus the net effect of Akt activity is promoting cell survival *(121).* Similarly, Akt phosphorylation of the forkhead transcription factors FKHR and FKHRL1, results in either 14-3-3 binding or alterations in nucleocytoplasmic shuttling. By either mechanism, these proteins are localized to the cytoplasm after Akt phosphorylation, and are thus rendered transcriptionally silent *(109,110).* Akt likewise phosphorylates and inactivates caspase-9, GSK3 and IKKα. Caspase-9 is involved in apoptosis *(120);* GSK3 is involved in glycogen synthesis *(111);* IKKα regulates nuclear factor-κB, which is a transcription factor *(114).* 4EBP-1 is a translational repressor that is inactivated by Akt *(113).* However, full inhibition of this protein requires phosphorylation by a second kinase, FRAP/mTOR, as well (122). In contrast to the other substrates discussed thus far, Akt phosphorylation activates eNOS, and leads to increased nitric oxide (NO) production *(117–119).*

Downregulation of the PI3K–Akt pathway would therefore predictably promote proapoptotic activity and growth suppression. Thus, the ability of PTEN to dephospho-

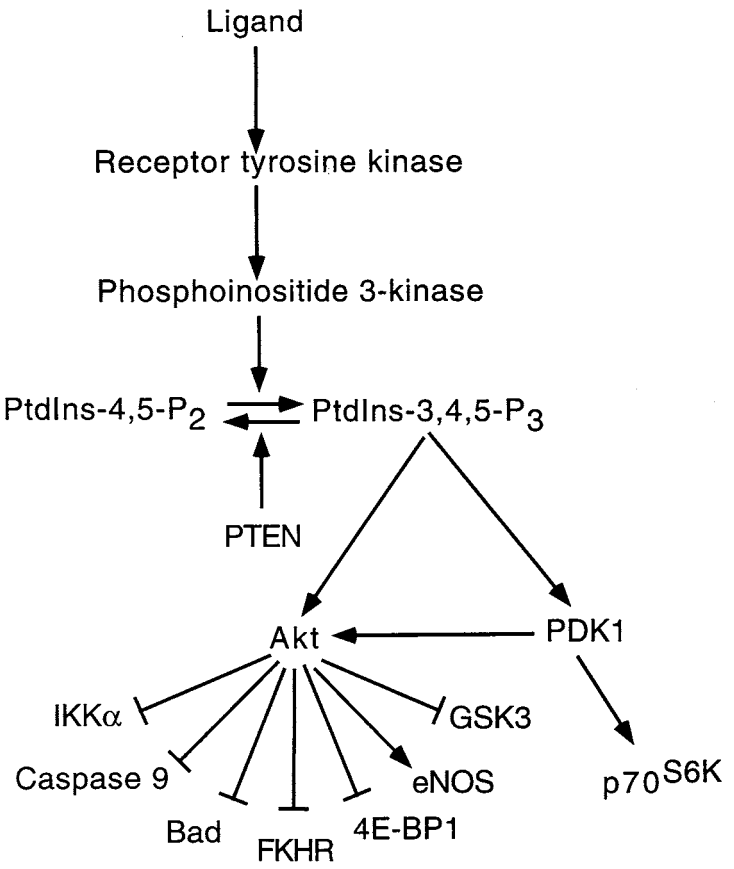


**Fig. 1.** PI3K–Akt pathway. Growth factor and survival factors bind and activate receptor TKs. Auto- or transphosphorylation allows receptor-mediated activation of PI3K. PI3K catalyzes the phosphorylation of PtdIns-4,5-$P_2$ to PtdIns-3,4,5-$P_3$. The latter activates both Akt family members and PDK1. PDK1 also participates in the activation of Akt. Akt phosphorylates and inactivates a number of substrates, as shown. In the case of eNOS, phosphorylation is activating. PDK1 phosphorylates and activates $p70^{S6K}$.

rylate PtdIns-3,4,5-$P_3$ and PtdIns-3,4-$P_2$ upstream of Akt would serve as a potent restraint to uncontrolled proliferation and prolonged cell survival.

### 3.2.3. PI3K–PTEN–Akt Pathway

Functional evidence for the role of PTEN in the regulation of the PI3K–Akt pathway was initially found by transfecting wt PTEN or a substrate-trapping mutant of PTEN (C124S) into a human embryonic kidney cell line (293) *(94)*. Introduction of wt PTEN led to decreased levels of PtdIns-3,4,5-$P_3$; introduction of the substrate-trapping mutant resulted in higher levels. Overexpression of PTEN also reduced the levels of PtdIns-3,4,5-$P_3$ in the presence of a constitutively active PI3K, suggesting that PTEN exerts its phosphatase activity on the products of PI3K, and not on PI3K itself *(87)*. In keeping with these data, loss of one or both copies of the murine *PTEN* gene was found to result in elevated PtdIns-3,4,5-$P_3$ levels *(123)*.

Multiple PTEN-deficient tumors cells have elevated basal levels of the phosphorylated form of Akt (i.e., the activated form). Reintroduction of wt but not phosphatase-defective forms, of PTEN into such cells reduces both phosphorylated Akt and Akt kinase activity *(87,124–126).* Override experiments have also demonstrated PTEN's placement in the PI3K–Akt pathway between PI3K and Akt. Specifically, phosphorylation of 4EBP1, a downstream target of Akt, is blocked by PTEN in the presence of a constitutively active PI3K, but not a myristylated form of Akt *(88),* and PTEN-mediated cell cycle arrest or apoptosis (discussed below) can be overridden by a constitutively active form of Akt *(124,127).* Together, these data suggest that PTEN functions downstream of PI3K and upstream of Akt.

Finally, although most disease-derived mutations of *PTEN* ablate both its protein and lipid phosphatase activity, a particular missense mutation in the catalytic motif (G129E) retains protein phosphatase, but not lipid phosphatase activity. This mutant was isolated from two independent CD families, one BZS family, and one endometrial carcinoma *(21,48).* This particular mutant fails to induce apoptosis in LNCaP cells, and fails to induce a G1 arrest in PTEN-null 786-O or U87-MG cells *(87,124,128).* Additional diseases- and tumor-derived mutations, which selectively retain activity for the phosphoinositides, have also been identified *(128).* These data indicate that protein phosphatase activity is not sufficient for these PTEN functions, nor is it sufficient for the suppression of the CD phenotype.

PTEN can regulate apoptosis in some cell lines *(87,127,129)* and cell cycle arrest in others *(86,124,128),* indicating that the PI3K–PTEN–Akt pathway may perform at least two distinct functions: one of cell survival and one of cell growth.

### 3.2.4. PTEN as a Cell Cycle Regulator

Several studies have demonstrated that PTEN is sufficient to arrest certain cells in G1 *(65,86,124,128).* Reintroduction of PTEN into PTEN-null glioblastoma, or renal carcinoma cells deficient in PTEN, resulted in growth suppression with a substantial increase in cells in the G1 phase of the cell cycle *(86,124,128).* This cell cycle block is overcome by expression of myristylated, and hence constitutively active, Akt, but not wt Akt, implying further that Akt exists downstream of PTEN. In cells arrested by PTEN, apoptosis was not observed *(124,128).*

Introduction of the G129E PTEN mutant or the G129R PTEN mutant, which is characterized by loss of both protein and lipid phosphatase activity into 786-O renal carcinoma cells, failed to produce a G1 arrest *(124,128).* Because the G129E PTEN mutant retained its protein phosphatase activity, this suggested that the presence of protein phosphatase activity, as measured in vitro, was insufficient to arrest cells in G1.

The cell cycle is regulated at specific checkpoints, most of which are controlled by cyclins and cyclin-dependent kinase (CDK) complexes, along with a set of cyclin-dependent kinase inhibitors (CDKIS) *(130).* Cyclin D–CDK4 acts during the G1 phase and is opposed by the CDKI, p16; cyclin E–CDK2 acts at the G1–S transition, and is probably primarily opposed by the CDKI, p27; cyclin A–CDK2 acts through S phase and the G2–M transition *(130).* In cells susceptible to a PTEN-mediated G1 arrest, re-expression of PTEN leads to decreases in the activities of CDK2, cyclin A, and cyclin E *(86).* In addition, p27, which can arrest cells in G1 when overexpressed *(130),* levels are significantly increased *(86).* Treatment of the cells with PI3K inhibitors also increases levels of p27, and decreases levels of phosphorylated Akt, suggesting that

p27 may be another target of the PI3K–PTEN–Akt pathway *(86)*. Whether these are direct effects, or, alternatively, are related to changes in the cell cycle profile of the treated cells, is not yet known.

In keeping with the data above, *PTEN–/–* ES cells exhibit decreased levels of p27 and an associated increase in CDK2 and cyclin E and A activity *(65)*. Only levels of p27 were affected when asynchronously growing cells were examined. These data suggest that the loss of *PTEN* and consequent activation of the PI3K–PTEN–Akt pathway may selectively target p27, thereby decreasing time in G1, accelerating entry into S phase, and, ultimately, increasing cell proliferation.

### 3.2.5. PTEN as Regulator of Apoptosis

As mentioned previously, a number of Akt substrates are components of apoptotic signaling pathways. Another mechanism by which PTEN acts as a tumor suppressor is as a mediator of apoptosis. Certain tumor-derived cell lines are susceptible to PTEN-induced apoptosis. For instance, in some PTEN-deficient breast cancer cell lines (MCF7, MDA-MB-468, and ZR75-1), adenoviral transfer of PTEN results in apoptosis in 75–80% and >90% of the cell populations, respectively *(127,131)*. This PTEN-mediated apoptosis can be rescued by co-expression of myristylated Akt or Bcl-2, but not FAK or PI3K *(127)*. In one study, apoptosis was associated with reduced endogenous levels of the c-*myc* oncogene; PTEN was shown to inhibit c-*myc* promoter activity in vitro *(131)*.

PTEN-mediated cell death or inhibition of cell survival may not simply reflect the induction of apoptosis. For example, adenoviral introduction of PTEN into LNCaP prostate carcinoma cells results in a dose-dependent decrease in Akt phosphorylation and the induction of cell death. However, the number of cells undergoing apoptotic cell death is significantly less, compared to the expression of p53 in the same cell line. Both p53 and PTEN infection result in decreases in caspase precursors, and treatment with caspase inhibitors partially rescues cells from apoptosis *(129)*. However, in experiments that measure cell growth, using a metabolic assay, LNCaP cells infected with wt PTEN were found to be significantly more growth-inhibited than their wt p53-infected counterparts *(129)*. Furthermore, although overexpression of Bcl-2 decreases PTEN-mediated apoptosis, the growth inhibition that is observed remains unperturbed. These data raise the possibility that alternative pathways to cell death may be important, or that growth inhibition in these cells reflects both antiproliferative and proapoptotic functions of PTEN.

In keeping with the above data, *PTEN–/–* mouse embryonic fibroblasts (MEFs) have a reduced sensitivity to apoptotic stimuli such as UV irradiation and osmotic stress. This resistance can be restored either by re-expression of PTEN or by treatment with PI3K inhibitors *(123)*. Furthermore, certain *PTEN–/–* mice develop lymphoid aggregates. Lymphocytes from these mice have diminished Annexin V staining, suggesting that there is an in vivo defect in lymphoid apoptosis *(90)*. These data suggest that, in certain tissues, *PTEN* is required for the appropriate regulation of cell survival signals.

### 3.2.6. Evidence for a PI3K-Independent Survival Pathway?

In one study of PC cells (LNCaP) deficient in PTEN, serum, androgen, or epidermal growth factor stimulation was able to rescue cells that were inhibited by PI3K inhibitors *(131)*. In another study of glioblastoma cells, growth factor stimulation with

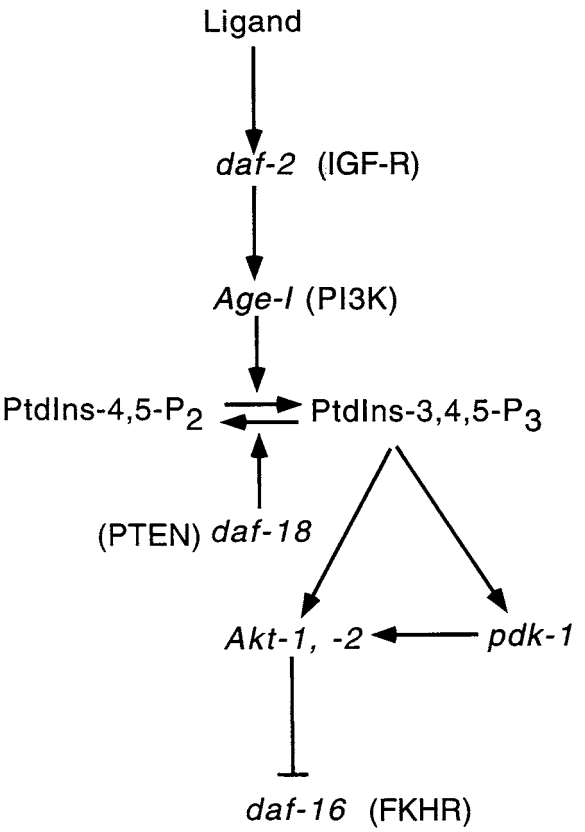


**Fig. 2.** PI3K–Akt pathway in *C. elegans.* The corresponding genetic alleles from *C. elegans* are shown positioned in the PI3K–Akt pathway shown in Fig. 1. The homologous mammalian proteins are shown in parentheses.

PDGF or insulin was able to bypass the PI3K–PTEN–Akt pathway, and generate activated Akt in the presence of PTEN *(87).* In contrast, Wu et al. *(88)* and Davies et al. *(129)* demonstrated that, in Rat-1 fibroblast cells and LNCaP cells, respectively, transfection of wt PTEN was unable to produce phosphorylated Akt, despite serum stimulation. Growth factor concentrations and length of stimulation differed in those studies. These data may suggest that there exists either a threshold level of certain growth factors that may be required to bypass the PI3K–PTEN–Akt pathway, or that an alternate pathway for PI3K activation by growth factors exists, and that such a pathway could be cell-type-specific.

### 3.2.7. PI3K/Akt Pathway in *Caenorhabditis Elegans*

Studies in *C. elegans* have provided genetic evidence that links the components of the PI3K–Akt pathway and PTEN (Fig. 2). In *C. elegans,* this pathway is involved in insulin receptor-like signaling, longevity, and dauer arrest (Fig. 2). Briefly, in *C. elegans,* the PI3K–Akt pathway involves the insulin receptor-like factor, DAF-2 *(132),* which interacts with an unidentified ligand and activates the PI3K homolog, AGE-1 *(133).* This signaling may be stimulated under reproductive growth conditions, and

activates the Akt homologs, AKT-1 and AKT-2, which subsequently inactivate DAF-16 *(134),* a homolog of the mammalian forkhead family of transcription factors. DAF-16 probably acts as a regulator of transcription *(135).* Null mutations in *daf-2* and/or *age-1,* which increase longevity and produce the dauer state, a state of hibernation that is normally entered upon under circumstances of starvation or overcrowding, may be suppressed by null mutations of *daf-16 (135,136).*

In addition, *daf-18* mutations have also been found to partially or completely suppress the *daf-2* and/or *age-1* mutants, respectively *(137–139). daf-18* is the homolog of *PTEN,* and has been shown to antagonize the DAF-2–AGE-1 pathway *(140–143).* Furthermore, loss of function mutations of *age-1* and *daf-2,* which produce a constitutive dauer phenotype, may be completely suppressed by certain *daf-18* mutations *(141–143).* In *C.elegans,* levels of PtdIns-3,4,5-$P_3$ prevent dauer arrest. Certain *daf-18* mutations, which suppress either of the loss-of-function mutants, rescue reduced levels of PtdIns-3,4,5-$P_3$ and prevent dauer arrest *(140).* In addition, although *daf-2* and *age-1* mutations increase life-span, mutations of *daf-18* decrease longevity *(141).*

Overall, these studies corroborate the role of PTEN as a downregulator of the PI3K–Akt pathway, acting between PI3K and Akt. They also suggest that PTEN may, in addition, also modulate insulin signaling and longevity in mammalian cells, as well, although no studies have demonstrated this to date *(144).*

#### 3.2.8. Summary

Current data support the notion that one of the primary mechanisms by which PTEN acts as a tumor suppressor is through its inhibition of PI3K signaling. Both regulation of cell survival and regulation of cell cycle progression can be ascribed to either loss of PTEN in primary tissues of re-expression of PTEN in tumor cells. This suggests that PTEN is both necessary and sufficient for these processes in specific contexts. Remaining questions include: What downstream targets of Akt or PI3K mediate these effects? And, what determines whether a particular cell is more inclined to depend on this pathway for survival, as opposed to proliferation? With respect to the latter question, it is clear that this is not simply a reflection of tumor-specific mutational events, because primary tissues in the mouse differ with respect to whether proliferation or cell survival is the net effect. Furthermore, a strict tissue-specific dependence does not exist because certain tumor cell lines, derived from different glioblastoma tumors, undergo either anoikis or cell cycle arrest, with reintroduction of PTEN *(86,128,145).* One possibility is that, during the process of differentiation, cells undergo a transition from a reliance on PI3K signaling for proliferation to a reliance on PI3K for survival. Differences in cell lines derived from similar tissue types could be accounted for if such tumors arose from different populations of committed stem cells. For example, such a switch could revolve around the loss or gain of specific Akt substrates. This model would not necessarily preclude the possibility that additional tumor-derived mutations could further influence this balance.

### *3.3. PTEN as Protein Phosphatase*

The protein phosphatase activity of PTEN, although not sufficient for PTEN function in regulating apoptosis or growth suppression, is linked to alterations in cell migration, spreading, and motility, and focal adhesion formation *(146–148).* Overexpression of PTEN in fibroblasts or glioblastoma lines has been shown to reduce or inhibit inte-

grin-mediated cell spreading and focal adhesion formation *(146)*. This is associated with a decrease in tyrosine phosphorylation of (FAK), as well as its downstream target, p130 Crk-associated substrate ($p130^{Cas}$); PTEN can dephosphorylate FAK in vitro. Furthermore, in contrast to the studies of apoptosis and cell cycle regulation, the G129E mutant of PTEN which lacks lipid phosphatase activity but retains protein phosphatase activity, is comparable to wt PTEN in these experiments *(146)*. Thus, this PTEN function is genetically distinguishable from the regulation of P13K signaling.

Subsequent studies have revealed that PTEN also decreases tyrosine phosphorylation of the adapter protein, Shc, and downregulates its downstream activation of mitogen-activated protein kinase (MAPK) *(148)*. This particular function appears to be involved in random cell motility *(147,148)*. In contrast, the FAK–$p130^{Cas}$ pathway appears to be involved in a parallel pathway that directs persistent directional cell migration *(148)*. Tamura et al. *(149)* found that overexpression of FAK in PTEN-reconstituted cells inhibited P13K activity, but did not fully restore PtdIns-3,4,5-$P_3$ activity, compared to PTEN-deficient cells. This suggested that PTEN might have multiple effects on cellular physiology, and possibly on the P13K–Akt pathway: the regulation of PtdIns-3,4,5-$p_3$, through its lipid phosphatase activity, and the ability to dephosphorylate FAK and Shc, via its activity as a protein phosphatase.

In contrast to these data, *PTEN–/–* ES cells do not have excessive phosphorylation of FAK, nor obvious alterations in MAPK signaling *(65)*. Thus, if PTEN is involved in the regulation of these pathways, it is not necessary for appropriate regulation of the pathway. To date, mutants retaining protein phosphatase activity have been noted, but no mutant retaining only lipid phosphatase activity has been described. Therefore, although protein phosphatase activity is clearly not sufficient for inhibition of cell growth, it has not been possible to ask whether such activity is necessary.

### *3.4. Structure and Function*

The minimal phosphatase domain of PTEN has been mapped to the region between amino acid residues 10 and 353 (Ramaswamy et al., unpublished data). This domain not only inhibits Akt kinase activity, but is also sufficient to induce a G1 arrest. The carboxyl-terminal (C-terminal) 50 residues (354–403) contains a PDZ-binding site *(94)*. However, this domain is not required for suppression of colony formation in soft agar, nor for the induction of apoptosis in LNCaP cells (Ramaswamy et al., unpublished data).

Although most mutations isolated occur in or near the catalytic motif *(87),* there are a small number of mutations that are found within these last 50 amino acids. The C-terminal PDZ-binding site would be predicted to interact with proteins containing PDZ domains. PDZ-domain proteins, named for the first three members of the class (PSD-95, disks-large, and ZO-1), are protein–protein interaction domains that are thought to direct the assembly of multiprotein complexes at the cell membrane *(97)*. More recently, these domains have been implicated in the regulation of membranetrafficking of signaling receptors *(150)*. The presence of tumor-derived mutations raises the possibility that the C-terminus is required for tumor suppression in vivo. In keeping with this notion, deletion of the C-terminus leads to an approx fourfold decrease in the half-life of PTEN (Vasquez et al., unpublished data, and ref. *151*). Taken together, these data suggest that, although the C-terminus may be unnecessary for phosphatase function alone, it is important for regulating the stability and activity of the protein.

Whether the PDZ-binding domain and/or interaction with a PDZ domain containing protein is required for PTEN stability, and in turn for PTEN function as a tumor suppressor, remains to be determined.

## 4. MOUSE MODELS

The importance of *PTEN* in development and tumorigenesis has been elucidated by the study of PTEN–/– mice generated by multiple groups *(64,90,91,123).* These mice do not survive beyond embryonic d 9.5, and are characterized by abnormal patterning and growth of cephalic and caudal regions *(123,152).* Bromodeoxyuridine (BrdU) labeling of *PTEN*–/– embryos demonstrates marked increases in BrdU incorporation, suggesting that lethality and alterations in the patterning and growth of the cephalic and caudal regions may arise as a consequence of increased levels of cell proliferation *(123).*

Studies of *PTEN*+/– chimeras and MEFs, as noted earlier, have demonstrated that they have an increased resistance to apoptotic stimuli *(123),* and behave with the ability to proliferate in an anchorage-independent manner *(64).* These cells also appear to have higher levels of activated Akt *(90,123).*

Studies of *PTEN*+/– mice have reported different phenotypes. Suzuki et al. *(91)* and Stambolic et al. *(123)* have found a high incidence of T-cell lymphomas associated with LOH of the wt *PTEN* allele. Podyspanina et al. *(90)* have reported phenotypes similar to CD in humans with gastrointestinal polyps, follicular and papillary thyroid carcinomas, endometrial hyperplasia, prostate intraepithelial neoplasia, and lymphadenopathy associated with an apoptotic defect. Similarly, Di Cristofano et al. *(64)* have reported dysplastic changes of the colon, prostate, and skin, as well as marked lymphadenopathy. In that particular study, the observed changes appear to be associated with the retention of the wt allele.

These varying phenotypes may be explained by differences in breeding backgrounds or *PTEN* genotypes. However, all groups have reported a high incidence of lymphomas. Recent data from Di Cristofano et al. *(64)* have suggested that, in *PTEN*+/– mice, the development of lymphomas is secondary to a defect in Fas-mediated apoptosis, and possibly haploinsufficiency. This provides further evidence that PTEN is necessary to regulate the cell survival pathway, and, in mice, one of these downstream targets includes the death receptor, Fas.

## 5. CONCLUSION

The identification of *PTEN* as a TSG has led to the surprising discovery that it functions as both a protein and lipid phosphatase *(94,146).* Through its specific activity against the phosphoinositides, PtdIns-3,4,5-$P_3$ and PtdIns-3,4-$P_2$, it regulates the P13K–Akt pathway, controlling both cell survival and cell proliferation *(97,144).* Its protein phosphatase function appears to be important in another aspect of tumorigenesis, i.e., FAK-mediated cell spreading and motility *(146).*

*PTEN* mutations occur in many tumor types, and are associated with different stages of tumorigenesis in certain tumor types (e.g., glioblastomas vs endometrial carcinomas). Currently, PTEN functions to regulate at least two distinct pathways: P13K–Akt and FAK, for at least three different purposes: cell cycle, cell survival, and cell motility regulation. PTEN antagonizes the P13K–Akt pathway, and, therefore, indirectly,

through Akt, modulates its many downstream targets, many of which are proapoptotic factors, as well. Thus, several questions remain. What are the critical downstream targets of Akt that are required for tumorigenesis in the absence of PTEN? What are the mechanisms that underlie PTEN's role as a regulator of cell survival and cell cycle progression? Does PTEN differentially modulate the numerous targets of Akt in a tumor- and/or tissue-specific manner? Is PTEN itself regulated? And, what are the genetic alterations that, together with PTEN loss, cooperate to fully transform cells? Answers to these and other questions will probably be forthcoming in the near future.

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# 11 Neurofibromatoses

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***Contents***



## 1. INTRODUCTION

Neurofibromatosis type 1 (NF1, or von Recklinghausen's NF; formerly, also, peripheral NF), and neurofibromatosis type 2 (NF2, formerly, bilateral acoustic or central NF) are inherited neurocutaneous disorders or phakomatoses. Diagnostic criteria for the two syndromes were decided upon at an National Institutes of Health Consensus Development Conference in 1987, and are listed in Table 1. The recognition of NF1 and NF2 as distinct clinical entities has since been validated by the mapping of the *Nf1* and *Nf2* genes to different loci, and by the identification of different functions for the encoded proteins. Thus, the protein encoded by the chromosome (chr) 17q11.2 *Nf1* gene, termed "neurofibromin," functions as a guanosine triphosphatases GTPase, activating protein (GAP) for Ras, and may also play a role in signaling mediated by adenosine-3′,5′-monophosphate (cyclic AMP, or cAMP); the product of the chr 22q12.2 *Nf2* gene, named "merlin" or "schwannomin," is a member of the ezrin-radixin-moesin (ERM) family of membrane–cytoskeleton linker proteins.

This chapter summarizes what is currently known about the functions of neurofibromin and merlin-schwannomin, and discusses how this emerging understanding may lead to novel therapies. The work leading up to the identification of the *Nf1* and *Nf2* genes is discussed only briefly. This and other early work has been covered by several prior reviews (1–5).

## 2. NEUROFIBROMATOSIS TYPE 1 (NF1)

### *2.1. The Disease*

Although descriptions and illustrations of individuals with NF1-like symptoms are known from throughout history, the first comprehensive description of NF1 was pro-

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**Table 1**
**Diagnostic Criteria for NF1 and NF2**

| |
|---|
| Diagnostic criteria for NF1 are met if an individual has two or more of the following: |
| Six or more café-au-lait spots |
| 0.5 cm or larger before puberty |
| 1.5 cm or larger after puberty |
| Two or more cutaneous or subcutaneous neurofibromas, or one plexiform neurofibroma |
| Freckling in the axillary or inguinal regions |
| Optic glioma |
| Two or more Lisch nodules (benign iris hamartomas) |
| Distinctive bony lesion |
| Sphenoid wing dysplasia |
| Dysplasia, or thinning of long bone cortex, with or without pseudoarthrosis |
| First-degree relative with NF1 by the above criteria |
| Diagnostic criteria for NF2 are met if an individual has one of the following: |
| Bilateral vestibular schwannoma (VS) |
| Family history of NF2 plus a unilateral VS diagnosed before 30 yr of age, or any two of the following: meningioma, glioma, schwannoma, juvenile posterior subcapsular lenticular opacities, or juvenile cortical cataract |
| Individuals with the following should be evaluated for NF2: |
| Unilateral VS before 30 yr, plus at least one of the following: meningioma, glioma, schwannoma, juvenile posterior subcapsular lenticular opacities, or juvenile cortical cataract. |
| Two or more meningiomas, plus unilateral VS diagnosed before 30 yr, or one of the following: glioma, schwannoma, juvenile posterior subcapsular lenticular opacities, or juvenile cortical cataract |

Rare related syndromes include familial café-au-lait spots, schwannomatosis, Watson syndrome (WS), and multiple meningiomas. NF1 and NF2 also occur in a nongeneralized form, called segmental NF1 or NF2, reflecting somatic mosaicism. Adapted with permission from ref. *6*.

vided by von Recklinghausen toward the end of the nineteenth century *(7)*. Several subsequent studies contributed to current understanding of NF1 as a dominantly inherited disorder with complete penetrance, but highly variable expressivity *(8,9)*. The disease has an estimated prevalence of 1/3500 in all ethnic groups *(10)*, making it about 10-fold more common than NF2, and ranking NF1 among the most frequent genetic diseases of man. 30–50% of NF1 cases occur sporadically, suggesting that approx 1/10,000 gametes harbor a *de novo Nf1* mutation *(10)*. The large size of the *Nf1* gene (*see* Subheading 2.2.) provides at least a partial explanation for this high mutation rate. The hypothesis that gene conversion between the *Nf1* gene and several *Nf1* pseudogenes also contributes remains speculative *(11)*. Similar to what has been found for several other genetic diseases, the majority of *Nf1* mutations occurs in the male germ line *(12,13)*. However, large deletions are predominantly of maternal origin *(14,15)*.

Loss of *Nf1* gene function is probably the most common cause of inherited increased cancer risk. NF1 is more than just a tumor predisposition syndrome, however, because between 30 and 65% of patients exhibit learning disabilities, and an estimated 20%

have skeletal defects *(see____).* Prenatal diagnosis in familial cases has been possible since the location of the *Nf1* gene was established *(16,17),* but the utility of genetic counseling is limited by the highly variable expressivity of NF1 symptoms. This unpredictability is among the most troubling aspects of the disease, from a patient's perspective, and does not reflect the nature of the *Nf1* mutation, because most studies have failed to reveal obvious genotype–phenotype correlations *(9).* Rather, the clustering of uncommon symptoms in monozygotic twins, and, to a lesser extent, in first-degree relatives with NF1, supports the notion that much variability reflects the action of symptom-specific modifier genes *(18).*

The most common symptoms of NF1 are areas of abnormal skin pigmentation, called café-au-lait spots; abnormal freckling in skin-fold areas; pigmented iris hamartomas, known as Lisch nodules; and cutaneous or subcutaneous neurofibromas *(8,9).* Each of these symptoms appears in an age-dependent manner. Thus, although café-au-lait spots are also found in 10% of normal individuals, and occur at increased frequency in NF2, McCune-Albright syndrome (MAS), and several other genetic disorders, the presence of six or more café-au-lait spots over 0.5 cm in size, in prepubertal individuals, is highly suggestive of NF1 (Table 1). Like café-au-lait spots and abnormal freckling, Lisch nodules have no associated morbidity, and are present in the eyes of most NF1 patients at puberty. Neurofibromas typically first appear in young adolescents, and increase in number later in life. Thus, adult NF1 patients can have from just a few to hundreds or even thousands of these benign tumors, which arise from peripheral nerve sheaths, and which consist of Schwann cells, (perineurial) fibroblasts, infiltrating mast cells, and other cellular elements *(8).* NF1 has been called a neurocristopathy *(19),* to stress that several of its symptoms involve cells originating from the embryonic neural crest. However, NF1 patients also suffer from skeletal abnormalities, and are at increased risk of developing juvenile malignant myeloid disorders. Moreover, it remains controversial whether defects in Schwann cells, nerves, or perineurial fibroblasts are responsible for neurofibroma formation *(20–22).*

Although the most common symptoms of NF1 are in large part benign, patients also suffer from a variety of less frequent, but potentially more serious, problems (*8,9; see* ref. *23* for frequencies of the most common symptoms). Thus, an estimated 50% of patients exhibit macrocephaly (head circumference above the ninety-fifth percentile), and about 30% are below the fifth percentile for height. Patients are also at increased risk of exhibiting distinct skeletal defects, notably scoliosis, sphenoid wing dysplasia, or thinning of the long bones, with or without pseudoarthrosis. Learning and behavioral problems also occur with high frequency in NF1, with different studies finding 30–65% of children affected *(24).* The cognitive phenotype includes both a specific learning defect (a significant discrepancy between ability and performance, sometimes likened to attention deficit disorder), and a somewhat lower full-scale IQ (typically around 90). No consensus exists whether the cognitive deficit is linked to the occurrence of $T_2$-weighted hyperintense lesions in brain magrative resonance images *(24).* These transient lesions, which can be detected in the brains of up to two-thirds of children with NF1, appear to reflect the presence of intramyelinic edema *(25).* Mental retardation (IQ <70) is relatively uncommon in NF1. Gliomas (pilocytic astrocytomas), most frequently of the optic pathway, are another feature of NF1. Optic gliomas occur in up to 20% of children with NF1, but do not usually lead to visual loss. One recent study *(26)* reported an association between the occurrence of optic gliomas and other

central nervous system (CNS) tumors. Among the most serious complications, 20–30% of patients develop plexiform neurofibromas alongside deeper-lying nerves. These lesions are often congenital, and can cause severe overgrowth of surrounding soft tissue and bone. Malignant peripheral nerve sheath tumors (MPNSTs), previously called "neurofibrosarcomas" or "malignant schwannomas," are strongly associated with NF1, and have a tendency to arise from plexiform neurofibromas. Other tumors, notably juvenile malignant myeloid disorders, pheochromocytoma, and rhabdomyosarcoma, also occur at increased frequency, and the lifelong risk of malignant tumors in NF1 has been estimated to be around 5% (*see* ref. *23* and references therein).

The variability of NF1 symptoms remains a source of confusion, especially because other genetic disorders have symptoms that overlap with those of NF1. Thus, a proportion of NF1 patients have facial features that resemble those seen in mild cases of Noonan syndrome, but current evidence argues against the existence of a distinct NF1–Noonan syndrome *(27).* Watson syndrome (WS) is a rare genetic disease characterized by café-au-lait spots, short stature, dull intelligence, and pulmonary valve stenosis. The genes responsible for WS and NF1 are tightly linked *(28),* and a 42-bp duplication in the *Nf1* coding region has been found in a WS family *(29).* Thus, although Lisch nodules and neurofibromas occur infrequently in WS *(28),* and although heart defects are rare in NF1 *(23),* WS is probably a subtype of NF1. It is interesting to speculate that WS mutations result in a mutant form of neurofibromin with partial dominant-negative characteristics, since murine *Nf1* knockouts also exhibit defective heart development (*see* below). A patient diagnosed with multiple lentigines syndrome, and a patient with encephalocraniocutaneous lipomatosis, also harbored *Nf1* mutations *(30,31).* However, these patients may have been misdiagnosed, or may have had NF1 in addition to these other diseases. Finally, a recurring theme is the equation of NF1 with Elephant Man Disease, even though the severely disfigured "Elephant Man," Joseph Merrick, is more likely to have suffered from Proteus syndrome *(32).*

### *2.2. Characterization of Nf1 Gene*

The gene responsible for NF1 was mapped to chr 17q11.2 in 1987 *(16,17),* and identified in 1990 *(33–35).* The gene consists of 57 constitutive and 3 alternatively spliced exons, spans close to 350 kb of genomic DNA, and is transcribed into two 11–13-kb mRNAs with different 3′-untranslated segments *(36). Nf1* mRNA, without any of the alternatively spliced exons, encodes a protein of 2818 amino acids (aas), with a calculated molecular mass of 317 kDa and an apparent molecular mass in sodium dodecyl sulfate (SDS) gels of 220–280 kDa. This protein, termed "neurofibromin," harbors a centrally located 360-aa segment related to the catalytic domains of Ras-specific GTPase activating proteins (RasGAPs) *(37,38),* and shares more extensive, but relatively low-level, similarity, extending over nearly 1500 aas with two RasGAPs (Ira1p and Ira2p) from *Saccharomyces cerevisiae* (Fig. 1). The subsequent confirmation that neurofibromin has RasGAP activity suggested that defective Ras regulation may be responsible for at least some of the diverse symptoms of NF1 (Fig. 2).

The large size of the *Nf1* gene, and the presence of several related pseudogenes, have complicated the search for mutations, and, in most early studies, only small segments of the 8454 bp open reading frame were analyzed. More recently, protein-based assays have allowed the detection of mutations throughout the coding region. In two such studies *(39,40),* around 70% of patients were found to harbor truncating mutations. A database

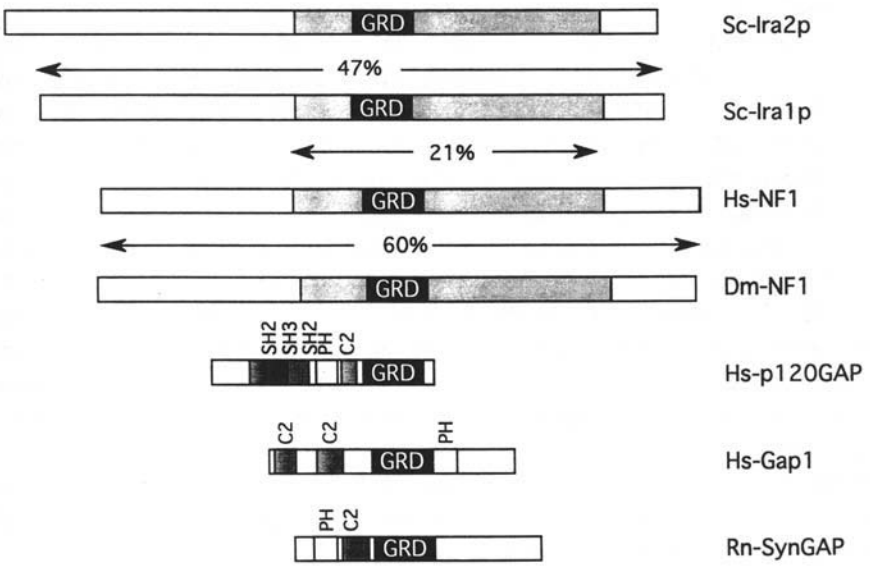


**Fig. 1.** Schematic structure and sequence similarity between RasGAPs. The proteins shown are (from top to bottom) *S. cerevisiae* Ira2p and Ira1p *(196); Homo sapiens* NF1; *D. melanogaster* NF1; human p120GAP *(197),* a representative member of a small family of human proteins related to *Drosophila* Gap1 *(198);* and *Rattus norvegicus* SynGAP *(199,200).* Human and *Drosophila* neurofibromin are 60% identical over their entire length. The human protein also shares around 20% identity with Ira1p and Ira2p between residues 900 and 2350.

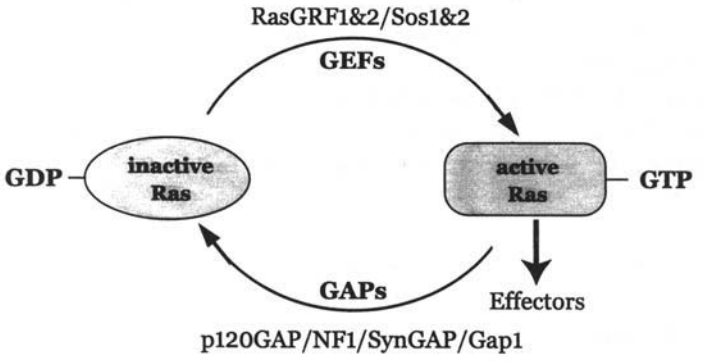


**Fig. 2.** Ras GTPases function as binary switches in signal transduction, by cycling between inactive guanosine diphosphate (GDP)- and active GTP-bound conformations. Ras proteins have a high affinity for both GDP and GTP. Cycling of mammalian Ras is promoted by at least four guanine nucleotide exchange factors, which activate Ras by promoting GTP for GDP exchange, and by as many as six GAPs, which stimulate the low intrinsic GTPase activity of Ras, thus promoting its inactivation. Active Ras interacts with several effector proteins, including Raf, phosphatidylinositol-3 kinase, and Ral-GDS, among others. Evidence exists that p120GAP and other RasGAPs may have effector functions in addition to their role as negative regulators of Ras-mediated signal transduction (*see* ref. *3* for further details).

maintained by the NF1 Genetic Analysis Consortium (http://www.nf.org/nf1gene) currently lists over 240 mutations, with no apparent hot spots among them. More than half of the mutations are deletions and insertions, which probably represents a ascertainment bias. Also listed are over 40 nonsense and around 30 missense mutations scattered throughout the coding region. Missense mutations are interesting from a functional point of view, although some may represent rare polymorphisms and others may affect protein stability. No clear genotype–phenotype correlations can be distilled from the mutation data, except that patients with large deletions appear more likely to exhibit a severe phenotype that includes facial dysmorphic features and large numbers of early-onset neurofibromas *(41,42).* Genetic linkage to the *Nf1* locus has been established in some, but not all, families with spinal NF *(43),* or familial café-au-lait spots *(44),* and a typical truncating *Nf1* mutation has been found in a family with spinal NF *(45).* Whether the predominant spinal location of neurofibromas in this family reflects a genetic background effect remains to be established.

The *Nf1* gene harbors three other genes transcribed in the opposite direction within one of its introns *(35).* One embedded gene encodes oligodendrocyte-myelin glycoprotein (OMG), a 110-kDa glycosylphosphatidylinositol-linked protein expressed in the CNS *(46).* Expression of OMG inhibits the proliferation of fibroblasts, suggesting a role in growth control *(47).* The other intronic genes are *EVI2A* and *EVI2B,* whose murine homologs were first identified in the midst of a cluster of viral integration sites in retrovirus-induced murine myeloid leukemia (ML). This initially suggested a role for *Evi2a* or *Evi2b* as myeloid leukemia oncogenes, but it is currently believed that retrovirus-mediated inactivation of *Nf1* is responsible *(48).* Whether loss of the embedded genes contributes to the severe phenotype in Nf1 patients, who harbor large deletions, remains unknown.

Tissue-specific alternatively spliced mRNAs predict neurofibromin variants that include 10 extra aas upstream of the IRA-related segment *(49),* 21 additional residues within the GAP-related domain (GRD) *(50),* or 18 extra aas close to the C-terminus *(51).* The 21-aa GRD insert reduces, but does not abolish, GAP activity *(52),* and the functional significance of any neurofibromin variant remains to be established. Human *Nf1* mRNA is also subject to RNA editing *(53),* and unequal expression of *Nf1* alleles in patient-derived cell lines, possibly reflecting nonsense-mediated mRNA decay, has been reported *(54).* Whether these phenomena contribute to NF1 pathogenesis remains unclear.

### *2.3. Structure and Function of Neurofibromin*

*Nf1* homologs from mice, the Japanese puffer fish, *Fugu rubripes,* and the fruit fly, *Drosophila melanogaster,* encode proteins that share, respectively, 98.5, 91.5, and 60% overall sequence identity with human neurofibromin *(55–57).* Although participation in protein complexes is among the most obvious reasons for such a high degree of evolutionary conservation, no obvious protein interaction motifs are evident in neurofibromin, and only Ras-related GTPases (*see* below), α- and β-tubulin *(58),* and an as-yet-unidentified 400–500 kDa protein *(59)* have been found to interact with human neurofibromin.

Many cell lines express neurofibromin, and the protein is widely expressed during mouse fetal development *(60,61).* By contrast, neurofibromin is most abundant in the peripheral nervous system and CNS of adult rats, with highest expression observed in

neurons, oligodendrocytes, and nonmyelinating Schwann cells *(62).* No consensus exists as to neurofibromin's subcellular localization, with different groups reporting localization to the smooth endoplasmic reticulum in Purkinje cells *(63),* to capped immunoglobulin in activated B-lymphocytes *(64),* to microtubules in fibroblasts *(65),* or to mitochondria *(66).* Mitochondrial staining was seen in six cell lines, using four antibodies and two fixation methods. An estimated 20% of neurofibromin co-purified with mitochondria *(66),* consistent with findings that neurofibromin is distributed between cytosolic and particulate fractions *(67).* Neurofibromin not only acts as a GAP for H-, K-, and N-Ras, but also inactivates R-Ras *(68).* This is especially intriguing, because both conventional p21 Ras and p23 R-Ras have been reported to interact with Bcl-2 *(69,70),* an antiapoptotic protein that localizes to mitochondrial, microsomal, and nuclear membranes *(71).* However, although a role in apoptotic signaling may help explain why *Nf1*-deficient neurons survive in the absence of neurotropic factors *(22),* or why it is hard to express neurofibromin in transfected cells *(72),* it cannot be ruled out that neurofibromin may yet have other GTPase targets. p120 RasGAP also stimulates GTP hydrolysis by Rab5 *(73).*

Notwithstanding this speculation, there is ample evidence that downregulation of conventional Ras is among the important functions of neurofibromin. Thus, baculovirus-produced neurofibromin stimulated the GTPase activity of H- or N-Ras in vitro *(58,74),* and expression of an NF1-GRD protein rescued the heat shock sensitivity of yeast *ira1* or *ira2* mutants *(75,76).* Moreover, three NF1 patient-derived MPNST cell lines expressed either very little or no neurofibromin. These cells contained up to 50% nonmutant Ras in the active GTP-bound conformation, and expression of a RasGAP catalytic segment inhibited their proliferation concomitant with a reduction in Ras-GTP *(77,78).* Less drastically elevated Ras-GTP levels were also found in neurofibromas *(79),* and in neurofibromin-deficient ML bone marrow *(80).* Mutations that activate Ras or inactivate neurofibromin define mutually exclusive subsets of ML, providing genetic evidence that loss of *Nf1* contributes to myeloid leukemogenesis by activating Ras *(81).*

A more complex situation exists in melanoma (mel) and neuroblastoma (NB). Although neither of these neural-crest-derived tumors occurs at increased frequency in NF1, several NB or mel cell lines did not express neurofibromin, which in some cases reflected loss of both *Nf1* alleles *(82–84).* Although loss of heterocygosty (LOH) at the *Nf1* locus was not found in primary mels *(85),* and is uncommon in primary NBs (G. Cowley and Bemards, unpublished observation), a NB tumor from an NF1 patient harbored a homozygous *Nf1* gene deletion *(86),* and heterozygous loss of *Nf1* strongly enhanced NB formation in MYCN overexpressing transgenic mice *(87).* Unlike MPNSTs, neurofibromin-deficient NBs and mels showed no increased levels of Ras-GTP *(82,83),* and re-expression of neurofibromin inhibited the growth of a mel line without altering its Ras-GTP content *(72).* Taken together with findings that Ras mutations are infrequent in neural-crest-derived tumors *(88),* which may reflect the fact that oncogenic Ras induces cell cycle arrest and differentiation in these cells *(89),* these findings suggest that loss of neurofibromin may contribute to NB and mel transformation through a mechanism that does not involve Ras, possibly by disrupting differentiation *(72,82).* However, it is important to note that only steady-state Ras-GTP levels were measured in these studies. Thus, it remains possible that *Nf1*-deficient NBs or mels exhibit transient defects in Ras regulation, similar to what has been observed in neurofibromin-deficient myeloid cells *(80,90,91).*

### 2.4. Nf1 Gene as Tumor Suppressor

The focal nature of many NF1 lesions suggests that additional genetic or epigenetic events at the somatic level are required to trigger their development. There is accumulating evidence that, at least in neurofibromas and NF1-associated malignant tumors, one additional genetic hit involves the inactivation of the remaining *Nf1* allele. Thus, an NF1 patient-derived neurofibrosarcoma showed LOH of all chr 17 polymorphisms and a 200-kb deletion affecting the remaining *Nf1* allele *(92).* This observation followed several reports of LOH of closely linked genetic markers in NF1-associated MPNSTs *(93,94).* Pheochromocytomas occur in approx 1% of NF1 patients, and LOH at the *Nf1* locus was found in each of seven NF1-associated tumors. In all cases in which this could be determined, LOH involved the wild-type (wt) *Nf1* allele, providing strong support for a tumor suppressor role in these adrenal tumors *(95).* Children with NF1 have a 200–500-fold enhanced risk of developing malignant myeloid disorders, particularly juvenile myelomonocytic leukemia (formerly known as juvenile chronic myelogenous leukemia) and monosomy 7 syndrome *(96,97).* Providing support for a tumor suppressor role, bone marrow cells from several children with NF1 and ML showed homozygous inactivation of the *Nf1* gene *(98).* Moreover, approx 10–20% of heterozygous *Nf1* knockout mice developed pheochromocytoma; a similar fraction developed ML (in all cases, with loss of the second *Nf1* allele; *99*), and irradiated mice whose hematopoietic system was reconstituted with *Nf1–/–* fetal liver cells developed granulocyte-macrophage colony-stimulating factor hypersensitive ML at high frequency *(90).*

Whether *Nf1* serves as a frequent tumor suppressor in other cancers remains uncertain. Of special interest is a possible role in astrocytoma, the most common and malignant of human brain cancers. A role in astrocytoma was initially suggested by the frequent occurrence of optic pathway and other gliomas in NF1, and by the detection of aa substitutions at residue 1423 (a conserved lysine within the GRD), which strongly reduced the catalytic activity of neurofibromin, in one case each of anaplastic astrocytoma, colon adenocarcinoma, and myelodysplastic syndrome *(100).* However, a subsequent study detected LOH at the *Nf1* locus in only 1/22 astrocytomas *(101);* others detected elevated *Nf1* mRNA or protein levels, rather than loss of expression, in pilocytic astrocytomas and reactive astrocytes *(102–104).* Thus, although other studies detected more frequent LOH *(105,106),* a role for *Nf1* as a common astrocytoma tumor suppressor remains to be proven.

Lisch nodules and optic gliomas are not easily accessible, and the analysis of neurofibromas is complicated by the fact that these tumors consist of several cell types, not all of which may be transformed *(107,108).* However, contrasting numerous earlier studies *(93,94,109–111),* which had failed to detect loss of chr 17 polymorphisms in neurofibromas, three groups recently reported evidence favoring a double-hit mechanism in these benign tumors. Thus, Colman et al. *(112)* detected LOH of *Nf1* intragenic and other chr 17q polymorphisms in eight neurofibromas from two sporadic patients, out of a total of 22 tumors from five patients. That study did not identify the constitutive mutation in these patients, although the deleted allele was always of maternal origin, consistent with the idea that most sporadic *Nf1* mutations are of paternal origin *(12,13).* Subsequently, Sawada et al. *(113)* identified a 4-bp deletion in *Nf1* exon 4b in a neurofibroma from a patient, which harbored a large constitutive deletion. DNA from microdissected tumor tissue showed both deleted and wt polymerase chain reaction products, consistent with the idea that neurofibromas consist of an admixture of normal

and mutant cells. Finally, Serra et al. *(114)* detected LOH in 25% of 60 neurofibromas from 17 patients. Why patients, with large deletions centered on the *Nf1* gene, have a tendency to develop large numbers of early-onset neurofibromas remains to be explained.

The basis of NF1-associated pigmentation defects remains incompletely understood. Early studies noted an increased occurrence of macromelanophores or melanin macroglobules in café-au-lait patches of NF1 patients *(115,116),* but similar structures also occur in normal skin *(117).* Moreover, although melanin macroglobules can be observed in melanocytes, it remains possible that abnormal clearance of these structures by dentritic Langerhans cells or macrophages also contributes to the pigmentation defects *(118).* Cultured melanocytes from NF1 patient café-au-lait spots expressed neurofibromin, and showed no evidence of LOH at the *Nf1* locus *(119).*

### *2.5. Genetic Models*

Of the many avenues of research aimed at ultimately designing rational therapy for human genetic disorders, few have been as fruitful as the generation and study of animal models. Thus, the first generation of *Nf1*-mutant knockout mice has proved invaluable, among several other things, in confirming a tumor suppressor role for neurofibromin; genetic analysis of *Drosophila Nf1* has provided the most compelling evidence so far for a Ras-independent role for neurofibromin in neuropeptide-mediated signal transduction.

Mice heterozygous for *Nf1*-null alleles do not exhibit pigmentation defects, iris abnormalities, or benign neurofibromas, and thus do not provide an accurate model for several aspects of the human disease *(99,120).* However, 75% of heterozygotes succumbed to a variety of tumors by 27 mo of age, compared to 15% of a comparable set of wt animals, confirming a tumor suppressor function for murine *Nf1 (99). Nf1* heterozygotes also showed learning and memory defects resembling those of NF1 patients. Thus, an impaired performance in the Morris water maze, which assesses spatial learning, was not fully penetrant, and could be overcome by increased training. The defect in spatial learning appeared specific, because associative learning was not impaired *(121).*

*Nf1* homozygous mutant mice die around d 13.5–14.5 of fetal development. A complex heart defect, resulting in generalized edema, is believed to be the proximal cause of embryonic death *(99,120).* A developmental delay and hyperproliferation of sympathetic and parasympathetic ganglia was observed in only one study, perhaps reflecting a difference in genetic background *(120).* Underlying the heart defect may be a failure of endocardial cushions to properly differentiate. Endocardial cushions are the precursors of heart valves, and form by a process of epithelial-to-mesenchymal conversion. Neurofibromin appears necessary to regulate this process, since, in its absence, mouse hearts develop overabundant endocardial cushions, obstructing the normal flow of blood *(122).* Enhanced invasiveness of *Nf1*-deficient cardiac cushion cells was rescued by expression of a dominant-negative RasN17 mutant, and mimicked in wt cells by expressing activated Ras *(122).*

Several explanations as to why murine *Nf1* heterozygotes do not develop neurofibromas or other common signs of human NF1 can be envisaged. Among other possibilities, the number of target cells that can give rise to neurofibromas or other lesions may be smaller in the mouse, or the animals may not live long enough to develop the

lesions. Differences in the mutation rates of the human and murine *Nf1* homologs may also contribute, as may differences in synteny between human and murine genes. To circumvent these limitations, more sophisticated second-generation conditional *Nf1* mutants are being generated. Such mice may provide a more accurate model of human NF1, given that neurofibroma-like lesions have been found in chimeric mice made with homozygous *Nf1*-deficient embryonic stem cells (T. Jacks, personal communication).

The fruit fly, *D. melanogaster,* and the nematode, *Caenorhabditis elegans,* have been used widely to genetically dissect complex biological processes. The recently completed *C. elegans* genome sequence predicts several RasGAP-like proteins, but no *Nf1* homolog. In contrast, a *Drosophila Nf1* homolog consists of 17 common and two alternative C-terminal exons, and predicts proteins of 2764 and 2802 aas that share 60% overall sequence identity with human neurofibromin *(57).* However, unlike the widespread developmental defects observed in transgenic flies expressing activated Ras1 or Ras2 (a *Drosophila* R-Ras homolog) *(123,124),* homozygous *Drosophila Nf1*-null mutants are 15–20% reduced in size during postembryonic development, but are otherwise normal. Biochemical analysis confirmed that *Drosophila* NF1 is a potent RasGAP, but genetic studies revealed no defects in Ras-mediated signaling downstream of several receptors with tyrosine kinase activity, even in sensitized genetic backgrounds. Moreover, although expression of an inducible *Nf1* transgene restored the size of mutants to that of wt flies, manipulating *Ras1* or *Ras2* gene dosage or activity did not modify the size defect. Thus, the most obvious defect observed in *Drosophila Nf1* mutants appears to reflect a Ras-independent function of the protein *(57).*

*Drosophila Nf1* mutants are also somewhat sluggish, and exhibit a diminished escape response, which did not reflect any obvious anatomical defect of the musculature or the nervous system *(57).* The behavioral defect may, however, result from abnormal neurotransmission, because a neuropeptide-elicited delayed rectifying potassium current at the larval body wall neuromuscular junction was absent in *Nf1*-null mutants. The authors analyzed signaling by the PACAP38 neuropeptide, because an increase in potassium conductance mediated by PACAP38 had been found to require both intact Ras-Raf and cAMP-mediated signaling pathways *(125).* However, the electrophysiological defect in *Nf1* mutants was not mimicked or modified by manipulating Ras, but was rescued by forskolin-mediated activation of adenylyl cyclase, or by providing cAMP *(126).* The reduced size of *Nf1* mutants was similarly rescued by expressing a constitutively active cAMP-dependent protein kinase A (PKA) transgene, or mimicked in flies carrying hypomorphic PKA alleles *(57).* Thus, *Drosophila Nf1* functions either upstream of adenylyl cyclase or in a parallel convergent pathway. In this context, it is worth noting that *Saccharomyces cerevisiae* cerevisiae Ras directly activates adenylyl cyclase *(127),* and that yeast adenylyl cyclase may be mislocalized in an *iral* mutant *(128).* However, yeast and metazoan adenylyl cyclases are very different in structure, and the significance of this observation remains unclear.

### *2.6. NF1: Current Picture and Future Directions*

The fact that all *Nf1*-deficient phenotypes in *Drosophila* are rescued by increasing cAMP–PKA signals is a departure from what had been found in mammals, in which most studies had emphasized the Ras-related role of neurofibromin. However, the

idea that neurofibromin may play dual roles in Ras and cAMP signaling is attractive for at least two reasons: First, the RasGAP catalytic domain accounts for only 10% of neurofibromin, and the rest of the protein is equally well conserved *(55,57);* second, although inappropriate Ras activity provides a ready explanation for NF1-associated tumors, other symptoms may be more easily understood in terms of defective cAMP signaling. This is especially true for NF1-associated learning disabilities, since mutations that affect cAMP signaling are known to cause learning and memory defects in *Drosophila,* mammals, and several other organisms *(129,130).* Defects in cAMP signaling may also contribute to other NF1 symptoms, however, and patients with MAS exhibit café-au-lait spots, skeletal defects (polystotic fibrous dysplasia, frequently leading to pseudoarthrosis), and endocrinologic abnormalities, including female precocious puberty, all of which are also seen in NF1. However, although *Drosophila Nf1*-deficient phenotypes are rescued by increasing cAMP–PKA signaling, MAS is caused by somatic mutations that constitutively activate the α-subunit of the adenylyl-cyclase-stimulating heterotrimeric Gs protein *(131,132).* Perhaps similar defects are caused by either too little or too much cAMP signaling: *D. rutabaga* (adenylyl cyclase) and *dunce* (cAMP phosphodiesterase) mutants have similar learning and memory defects.

The question whether defective cAMP signals explain some features of human NF1 is an important one, because different therapeutic strategies may be used to downregulate Ras or increase cAMP. Thus, farnesyl transferase inhibitors show great promise as anti-Ras agents *(133),* and have already been used to modify the in vitro properties of *Nf1*-deficient murine Schwann and human MPNST cells *(134,135);* cAMP phosphodiesterase inhibitors are among agents that may be employed to restore cAMP signals. So far, however, most research has focused on the Ras-related role of neurofibromin, with a recent study *(136)* suggesting that defective Ras regulation may provide an adequate explanation for all aspects of NF1. The authors of that study based their conclusion on the identification of a disease-associated missense mutation, which altered the catalytically important arginine finger residue in the NF1–GRD *(137).* The mutant protein had severely impaired GAP activity, but its interaction with Ras, and its stability, appeared normal. Several family members with this mutant allele had severe NF1, including learning disabilities, suggesting that loss of GAP activity may explain all features of NF1 *(136).* However, others found that missense mutations within the GRD may affect the subcellular localization of neurofibromin by disrupting its association with microtubules *(138).* Thus, although the cumulative evidence indicates that loss of RasGAP activity plays an important role in several NF1 symptoms, it cannot be concluded that other functions of neurofibromin are not also important. Most of the 30 or so disease-associated missense mutations occur outside of the RasGAP catalytic domain of neurofibromin.

Nine yr after the *Nf1* gene was identified, a global understanding about neurofibromin's role in signal transduction has been achieved. Among the most important remaining questions is the identity of the specific Ras- and/or cAMP-mediated signaling pathways that are responsible for NF1 symptoms. Another important question concerns the identity of modifier genes that determine the clinical severity of NF1. By employing a combination of biochemical, cell biological, and genetic approaches in man, mice, and flies, it should be possible to provide answers to these questions in the not too distant future.

## 3. NEUROFIBROMATOSIS TYPE 2

### *3.1. The Disease*

NF2 (non-von Recklinghausen disease) is less common than NF1, affecting about 1/35,000 individuals, and is symptomatically distinct (*139;* Table 1). However, the disease features a similar dominantly inherited predisposition to developing nervous system tumors, indicating that the gene responsible is also a tumor suppressor. The symptoms of NF2 generally appear during the second and third decades of life, and, like those of NF1, are progressive. The hallmark of NF2 is the development of Schwann cell tumors of the eighth cranial (acoustic) nerves, usually causing loss of hearing. NF2 patients frequently develop multiple schwannomas of the cranial and spinal nerves, and are also predisposed to developing meningiomas, ependymomas, and posterior subcapsular cataracts. In contrast to neurofibromas, which are mixed tumors composed of several cell types including Schwann cells, schwannomas are homogeneous tumors derived from Schwann cells or their precursors *(140).* Moreover, schwannomas are benign tumors that rarely, if ever, progress to malignancy. However, they are associated with a high degree of morbidity in NF2, because of their multiplicity and intractable location at the boundary between the peripheral nervous system and CNS *(139).* Unfortunately, inconsistent histological diagnosis of schwannomas and neurofibromas has historically led to some diagnostic confusion; there is, in fact, probably no true overlap between NF1 and NF2.

### *3.2. The Gene*

LOH studies localized the *Nf2* locus to human chr 22q12: The gene was subsequently identified by positional cloning in 1993 *(17,141–144).* Genetic studies have consistently revealed loss of the remaining wt allele in NF2-associated tumors, and somatic inactivation of both *Nf2* alleles in sporadic schwannomas and approx one-half of sporadically occurring meningiomas *(5).* Most mutations in the *Nf2* gene are deletions, or frameshift or nonsense mutations predicted to encode nonfunctional protein. Moreover, immunohistochemical absence of the NF2 antigen has been detected in several NF2-associated tumors, even when the mutation was a missense or truncating mutation predicted to encode detectable protein, suggesting that mutant *Nf2* protein is particularly unstable *(145).* Moreover, investigation of the nature of the mRNA transcript, produced from alleles carrying *Nf2* splice-site mutations, revealed the use of cryptic splice sites that resulted in frameshifts and protein truncation, instead of the expected excision of a single exon *(146).* Thus, no clear genotype–phenotype correlations have been made. Taken together, this genetic behavior is consistent with the notion that the *Nf2* locus encodes a tumor suppressor whose normal growth-suppressing function must be lost, for tumor formation to occur. The normal growth-suppressing function of NF2 appears to be specific for, or specifically relied upon in, the Schwann and meningeal cell lineages, because *Nf2* mutations have not consistently been identified in other types of human cancers, with one exception; Cell lines derived from malignant mesotheliomas of the lung lining, which are associated with asbestos exposure, frequently harbor *Nf2* mutations *(147,148).* The fact the NF2 patients are not predisposed to mesothelioma development may reflect the rare co-occurrence of asbestos exposure and germ-line *Nf2* mutation or the requirement for other genetic alterations, perhaps preceding Nf2 loss in mesothelioma formation.

In general, there is much less variability in the expressivity of NF2, compared to NF1, rendering disease course more predictable, and suggesting less genetic modification. However, two related disorders have been described: Schwannomatosis is likely to involve loss of *Nf2* gene function, either through somatic mosaicism (segmental NF2), or, when familial, through an apparently inherited predisposition to develop mutations at the *Nf2* locus *(149).* On the other hand, it has been reported that meningiomatosis, or multiple meningiomas, as inherited in an autosomal dominant fashion, is not linked to the *Nf2* locus on chr 22, and may result from mutation of an as-yet-unidentified tumor suppressor gene *(150).* This is supported by the fact that a large subset of sporadic meningiomas do not exhibit obvious mutation at the *Nf2* locus *(151).*

Transcription from the *Nf2* gene produces transcripts of 2.6, 4.4, and 7 kb, which probably differ with respect to the lengths of 5′ and 3′ untranslated regions *(143,144).* These transcripts encode a 595- or 590-aa protein (*see* below) with an apparent mol wt of ~69 kDa on SDS-polyacrylamide gel electrophorotic. The *Nf2* mRNA and protein is widely expressed during development and in the adult, with particularly high levels found in the developing nervous system *(61,152,153).* Although several alternatively spliced forms have been reported, the expression of only one has been studied extensively. Alternative use of the extreme carboxy, (C)-terminal exon is controlled in a tissue-specific and temporal fashion; isoform I lacks exon 16 and uses exon 17 as its final exon; isoform II contains exon 16, which carries its own stop codon (Fig. 3). Isoform II is the predominant isoform expressed in most adult tissues, including the adult brain; isoform I is expressed in the fetal brain and some adult renewal tissues, such as the spleen, kidney, and ovary (*152;* McClatchey, unpublished observations). Recent experimentation suggests that the biochemical properties of the proteins encoded by these two mRNAs may be functionally distinct, supporting the significance of their different expression patterns (*154; see* below).

### *3.3. Structure and Function of Merlin/Schwannomin*

The cloning of the *Nf2* gene led to the surprising discovery that the encoded protein is a member of the band 4.1 superfamily of cytoskeleton–membrane linker proteins *(155).* This places the NF2 protein in an intriguing physical niche within the cell for a tumor suppressor. Many tumor suppressors function as transcription factors or components of the cell cycle machinery controlling cellular proliferation from within the nucleus, or, like neurofibromin, function in the cytoplasm to regulate signal transduction pathways that directly control growth-promoting genes in the nucleus. In contrast, band 4.1 family members were previously thought to function primarily in providing cytoskeletal integrity, and perhaps reorganization, secondary to nuclear decision-making. In particular, the NF2 protein is most closely related to the ERM proteins, and was thus dubbed merlin (moesin, ezrin, radixin-like protein) by one group, and schwannomin by another *(156).* The ERM proteins associate with membrane partners via their amino (N)-terminus, and directly with actin via their C-terminus; they are localized to areas of active cortical cytoskeletal remodeling. Merlin also localizes similarly to cortical actin structures, particularly membrane ruffles, microvilli, and the cleavage furrow, and can bind to some ERM membrane partners via its N-terminus, but lacks the C-terminal actin-binding domain of the ERMs *(157–160).*

What little is known about the molecular function of merlin is modeled on an emerging, but still relatively poor, understanding of ERM function. For example, a number of

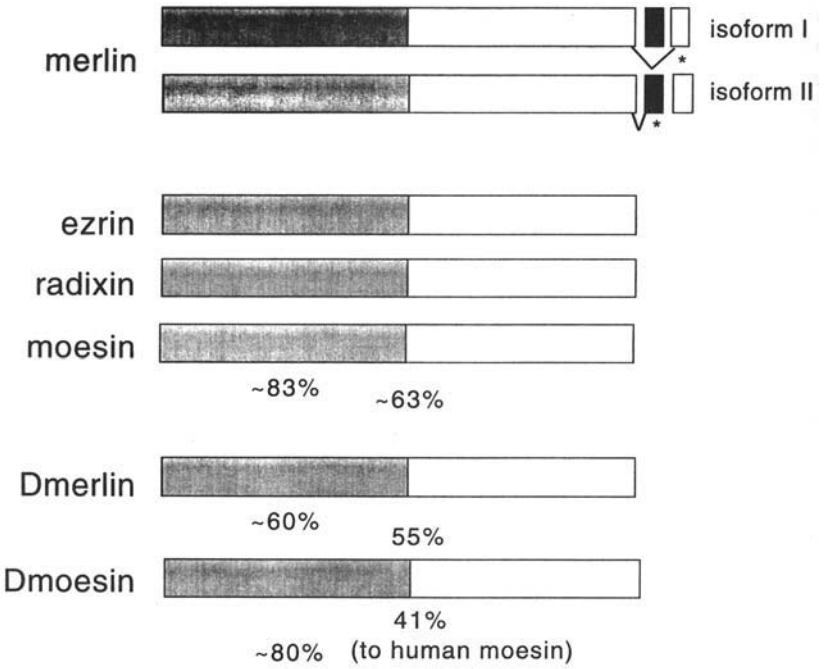


**Fig. 3.** The NF2 protein, merlin, belongs to the ERM subfamily of the band 4.1 superfamily of cytoskeleton–membrane linkers. These proteins share similarity primarily within their N-terminal halves (gray boxes). Thus, the ERMs share 83% aa identity to one another within their N-terminal halves, and ~63% identity throughout their entire lengths. Similarity to the *Drosophila* proteins, Dmerlin and Dmoesin, are also shown *(194).* Dmerlin and Dmoesin share 55 and 41% aa identity to human merlin across their entire lengths; Dmerlin shares 60% identity to the human merlin N-terminus, and Dmoesin shares 80% identity to the human moesin N-terminus.

ERM membrane-interacting proteins have recently been described, including the hyaluronate receptor, CD44; CD43; the integrins, ICAM-2 and ICAM-3; the myosin-binding subunit of myosin phosphatase; PKA; and a co-factor for the $Na^+/H^+$ exchanger, hNHE-RF/EBP50 *(161–166).* Thus far, merlin has similarly been reported to bind to CD44 and hNHE-RF/EBP50 in vitro *(167,168).* In addition, the C-terminus of merlin isoform II specifically has been reported *(169)* to bind to the actin-binding protein βII-spectrin/fodrin, suggesting an indirect mechanism whereby merlin associates with the actin. Although the consequences of these interactions are not yet known, an emerging theme posits that the ERM proteins function to locally organize signaling components for rapid and local response. For example, several lines of experimentation suggest that the ERM proteins control the formation of specialized membrane protrusions, such as microvilli and uropods, and the relocalization and concentration of cell surface molecules to them *(163,170,171).* Similarly, delocalization of the ERMs has been suggested to causally precede microvillar breakdown at the initial stages of apoptosis *(172).*

The ERM proteins are capable of intramolecular, monomeric or intermolecular, oligomeric head-to-tail association *(173–175).* In addition, phosphorylation of the ERMs on serine/threonine and tyrosine (Tyr) residues occurs in response to various

stimuli, and phospholipid (4,5-$PIP_2$) binds to the ERM N-terminus *(156)*. Phosphorylation, and/or 4,5-$PIP_2$ binding, is thought to activate the ERMs, relieving this conformation, and causing relocalization to the membrane and interaction with membrane and cytoskeletal partners. In support of this idea, only the N-terminal halves, and not full-length ERM proteins, can bind to CD44 in vitro; however, interaction between full-length ERM and CD44 can occur in vitro in the presence of 4,5-$PIP_2$ *(161)*. Similarly, threonine phosphorylation of the C-terminus of the ERMs disrupts the intramolecular association of their N- and C-terminal halves *(176)*. Tyr phosphorylation of the ERMs occurs in response to epidermal growth factor, platelet-derived growth factor, and hepatocyte growth factor (HGF) treatment *(177–179)*. In fact, ezrin has been reported to be a direct substrate of the HGF receptor, Met *(179)*. Despite the strong aa identity between the ERMs, they were differentially phosphorylated in response to each growth factor *(178)*. Regulated localization of the ERMs may lead to their differential use as substrates. The relative contributions of these posttranslational modifications to ERM activation remains an important area of investigation.

Merlin is phosphorylated on serine/threonine residues, but, to date, Tyr phosphorylation of merlin has not been detected *(180)*. Indeed, the known ERM Tyr phosphorylation sites are not conserved in merlin. In contrast to the ERMs, unphosphorylated merlin is associated with various forms of growth arrest, and thus with the active growth-suppressive form of merlin *(180)*. For example, total merlin levels, and particularly levels of unphosphorylated merlin, increase with increasing cell density, serum deprivation, or loss of adhesion. This unphosphorylated form is predominantly found in the detergent insoluble (cytoskeletal) compartment; stimuli that lead to phosphorylation of merlin lead to the appearance of phosphorylated merlin in the cytosol. Merlin isoforms I and II have C-termini with very different predicted secondary structures. In fact, emerging evidence suggests that merlin isoform I, and not II, is capable of head-to-tail interaction *(154)*. Moreover, some evidence suggests that isoform I specifically can suppress the growth of rat Schwann cells in vitro. Together, these observations lead to the similar, yet contrasting models for ERM/merlin function depicted in Fig. 4. Much more information is necessary to complete these simplistic models.

Several lines of recent evidence link ERM function to signaling pathways controled by the Rho GTPases, a subfamily of the Ras GTPase superfamily *(181)*. The prototypes of this subfamily, RhoA, Rac 1, and Cdc42Hs, effect specific forms of cytoskeletal remodeling, in response to various stimuli. Thus, activation of RhoA, Rac 1, and Cdc42 lead to the formation of stress fibers, membrane ruffles, and filopodia, respectively *(181)*. In addition, the function of these proteins has been linked to such cellular activities as adhesion, migration, proliferation, and transformation. A requirement for the ERM proteins in Rho- and Rac-dependent actin remodeling in permeabilized cells has been demonstrated *(182)*. Activation of RhoA, but not Rac 1 or Cdc42Hs, effects phosphorylation and relocalization of the ERM proteins *(183)*. In fact, the ERM proteins have recently been identified as direct substrates of the RhoA effector, Rho kinase (RhoK) *(176)*. Coordinated regulation of the phosphorylation state of the ERM C-terminus by RhoK and myosin light chain phosphatase, whose regulatory subunit can bind to the ERMs, has been proposed *(164)*. Finally, it has been reported that, in vitro, the ERM proteins can bind directly to both negative (RhoGDI) and positive (Db1) regulators of RhoA, suggesting that the ERMs may function to control the localization of regulators of Rho GTPase signaling pathways *(184,185)*. The consequences of RhoA-

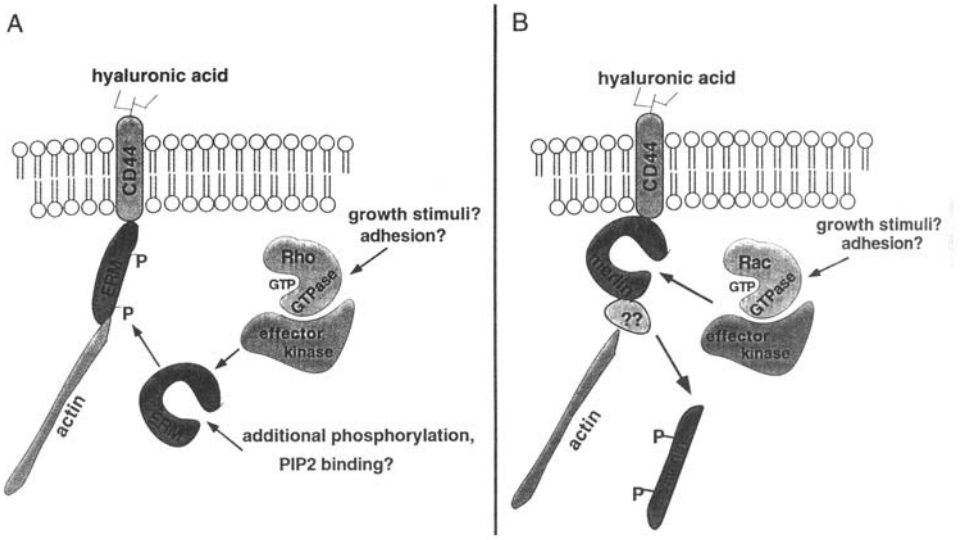


**Fig. 4.** Contrasting hypothetical models of ERM **(A)** and merlin **(B)** regulation. The ERM proteins and merlin isoform I can exist in an intramolecular/monomeric or intermolecular oligomeric head-to-tail conformation. Phosphorylation, and possibly 4,5-PIP2 binding, is thought to relieve this conformation. For the ERM proteins, this activates them, and strengthens their association with the cytoskeleton, and membrane partners, such as CD44. In contrast, phosphorylation appears to inactivate merlin, decreasing the strength of its cytoskeletal association. Rho kinase is one known effector of ERM phosphorylation; in contrast, merlin appears to be phosphorylated in response to Rac activation.

mediated ERM phosphorylation to the cytoskeleton, cellular movement, and/or proliferative signaling remain to be determined.

Preliminary evidence indicates that merlin is phosphorylated in response to activation of Rac, and not Rho (R.J. Shaw, A. Yaktine, T. Jacks, McClatchey, unpublished); Rac induces the formation of membrane ruffles, a structure to which merlin preferentially localizes *(158)*. In this case, phosphorylation would be predicted to inactivate merlin, removing it from tight cytoskeletal association; indeed, preliminary experimental evidence supports this (R.J. Shaw, A. Yaktine, T. Jacks, McClatchey, unpublished). Much attention is currently focused on determining the role of merlin and merlin phosphorylation in Rho GTPase signaling, and on identifying the kinase(s) responsible for merlin phosphorylation. It will be particularly interesting to determine whether merlin functions to regulate any of the known downstream components of Rac (or Rho or Cdc42) signaling, such as jun-N-terminal kinase or nuclear factor F-κB activation, cell cycle entry, or targets of the serum response element.

At the cellular level, roles for the ERMs have been identified in cell adhesion, migration, and morphology *(156)*. Antisense oligonucleotide impairment of ERM expression suggests redundant functions for the three proteins *(186)*. Inactivation of single family members produces little effect; however, loss of expression of all three ERM proteins (as determined by Western blot analysis) leads to the disappearance of microvilli from the surface of cells, and impaired cell–cell and cell–substrate adhesion. A positive role for the ERMs in cell migration is suggested by evidence that ezrin func-

tion is both necessary and sufficient for HGF-mediated cell migration in vitro *(179)*. This is also consistent with a relationship between the ERMs and the Rho GTPases, which are known to promote cell migration *(181)*.

Both antisense studies and the study of the properties of Nf2-deficient cells suggest similar cellular functions for merlin. Antisense disruption of merlin expression leads to loss of adhesion and altered morphology of Schwann-like and glioma cells in vitro *(187)*. Primary Nf2-deficient mouse embryo fibroblasts (MEFs) exhibit defects in adhesion, manifested by reduced spreading upon replating and by withdrawal of serum (R.J. Shaw, I. Saotome, T. Jacks, McClatchey, unpublished). Furthermore, in contrast to the ERMs, the study of *Nf2–/–* MEFs reveals a negative role for merlin in cell migration. In culture, *Nf2–/–* MEFs migrate to close monolayer wounds significantly faster than their wt counterparts (R.J. Shaw, I. Saotome, T. Jacks, McClatchey, unpublished).

### *3.4. Genetic Models*

As for NF1, the generation and study of animal models has proven a powerful approach to the study of NF2. In the case of NF2, mouse modeling has confirmed the tumor suppressor function of merlin, revealed several developmental contexts requiring merlin function, and implied a greater role for NF2, and perhaps its ERM family members, in cancer development and progression in humans, than previously thought *(188,189)*.

The mouse NF2 protein is 97% identical to human NF2: There are only 7 aa differences between the two proteins *(190)*. *Nf2* heterozygous mice are cancer-prone, but do not develop schwannomas; instead, they develop malignant osteosarcomas, fibrosarcomas, and hepatocellular carcinomas that exhibit *Nf2* LOH *(189)*. A large percentage of these tumors metastasize to distant sites, a phenomenon that is unusual in the mouse. This observation raises the possibility that loss of Nf2 contributes to metastatic potential in this setting. Indeed, initial experiments support a role for merlin loss in metastatic progression. This is particularly interesting, given the interaction of the ERM proteins and merlin with CD44, a molecule that has long been associated with metastatic progression in humans *(191)*. A role for merlin loss in metastatic progression is also consistent with the increased motility exhibited by *Nf2–/–* MEFs, and a relationship between merlin and Rac, which is known to promote cellular invasion and metastasis *(181)*. It will obviously be important to investigate whether merlin or the ERMs are altered during metastatic progression in humans.

*Nf2* heterozygous-mutant mice do not develop schwannomas, and *Nf2* mutations have not been found in human osteosarcomas, but explanations for differing human–mouse phenotypes have emerged from other studies *(192)*. In some cases, such as those of *Rb+/–*, humans and mice, it is clear that closely related family members are able to compensate for retinal loss of Rb in the mouse, and not in humans. In other cases, such as *Nf1+/–* humans and mice, it is clear that the rate of loss of the wt allele in mice is limiting. Thus, although *Nf1+/–* mice do not develop neurofibromas, chimeric mice, composed in part of *Nf1–/–* cells, develop many neurofibroma-like lesions (K. Cichowski, S. Shih, T. Jacks, in preparation). An extension of this idea is supported by the observation that mice carrying *Nf2* and *p53* or *Nf1* mutations in *cis* on the same chr (these three loci are all linked in mice, and not in humans), leads to dramatic acceleration of tumor formation (*189;* McClatchey, I. Saotome, T. Jacks, unpublished). These studies not only reveal cooperation between a *Nf2* mutation and mutations in both the

*p53* and *Nf1* genes in cancer development, but they also reveal the importance of linkage of cancer-predisposing mutations. Differences in mouse and human genome configuration may dramatically influence combinatorial mutation rates, and thereby account for differences in the spectra of tumors that develop. The identification of the molecular pathway, in which merlin functions, may reveal other targets of mutation during the development of schwannomas in humans or osteosarcomas in mice.

Cooperation between *Nf1* and *Nf2* mutations in mouse tumor development was somewhat unexpected, given the growing symptomatic distinction between NF1 and NF2 in humans. Mice carrying *Nf2* and *p53* mutations in *cis* exhibit a dramatic acceleration of osteosarcoma formation, developing multiple, early-onset osteosarcomas, but *Nf2–Nf1* compound *(cis)* heterozygotes reproducibly develop a tumor type resembling a MPNST (McClatchey, I. Saotome, T. Jacks, unpublished). The fact that Rac functions downstream of Ras signaling provides one possible explanation for this cooperativity *(181).* Rac function has been shown to be required for Ras-mediated transformation, and Ras and Rac activation cooperate in cell transformation. Loss of merlin and neurofibromin may similarly cooperate in activating the two connected pathways. In fact, merlin has been reported to suppress Ras-mediated transformation *(193).*

Gene targeting in the mouse has also revealed that the normal function of merlin is manifest during several stages of mouse embryonic development *(188).* A homozygous *Nf2* mutation in the mouse leads to developmental failure early, during embryonic development at gastrulation. The defect resides in the extraembryonic lineage, and may result from abnormal cell adhesion/invasion. In addition, mosaic embryos composed of *Nf2–/–* and wt cells reveal additional developmental requirements for Nf2, including during cardiac development (I. Saotome, T. Jacks, McClatchey, unpublished). In the developing myocardium, loss of Nf2 function is sufficient for hyperproliferation of myocardial precursors; chimeric hearts contain tumor-like lesions that are composed entirely of *Nf2–/–* cells, the nature of which is reminiscent of NF2-associated schwannomas. These developmental contexts reflect the growth-controlling situations that merlin evolved to perform, and thus important ones in which to study merlin function.

In *D. melanogaster,* homologs for merlin (Dmerlin) and, apparently, a single ERM protein (Dmoesin), have been identified *(194).* In addition, a merlin homolog can be identified in the recently sequenced *C. elegans* genome. Dmerlin shares 55% aa identity to that of human merlin, with particularly concentrated relatedness throughout the N-terminal halves (Fig. 3). Dmoesin shares ~58% aa identity with the ERM proteins. Conditional expression of exogenous Dmerlin in S2 cells reveals that the protein is initially localized at the membrane, but is rapidly internalized and localized to endocytic vesicles. Similarly, detection of endogenous protein in *Drosophila* embryos reveals both membrane and punctate, intracellular staining. This raises the intriguing possibility that merlin functions in the cytoskeletal reorganization that accompanies endocytic recycling. Impaired recycling of active signaling molecules, such as growth factor receptors, could lead to hyperactivation of the signaling pathways that they govern, and to uncontrolled cellular growth. In addition, somatic mosaic analysis in *Drosophila* does, in fact, reveal a cell-autonomous growth-suppressive function for Dmerlin, supporting its identity as a tumor suppressor *(195).* Additional studies have examined the ability of a series of mutant Dmerlin isoforms to localize subcellularly in vitro, and to transgenically rescue a null phenotype in vivo *(195).* These studies identified both

potentially dominant-active and dominant-negative merlin isoforms. Examination of the mammalian counterparts of these mutants may be particularly enlightening.

### *3.5. NF2: Current View and Future Directions*

Most current effort is placed on the identification of signaling pathway(s) in which merlin functions. Particular attention is focused on an assessment of the function of merlin in the signaling pathways governed by the Rho family of GTPases. This is being pursued chiefly through the attempted identification of additional interacting proteins, and through the comparison of Nf2-expressing and Nf2-deficient cells. Many published studies have compared primary human Schwann cells and cells derived from Nf2-deficient schwannomas, which suffer from the probability of additional mutations present in the tumor cells. The generation of cell lines that stably overexpress merlin, and the reintroduction of merlin expression to Nf2-deficient tumor cells, has proven difficult, probably because excess merlin is not tolerated in most cell types. The use of inducible systems, and the generation of primary Nf2-deficient cells and their wt counterparts, should allow the controlled evaluation of the cellular and molecular consequences of merlin function.

A determination of the relationship between merlin and the ERM proteins remains an important area of investigation. Although modeling of merlin function upon ERM function may be fruitful, several lines of evidence suggest that ERM and merlin functions are different, and may, in fact, be antagonistic. It will be interesting to compare the status of the ERM proteins in Nf2-expressing and Nf2-deficient cells. For example, are the ERM proteins phosphorylated, localized, or oligomerized differently under various conditions in Nf2-expressing vs Nf2-deficient cells?

Future investigations will also focus on the development of better models of Nf2-associated schwannoma and meningioma formation in mice. For example, the use of conditional targeting and chimeric analysis will allow the generation of adult animals that lack Nf2 in the Schwann and meningial cell lineages, and a determination of whether loss of the wt *Nf2* allele is rate-limiting for schwannoma formation. Alternatively, intercrossing of *Nf2*+/– mice with other mutant mouse strains may reveal genetic interaction in schwannoma or meningioma formation. The use of both mouse and invertebrate models will be invaluable toward identifying genetic modifier loci and other members of the merlin pathway.

The placement of merlin, within known signal transduction pathways, will suggest rational treatment possibilities. For example, given the anti-Ras function of merlin and the placement of Rho GTPases downstream of Ras, farnesyl transferase inhibitors may be expected to be effective in treating NF2-deficient tumors. Alternatively, geranyl transferase inhibitors, which would be predicted to disable some Rho family members, including Rac, may be effective. The identification of genes that are upregulated or activated in Nf2-deficient cells may also be targets. For example, if merlin normally antagonizes ERM function, then targeting the inactivation of the ERMs may have therapeutic value. Mouse models of Nf2-deficient tumor formation provide important vehicles in which to test these therapeutic strategies. Finally, the investigation of a broader role for NF2 and the ERMs in human cancer is an important avenue of inquiry that may help to decipher the role of the poorly understood membrane–cytoskeleton interface in cancer development and progression.

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# 12 Von Hippel-Lindau Disease

## *Clinical and Molecular Aspects*

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***CONTENTS***



### 1. INTRODUCTION

von Hippel-Lindau (VHL) disease (OMIM 193300) is a hereditary cancer syndrome characterized by the development of central nervous system (CNS) and retinal hemangioblastomas, renal cell carcinomas (RCCs), pheochromocytomas, and other lesions *(1)*. The first description of retinal angiomata affecting two siblings can be traced to Treacher Collins (1894) *(2)*. The German opthalmologist, Eugene von Hippel, described two unrelated kindreds with retinal angiomata in 1904 *(3)*. The familial nature of this disease was fully appreciated by neurophysiologist John Fulton in 1920 *(4)*. The Swedish opthalmologist, Arvin Lindau, observed the connection between retinal lesions and larger angiomatous lesions of the CNS in 1927 *(5)*. The term "Lindau disease" was coined by Melmon and Rosen in 1964, in a comprehensive review of the literature *(6)*.

Significant advancements in understanding VHL disease have occurred over the past decade. Cloning of the tumor suppressor gene (TSG), which, when altered, gives rise to VHL disease, was reported in 1993. The availability of the VHL gene has allowed for genetic, rather than purely clinical, diagnosis of VHL disease. As a result, the spectrum of clinical abnormalities associated with VHL disease is now better appreciated, and certain genotype–phenotype correlations are emerging. In addition, the VHL gene

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product (pVHL) has been identified in mammalian cells, and studies of its biochemical and biological functions have already yielded clues as to why VHL patients are cancer-prone. This chapter highlights classic and recently appreciated clinical features of the disease, describes new molecular insights into the function of pVHL, and suggests potential strategies for therapeutic intervention based on this information.

## 2. CLINICAL FEATURES OF VHL DISEASE

### *2.1. Hemangioblastoma and Retinal Angiomatosis*

CNS hemangioblastomas and retinal angiomas are histologically identical lesions. They are the commonest, earliest, and most characteristic lesions of VHL disease, occurring in 60–80% of VHL patients *(9–10)*.

VHL-associated hemangioblastomas are typically multifocal, and may occur synchronously or metachronously. They involve the cerebellar hemispheres (75%), spinal cord (20%), and brain stem (5%). Spinal hemangioblastomas occur most often in the cervical and lumbar area, are intradural, and often produce syringomyelia *(11,12)*. Rare cases of supratentorial, nerve root, peripheral nerve, and hepatic hemangioblastomas, as well as spinal leptomeningeal hemangioblastosis, have been reported *(13)*. Despite the fact that hemangioblastomas are nonmetastatic lesions, they cause considerable morbidity and mortality because of their space-occupying nature. Symptoms depend on the affected area, and develop once the tumor reaches a size critical for its location: headache, vomiting and cerebellar ataxia (infratentorial lesions, with increased intracranial pressure), orthostatic hypotension (brain stem lesions), spinal motor and sensory disturbances (spinal cord lesions), and pain (peripheral nerve lesions). Hemangioblastomas may be associated with polycythemia as a result of tumor-derived erythropoietin *(14)*. Sporadic, non-VHL-associated hemangioblastomas, in contrast to VHL-associated lesions, are typically unifocal, and rarely involve the spinal cord *(15,16)*.

Macroscopically, a typical cerebellar hemangioblastoma, presents as a cystic lesion harboring a solid component attached to its wall. The solid: cystic ratio varies. Purely cystic lesions do exist, but they are rare. Spinal hemangioblastomas are more often solid tumors or mixed solid tumors bearing small cysts *(11)*.

Histologically, hemangioblastomas consist of a mixture of stromal cells embedded within a rich and well-defined capillary network. Electron microscopy and immunohistochemical studies have addressed the origin of the stromal cell *(17)*. It appears that these cells are probably of mesenchymal origin. They may share a common ancestor with endothelial cells, but it is still unclear at which stage stromal and epithelial cells become committed to distinct differentiation programs. Stromal cells stain positively for neuron-specific enolase and vimentin, but negatively for epithelial membrane antigen and Ki-67 *(17)*. This immunocytochemical profile supports the mesenchymal origin of the stromal cell, and may help differentiate hemangioblastomas from RCCs metastatic to the CNS.

Retinal hemangioblastomas in VHL disease are also typically multifocal and bilateral. They occur, by decreasing order of frequency, in the temporal periphery, the nasal periphery of the retina, and in the posterior pole next to the optic nerve and the optic disk *(18)*. Left untreated, they cause retinal exudates, hemorrhage, and, ultimately, glaucoma and retinal detachment, leading to blindness. Decreased visual acuity in a VHL patient may also result from optic nerve atrophy caused by chronic obstructive hydrocephalus related to a cerebellar hemangioblastoma.

### 2.2. Renal Cysts and Clear Cell Renal Carcinomas

Between 60 and 85% of VHL patients will develop hundreds of bilateral renal cysts during their lifetime *(9,19,20).* The majority of these cysts will grow slowly over a period of years. Nevertheless, they usually remain clinically silent, rarely causing impairment of renal function *(21).* Macroscopically, they appear purely cystic, mixed cystic with a solid component, or multiloculated *(19).* It is currently believed that mixed and multiloculated cysts arise from the proximal renal tubule, and that purely cystic ones arise from the distal renal tubule *(22).*

RCC and CNS hemangioblastomas are the major causes of mortality in VHL patients *(8,23).* VHL-associated RCCs are clear cell or predominantly clear cell carcinomas, and arise from the epithelial lining of the proximal renal tubule *(24).* They are often multicentric and bilateral, and may form within a pre-existing mixed or multiloculated cyst, or *de novo* in the renal parenchyma *(25).*

### 2.3. Pheochromocytoma

VHL is phenotypically a heterogeneous disease. The development of pheochromocytomas tends to cluster within certain families (*see* Subheading 3.2.). VHL disease appears to be the most common cause of hereditary pheochromocytoma. Neumann et al. *(26)* examined the frequency and clinical characteristics of familial-type pheochromocytomas (MEN-2 and VHL) in an unselected group of 82 consecutive patients with documented symptomatic pheochromocytoma: 19% of patients had VHL, and 4% had MEN-2 disease. Compared to those with sporadic pheochromocytomas, VHL patients often have multifocal and bilateral lesions, were diagnosed at an earlier age, and were less likely to have histologically malignant disease. Prospective screening of a population at risk (VHL patients and their first-degree relatives), presented in the same study, revealed the presence of pheochromocytoma in 46% of the cases.

Many VHL-associated pheochromocytomas are asymptomatic *(26).* When symptoms occur, they include sweating, palpitations, anxiety, and headaches caused by catecholamine hypersecretion, and, if left untreated, may result in sequelae of uncontrolled hypertension, such as stroke or myocardial infraction.

### 2.4. Pancreatic Cysts and Neoplasms

The commonest pancreatic lesions associated with VHL disease include pancreatic cysts, microcystic serous adenomas (cystadenomas), and islet cell tumors. These lesions tend to cause less morbidity and mortality, compared to hemangioblastomas and RCCs. Many of these lesions may be clinically silent. Their estimated frequency varies from 10 to 60% among various series, depending primarily on the mode of detection (clinical, radiological, pathological) *(8,9,27–29).*

In VHL disease, simple cysts occur throughout the pancreas, without predilection for specific site. Their size ranges from a few millimeters to more than 10 cm. The serous adenomas are benign clusters of grape-like cystic lesions *(13,28,30).* Cysts and cystadenomas are asymptomatic in most cases. Excessive local growth may cause biliary duct obstruction, pancreatitis, and, in rare cases, exocrine and endocrine pancreatic insufficiency caused by cystic replacement of the parenchyma *(28–30).* VHL patients do not appear to be at risk for classic adenocarcinoma of the pancreas.

Islet cell tumors are unrelated to pancreatic cystic disease. These tumors are of neural-crest origin *(31)* and occur more frequently in patients with pheochromocytoma

*(27,33).* In most cases, these tumors grow slowly and remain asymptomatic *(27,29,31).* More rarely, they may exhibit more aggressive biology, manifested by rapid growth and metastasis, coupled with secretion of biologically active peptides, such as vasoactive intestinal peptide, calcitonin, insulin, glucagon, gastrin, or somatostatin *(13).* In the latter case, the type of symptoms depends on the endocrine function of the hypersecreted peptide.

### *2.5. Endolymphatic Sac Tumors*

Endolymphatic sac tumors (ELSTs) are locally invasive, but nonmetastatic, papillary adenocarcinomas arising from the endolymphatic sac, an ectodermal extension of the membranous labyrinth of the internal ear to the posterior surface of the petrous bone. Early symptoms consist of gradual or acute onset of decreased auditory acuity, tinnitus, and vertigo *(33).* Progressive tumor growth and invasion of local structures results in facial paresis and anesthesia, vocal cord paralysis manifested as hoarseness, and sternocleidomastoid muscle atrophy *(33,34).* Prospective audiologic evaluation at the National Cancer Institute revealed that 65% of VHL patients had pure tone threshold abnormalities, which, in one-half of cases, was bilateral *(33).* Many VHL patients with hearing loss do not have a radiographically detectable ELST at the time of examination. Nonetheless, it is possible that early auditory abnormalities herald the development of a microscopic ELST.

### *2.6. Papillary Cystadenomas of Mesonephric Origin*

The mesonephric duct forms during embryologic development. In males, it gives rise to ductus deferens and epididymis. In females, it involutes to the nonfunctional structures of the duct of epoöphoron and the duct of Gartner, extending along the adnexal area from the ovaries to the lateral wall of the vagina. These structures are the targets of tumor development in VHL patients.

Up to 60% of male VHL patients develop papillary cystadenomas of the epididymis *(8,35).* These are nonmetastatic tumors, ranging in size from 1 to 5 cm. They are mostly asymptomatic, unless they become inflamed or rupture. Infertility, although a rare complication, can arise if sperm delivery is impaired by bilateral tumors. Simple epididymal cysts are common in the general population, and do not raise suspicion for VHL disease. Papillary cystadenoma of the epididymis, however, is rare in the general population, and, if bilateral, is almost diagnostic for VHL disease.

Development of adnexal papillary cystadenomas of mesonephric origin has only recently been appreciated in female VHL patients *(36,37).* Consequently, it is still too early to estimate its real incidence in this patient population. Tumors may develop at any site along the mesonephric duct.

## 3. *VHL* GENE

### *3.1. Cloning, Structure, and Expression of VHL Gene*

The *VHL* gene was mapped to human chromosome (chr) 3p25 region, using linkage analysis *(38,39).* The identification of three unrelated patients, with small constitutional deletions in this area, was helpful in this regard *(38).* The gene consists of three exons (Fig. 1; *40*). Northern blot and reverse transcriptase-polymerase chain reaction analysis of mRNA, obtained from various cell lines with wild-type (wt) VHL, reveals

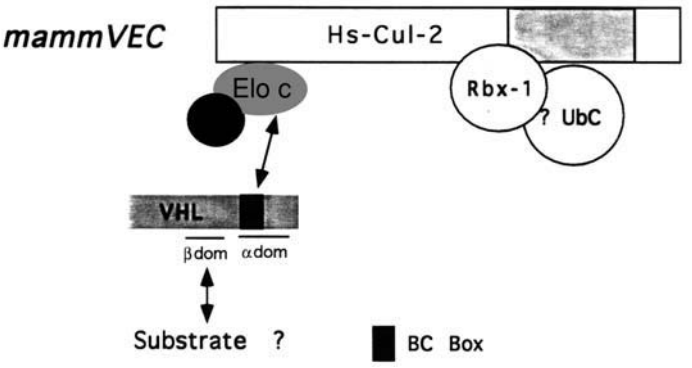


**Fig. 1.** **(A)** Structure of human *VHL* gene open reading frame (ORF), with representative mutations. Exon 1 encodes amino acids 1–113, exon 2 (shaded area) amino acids 114–153, and exon 3 aa 154–213. Arrowheads indicate missense mutations; vertical lines, nucleotide insertions; filled circles, stop codons; and letter D, deletions. Arrows point to the codon corresponding to the indicated aa. **(B)** Human pVHL30 and pVHL19. The crystal structure of pVHL 54–213 (third row) reveals two domains, α and β. BC box extends between aa 157 and 172, and corresponds to the H1 helix of the α-domain. Transcription-dependent nuclear localization domain (TDNL) encompasses exon 2. **(C)** Mouse and rat proteins drawn to scale with the human pVHL. The degree of homology between the two rodent species and the corresponding segments of the human protein are shown.

the presence of two mRNA species. The larger of these, which is approx 4.6 kb in size, corresponds to exons 1–3, and the smaller to an alternative spliced form consisting of exons 1 and 3 only *(41)*.

The VHL promoter contains putative binding sites for several transcription factors, including Sp1, AP-2, PAX, nuclear respiratory factor-1 and retinoic acid receptor, but neither a TATA nor a CCAAT box. Transcription is initiated around a putative Sp1 site located 60 bp upstream of the first AUG codon in the VHL mRNA *(42)*. The 3'UTR of *VHL* gene is ~3.6 kB in length, and contains multiple *Alu* repeat elements *(43)*.

*VHL* gene is expressed in every adult human tissue examined, and in all three germ layers during human and mouse embryonic development *(44,45)*. In adults, the *VHL* gene is strongly expressed in tumor-developing tissues (such as the renal proximal tubular epithelium and the CNS), as well as in tissues not known to be targets of tumor development, such as testis, lung, and liver *(46)*. Mouse and rat VHL have high homology to the human VHL gene (*47,48;* Fig. 1). No clear VHL homologs have been reported so far in yeast.

### *3.2. Germ-line Mutations and Genotype–Phenotype Correlations*

Germ-line mutations of the VHL gene can be detected in almost 100% of patients who carry a diagnosis of VHL disease, based on clinical criteria *(49)* (Table 1). Examples of missense or nonsense mutations, microdeletions, or microinsertions resulting in frameshifts, as well as large deletions encompassing the entire VHL gene, have been described *(50–58)*. A continuously updated list of VHL mutations is accessible through the internet at: http://www.umd.necker.fr *(59)*. All intragenic mutations reported thus far map downstream of the codon corresponding to amino acid (aa) Met 54. These mutations occur at several sites across the three exons. A cluster of nonsense and mis-

Table 1
Major Clinical Manifestations of VHL Disease

| *Horton (170)* | *Lamiell (8)* (*n = 50*) | *Maher (9)* *n = 554* | *Maddock (171)* *n = 152* | *n = 83* |
|---|---|---|---|---|
| | | *%Frequency (mean age of diagnosis)* | | |
| Hemangioblastomas | | | | |
| CNS | 44 (31) | 61 (28) | 72 (29) | 72 (30) |
| Retinal | 58 (28) | 57 (25) | 59 (25) | 41 (21) |
| Renal cancer | 28 (41) | 24 (39) | 28 (44) | 25 (38) |
| Pheochromocytomas | 10 (34) | 19 (27) | 7 (nr) | 12 (nr) |

Mean age at the time of diagnosis as reported in several series is shown. Diagnosis was made by clinical examination and/or imaging studies in both symptomatic patients and asymptomatic relatives at risk *(8,9,170,171).* nr, not reported.

sense mutations is noticeable at the 3′ end of the exon 1 and the 5′ end of the exon 3 (Fig. 1).

Hemangioblastomas *(56),* RCCs *(60),* and islet cell tumors of the pancreas *(31),* which develop in VHL patients, exhibit loss of heterozygosity (LOH) at the VHL locus, because of loss of the remaining wt allele. In the case of hemangioblastomas, it is the stromal cell component of the tumor that exhibits biallelic inactivation of the VHL gene *(61).* Lubensky et al. *(60,62),* using laser-capture microdissection, were able to detect LOH at the VHL locus in cells lining atypical renal cysts of VHL patients. This observation places loss of VHL function at the very initial steps of renal tumor development.

Clinical heterogeneity is an established feature of VHL disease *(52,55,63,64).* Patients with type I disease are predisposed to develop clear cell carcinoma, or CNS and retinal hemangioblastoma, but not pheochromocytoma. Families with type II disease are also predisposed to develop pheochromocytoma. Type II families can be subdivided into those with low (type IIA) or high (type IIB) risk for development of renal carcinoma. Several reports have established a correlation between specific VHL germline mutations and the clinical phenotype of the disease (Table 2). More than 90% of type II patients have missense mutations; large deletions, microdeletions/microinsertions, and nonsense mutations are typical in type I disease *(52,58).* Recently, missense germ-line *VHL* mutations that result in familial predisposition to pheochromocytoma, without the other stigmata of VHL disease, were described *(54,65–69).* These patients may constitute an additional VHL disease subtype (type IIC).

### *3.3. VHL Gene in Sporadic Neoplasms*

The Knudson two-hit genetic model for TSG predicts that familial cancers result from the inheritance of a germ-line mutation in a TSG, followed by somatic inactivation of the remaining wt allele *(70).* This is the case for several familial cancer syndromes, such as those arising from mutations in *Rb1, p53, WT1,* and, as described above, for *VHL.* A corollary to this model predicts that these genes would also be altered in the sporadic counterparts of the tumors encountered in their respective hereditary cancer syndromes. The *VHL* gene obeys this rule.

LOH at the VHL locus, with mutational inactivation of the remaining allele, was detected in 50–60% of sporadic clear-cell-type renal carcinomas *(41,71–73).* Inactiva-

Table 2
Genotype–Phenotype Correlations

| | *Classification* | *Phenotype* | *Germ-line mutations* |
|---|---|---|---|
| Type I | H + RCC | | Deletions 46%<br>Nonsense 10%<br>Missense 44%<br>N78H/S/T S80R/I S111R/N<br>R161P C162P/Y/T L184P/R |
| Type II | | | Missense 96% |
| IIA | H + Pheo + RCC (low) | | Y98H, Y112H |
| IIB | H + Pheo + RCC (high) | | V74G, R161G, R167W/Q/G |
| IIC | Pheo only | | L188V, G114S, F119S,<br>V84L, V166F |

Left column: VHL subtype. Middle column: Clinical phenotype. H, CNS and retinal hemangioblastoma; Pheo, pheochromocytoma; RCC, renal cell carcinoma; (low), low risk of RCC; (high), high risk of RCC. Right column: most frequent mutations encountered for each phenotype.

tion of *VHL* gene bears histologic specificity: VHL mutations do not occur in papillary RCCs, a histologic subtype not encountered in VHL families *(41)*. In an additional 25% of sporadic clear cell renal cancers, the *VHL* gene is silenced by hypermethylation in CpG islands spanning the area around the promoter and the 5′ end of the first exon. In total, both copies of *VHL* gene are inactivated in approx 75% of sporadic clear cell renal carcinomas.

The *VHL* gene was also found to be mutationally inactivated in 50–60% of sporadic hemangioblastomas *(74–77)*. No evidence of hypermethylation has been reported so far in sporadic hemangioblastomas. The *VHL* gene in sporadic pheochromocytomas, unlike the situation for RCCs and hemangioblastomas, does not appear to conform to Knudson two-hit model: so far, only two cases of VHL inactivation in sporadic pheochromocytomas have been reported *(66,78)*. Whether the *VHL* gene is hypermethylated in sporadic pheochromocytoma is not known. Lastly, LOH at the VHL locus was reported in 7/10 studied sporadic pancreatic microcystic adenomas *(79)*.

The status of *VHL* gene was also examined in several cell lines and tissue obtained from non-VHL-associated tumors (including breast, colon, prostate, ovarian, nonsmall cell lung cancer) *(41,80),* and in tumors suspected of harboring inactivation of a putative TSG on chr 3p (small cell, squamous cancer of the head and neck, esophageal) *(80–82)*. With rare exceptions, corresponding usually to advance tumor stage and consequent loss of genetic material because of genetic instability *(83),* neither VHL mutation nor LOH at the VHL locus have been reported in these settings.

## 4. VHL TUMOR SUPPRESSOR PROTEIN

### *4.1. Identification of pVHL Isoforms*

The human *VHL* gene encodes a 213-aa protein that migrates with an apparent mol wt of 28–30 kDa (pVHL30), following sodium dodecyl sulfate-polyacrylamide gel electrophoresis *(38,47,84)*. A second pVHL isoform, migrating with an apparent mol

wt of 19 kDa (pVHL19), has also been identified *(25,85,86)*. Mutational analysis and pulse-chase experiments indicate that pVHL19 is not a proteolytic product of pVHL30, but is probably generated by translational initiation at methionine 54. pVHL19 has been detected in every cell line producing pVHL30. pVHL19, as described below, is a biologically active molecule, and almost all tumor-derived VHL mutations map C-terminal to codon 54. This underscores the notion that both pVHL isoforms need to be inactivated in order for tumor formation to ensue.

It is possible that additional pVHL isoforms exist. Immunoprecipitation of cellular extracts, followed by Western blot analysis with monoclonal anti-VHL antibodies, reveals the presence of specific bands in addition to the ones corresponding to the isoforms mentioned above *(84,87)*. As described above, cells produce an alternatively spliced VHL mRNA consisting of exons 1 and 3 *(41)*. Whether this mRNA encodes a stable protein product is not known. Some intronic VHL mutations result in the exclusive production of this mRNA, suggesting that its protein product, if made, is defective as a tumor suppressor.

Several groups investigated the cellular localization of pVHL. Biochemical fractionation of expotentially growing cells suggested that endogenous pVHL30 resides primarily in the cytoplasm and, to a lesser extent, in the nucleus *(84,88)*. In the cytoplasm, pVHL30 was detected both in the cytosol and in fractions enriched for membranes, including the Golgi apparatus and the endoplasmic reticulum (ER). Immunocytochemical studies of paraffin-embedded human tissue and mammalian cell lines corroborated these observations *(89,90)*. pVHL19, on the other hand, appears equally distributed between the cytosol and the nucleus, and is not measurably present in the membranous fraction *(85)*. Recently, Lee et al. *(91)*. reported that pVHL30 shuttles between the cytoplasm and the nucleus. Exposure of cells to actinomycin D or 5,6-dichlorobenzimidazole, both general inhibitors of polymerase II-mediated transcription, inhibited pVHL30 nuclear export, and resulted in relocalization of pVHL30 to the nucleus. Exon 2-encoded aa are necessary for this function, and exon 2-deleted mutants appear biologically impaired, at least regarding their ability to regulate hypoxia-inducible proteins (*see* Subheading 6). The nature of physiologic stimuli that regulate this nucleocytoplasmic shuttling is currently under investigation.

In asynchronously growing cells, pVHL30, but not pVHL19, is phosphorylated on serine residues *(92)*. The sites of phosphorylation, the relevant kinase(s), and the functional consequences of pVHL phosphorylation are currently unknown.

### *4.2. pVHL Is a Gatekeeper Tumor Suppressor Protein*

Molecular studies in colon cancer support the notion that the development of cancer in humans is a multistep process *(93)*. It appears that the development of a fully malignant clone is shaped by the accumulation of mutations that are characteristic for a particular tumor type. Thus, it is anticipated that there will be a molecular signature for different tumors derived from different tissues. For a given tissue, there may be a critical gene, or genes, which regulate cell growth, and which must be inactivated early during the course of carcinogenesis, if a fully malignant clone is to emerge. For example, it appears that inactivation of the *APC* gene plays such a role in colorectal cancer. Reconstitution of the function of these genes, referred to as "gatekeepers," suppresses the malignant phenotype *(94)*. In contrast, inactivation of another family of TSGs (such

as *MSH1* and *MLH2*) leads to genomic instability and tumor development through secondary mutations affecting other genes (including gatekeepers). Reconstitution of the function of these caretaker genes cannot override these secondary effects, and fails to suppress the malignant phenotype. The distinction between these classes of TSGs has obvious implications with respect to therapeutic strategies based on reconstitution of tumor suppressor function.

Several groups studied the effects of reintroducing wt pVHL into renal carcinoma cell lines that lacked a wt VHL allele. With the exception of one report *(95),* restoration of pVHL function to such cells did not alter their ability to form colonies in soft agar, or to grow in monolayer culture under serum-rich conditions *(84,96,97).* In contrast to these cell culture experiments, restoration of pVHL function dramatically suppressed the ability of VHL-defective renal carcinoma cells to form tumor xenografts in nude mice *(84,96,97).* This disparity between the effects of pVHL in vitro and in vivo led to the hypothesis that pVHL mediates tumor suppression, at least in part, by mechanisms involving interactions between tumor cells and their surrounding microenvironment. The subsequent demonstration that pVHL plays a role in angiogenesis and cell matrix formation (*see* Subheading 5.3 and 6.1.) is consistent with this view.

Some cell–microenvironment interactions can be, in part, recapitulated by growing cells as multicellular spheroids. Under such conditions, Lieubau-Teillet at al. *(97)* showed that VHL–/– RCC formed compact spheroids, and exhibited a higher proliferative index (by H3 thymidine and bromodeoxyuridine incorporation) than their pVHL-expressing counterparts. In addition, RCC clones producing pVHL formed loosely arranged spheroids that exhibited morphological characteristics of renal tubular differentiation. In addition, these spheroids were associated with increased deposition of a fibronectin matrix, compared with spheroids formed by pVHL-defective cells (*see* Subheading 5.3).

The in vitro effect of pVHL on cellular proliferation was revisited by Pause et al. *(98).* RCC clones, expressing wt pVHL, exited the cell cycle upon serum withdrawal, when grown under certain cell-density conditions. In contrast, VHL–/– RCC clones continued to cycle under these conditions. The reason that cell density is critical for the ability of pVHL to mediate cell cycle arrest is unclear, but again underscores the potential importance of cell-microenvironment communication regarding tumor suppression by pVHL.

These series of experiments, along with the observation that pVHL inactivation is an early event in the generation of RCC, place pVHL in the gatekeeper group of tumor suppressor proteins. From the therapeutic standpoint, they also support the concept that pharmacological restoration of pVHL function(s) in an established clear cell renal carcinoma may lead to tumor regression.

### *4.3. Mice Models for VHL Disease*

Inheritance of an inactivated copy of *VHL* gene predisposes patients to develop VHL disease. To examine the role of pVHL in development and tumor suppression, Gnarra et al. *(96),* using gene knockout techniques, generated VHL +/– mice, thus simulating the germ-line genotype of the *VHL* gene in VHL patients. These heterozygous VHL+/– mice develop normally, and have been followed, up to the age of 2 yr, without any evidence of tumors or other stigmata of VHL disease. The response of heterozygous mice to carcinogenic stimuli is currently under investigation.

Mice with homozygous deletion of both VHL loci die *in utero* at 10.5–12.5 d of gestation. These VHL–/– embryos exhibit no developmental abnormalities at the time of death. Instead, the cause of death appears to be lack of endothelium and blood vessel formation in the embryonic component of the placenta, leading to whole embryo resorption. Failure of embryonic placenta vasculogenesis correlates with great reduction of vascular endothelial growth factor (VEGF) levels in the trophoblast. Because of the role of pVHL in negatively regulating VEGF expression in renal carcinoma cells (*see* Subheading 6.1.), these results may indicate that either VEGF regulation by pVHL differs in trophoblasts, compared to renal carcinoma cells, or that the temporal expression of a host of vasculogenic factors regulated by pVHL is critical for the mature formation of vessels.

The next generation of transgenic mice experiments is expected to focus on rescuing the VHL–/– embryos by applying embryo transfer techniques. This may allow the generation of viable VHL–/– offspring, and the study of disease development and progression in a mouse model. In parallel, efforts to develop mice, in which VHL can be conditionally inactivated in selected target tissues (e.g., kidney, cerebellum), are currently underway.

## 5. MECHANISMS OF TUMOR SUPPRESSION AND pVHL-ASSOCIATED PROTEINS

### *5.1. Elongins C and B*

In order to understand the biochemical mechanism(s) leading to tumor suppression by pVHL, several groups looked for cellular proteins that stably bind to pVHL. Initially, it was found that pVHL binds to elongins C and B *(87,99,100),* which form a tripartite enzymatic complex when bound to elongin A, called elongin or SIII. These three proteins were initially purified from rat liver nuclei as components of a multiprotein complex that enhances the overall rate of RNA polymerase II transcriptional elongation in vitro *(101).* This complex is evolutionary conserved, and members of it have been identified in yeast, *C. elegans,* and *Drosophila.*

The biochemistry of transcriptional elongation has been extensively studied, using in vitro assays of transcript elongation rates from artificial templates. In such assays, elongin A constitutes the catalytic subunit of the complex *(102,103).* The minimal domain required for the catalytic activity of elongin A maps between aa 521 and 690, although sequences extending between aa 400 and 773 are required for maximal catalytic activity *(102).* Elongin C binds directly to elongin A, and enhances its catalytic activity *(104).* Elongin B binds, in turn, through its N-terminal ubiquitin-like domain, to elongin C, and appears to stabilize the complex *(105).* Elongin/SIII is thought to suppress transient pausing of RNA polymerase II as it encounters impediments along DNA templates. Whether elongin/SIII is a general factor, or regulates specific genes in vivo, is not known.

Elongin C binds directly to pVHL aa 157–172 *(87).* This region constitutes a hotshot of naturally occurring, tumor-associated, mutations, supporting the concept that elongin binding is connected to the ability of pVHL to act as a tumor suppressor. pVHL (157–172) contains an elongin C/B-binding motif (termed "BC box") shared between pVHL and elongin A: (T,S)Lxxx(C,S)xxV(L,I) *(106).* Alanine scanning showed that the conserved aa of this motif are critical for binding of pVHL to elon-

gins C/B *(107).* These findings led to early speculation that pVHL tumor suppressor function was related to its ability to inhibit elongin/SIII. This model was initially supported by the observations that a) binding of elongins C/B to elongin A or pVHL was mutually exclusive and b) pVHL inhibited elongin/SIII transcriptional elongation activity in vitro.

Although it is still possible that some pVHL function(s) may relate to transcriptional elongation inhibition, possibly through nuclear–cytoplasmic shuttling, several recent observations, outlined below, challenge this view. First, elongins C/B are in vast excess of pVHL and elongin A, rendering sequestration of elongin B/C by pVHL improbable *(107a).* Second, elongins C/B reside primarily in the cytoplasm, as pVHL does, suggesting that they may perform additional function(s) unrelated to transcriptional elongation *(107b).* This hypothesis is further supported by the recent observation that pVHL/elongin B/C, but not elongin/SIII, binds to human cullin-2, as described below *(107b).*

### *5.2. Cullin-2*

The first member of the cullin family of proteins, *Caenorhabditis Elegans* cullin-1, was cloned by Kipreos et al. *(108)* as a gene that, when inactivated, gives rise to small, but differentiated, larvae. Database searches revealed five *C. elegans* and six human genes that are highly homologous to *C. Elegans* cullin-1. *(108).*

pVHL forms complexes with human cullin-2 (Cul-2) *(109,110).* The N-terminus of Cul-2 binds to elongin C *(92),* which, in turn, binds to pVHL and elongin B, as described in subheading 5.1. *(94).* Elongin A, in contrast to pVHL, does not associate with Cul-2 *(107b).*

Human Cul-1 and Cul-2 are highly homologous to yeast Cdc53, a protein that plays a role in ubiquitination of specific substrates (E3) *(108,109).* Based on this homology, it is reasonable to hypothesize that Cul-2 also plays a role in ubiquitination. Such a function, shown for human Cul-1 *(111–113),* remains to be proven regarding Cul-2. Protein degradation through ubiquitin-dependent proteolysis is a rapid and irreversible process regulating diverse cellular functions, such as progression through the cell cycle, kinetochore function, orderly sister chromatid separation, DNA repair, and response to nutritional starvation *(114,115).* Protein ubiquitination may lead to outcomes other than degradation, including receptor internalization or alteration in subcellar compartmentalization. Ubiquitin is activated by the ubiquitin activating enzyme (E1), and subsequently transferred to one of many ubiquitin-conjugating enzymes (E2s). In a third step, ubiquitin is covalently linked to the target protein by a ubiquitin ligase (E3).

Multiprotein complexes mediating the ubiquitination of various substrates have been identified in eukaryotes. Substrate specificity depends on members of the complex acting as receptors. These substrate receptors associate with specific E2 and E3 partner proteins *(116).*

In *Saccharomyces cerevisiae,* Cdc53 serves as a scaffold protein for the assembly of such complexes *(117).* These multiprotein complexes, referred to as SCF complexes, contain Skp1, Cdc53, and a receptor protein containing a co-linear motif known as an F-box (so named because the prototypical sequence is present in cyclin F). The substrate specificity and nomenclature for a given SCF complex is determined by the F-box protein (e.g., $SCF^{Cdc4}$, $SCF^{Grr1}$, or $SCF^{Met30}$) *(118,119).* SCF complexes containing Cdc4 bind and degrade yeast G1 cyclins (cln 1 and 2); complexes containing Grr1 bind and degrade the Clb5/Cdc28 inhibitor, p40/Sic1 *(119–121).* Met 30 con-

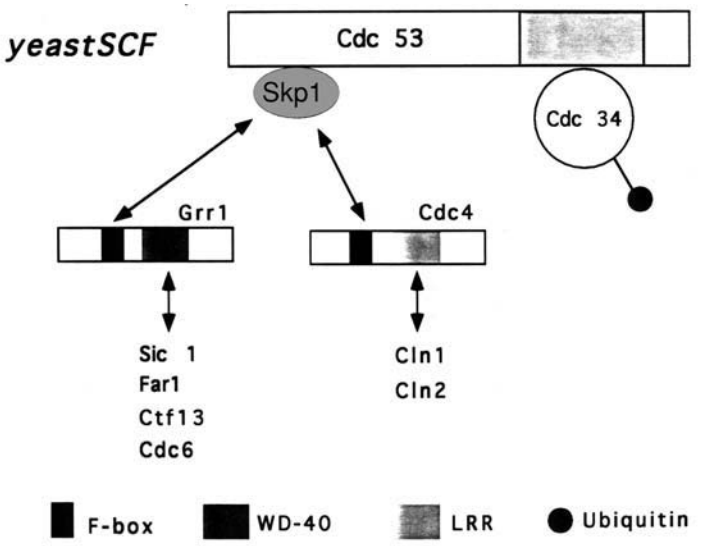


**Fig. 2.** Comparison of yeast SCF (ySCF) to mammalian SCF and VHL/elongins/Cul-2 (VEC) complexes. **(A)** Yeast $SCF^{Grr1}$ and $SCF^{Cdc4}$ complexes. Skp1 binds to the F-box domain of the receptors. Substrates bind to a second protein–protein interaction domain of the receptor. The shaded area corresponds to the cullin box. **(B)** Mammalian $SCF^{SKP2}$ and $SCF^{Trcp}$. Cyclin A/CDK2 bind to SKP2 through the indicated motif. **(C)** Mammalian VEC complex. Notice the modular similarities between VEC and the complexes depicted in (A) and (B).

taining SCF complexes target Swe 1 for degradation *(122).* A structurally similar multiprotein complex (anaphase promoting complex, [APC]) guides progression through mitosis. One of its subunits, APC2, bears C-terminal homology to cdc53, and it is regarded as the equivalent E3 scaffold ligase *(116).*

In human cells, complexes containing the F-box protein SKP2 and the human homologs of Skp1, Cdc34, and Cul-1, have been identified *(123,124).* An intriguing homology exists between such SCF complexes and the pVHL-containing complexes described above (Fig. 2). As mentioned above, the C-terminus of Cul-2 is similar to the C-termini of Cul-1 and Cdc53. This region contains a highly conserved subdomain referred to as a "cullin-box." The N-terminus of elongin C is homologous to Skp1 *(109,118).* Elongin B, bears an N-terminal ubiquitin-like domain. pVHL does not contain an F-box as originally defined by Bai et al. *(118).* Nonetheless, pVHL, by virtue of its association with elongin C, may play the role of an F-box protein, namely, to serve as the substrate recognition determinant in the complex.

From this point of view, the crystal structure of the ternary complex formed between pVHL and elongins C/B provides interesting insights. VHL has two domains: an amino-terminal β-domain (residues 63–154), rich in β sheet, and a smaller α-domain (residues 155–192), consisting of three helices, H1–H3. These three helices form a groove that binds to a helix formed by the C-terminus of elongin C. The previously mentioned BC box corresponds to the first H1 helix of the α-domain, making extensive contacts with elongin C. Residues from the other two helices contribute to the stability of the complex. Half of the reported tumor-derived mutations map to the residues mediating contact between the α-domain and elongin C. Structural comparison reveals

some loose similarity between the F box and the α-domain of pVHL. In addition, it appears that several residues located in the N-terminal portion of β-domain form a macromolecular patch that is likely to participate in protein–protein interactions. This patch may mediate interactions between pVHL and the substrate(s) of the pVHL–Elongins C/B–cul-2 (VEC) complex.

These observations raise the possibility that a primary role of VEC complexes is to regulate ubiquitination of specific protein substrates. Two recent reports support this hypothesis. Gorospe et al. *(125)* showed that, after prolonged exposure to low-glucose, VHL–/– cells undergo apoptosis, but their VHL+/+ counterparts do not. This differential response was attributed to the formation of hypoglycosylated and misfolded proteins. It was reasoned that VHL+/+ cells effectively degrade these misfolded proteins through the ubiquitination–proteasome pathway, but VHL–/– do not, resulting in apoptosis. Second, pVHL co-purifies with a protein called Rbx1 or ROC1 *(126,127)*. This protein interacts with both pVHL and Cul2, and has been implicated in the recruitment of ubiquitin-conjugating enzymes (E2) to SCF complexes *(128,129)*. If the rate of VEC complex in ubiquitination is validated, it will be important to identify their protein substrates, and to understand the stimuli that regulate this activity.

### *5.3. Fibronectin*

Wild-type pVHL30, but not tumor-derived pVHL mutants, bind (at least indirectly) to fibronectin in intact cells *(130)*. Immunofluoresence using confocal microscopy, as well as cellular fractionation experiments, suggest that the interaction of pVHL and fibronectin takes place in association with the ER. pVHL19, in contrast to pVHL30, is not found in the membranous compartment of cells (which includes the ER), and does not bind to pVHL *(85)*.

The association of fibronectin with pVHL has functional consequences. Renal carcinoma cells lacking wt pVHL fail to assemble an extracellular fibronectin matrix, as determined by immunofluoresence and ELISA assays. This defect can be corrected by reintroduction of wt, but not mutant, pVHL. In addition, extracellular fibronectin matrix assembly in VHL–/– embryos and VHL–/– mouse embryo fibroblasts is likewise defective, compared with their VHL+/+ counterparts *(130)*. This pVHL effect is specific for fibronectin, since it does not extend to other macromolecules of the extracellular matrix, such as laminin or collagen.

Several lines of evidence suggest that alterations in fibronectin play a role in cellular transformation. The assembly of fibronectin in multimeric complexes has been shown to exert antiproliferative and antimetastatic effects in various model systems *(131–133)*. Conversely, many transformed cells produce diminished amounts of fibronectin matrix, compared to their nontransformed counterparts. A potential role for fibronectin in renal carcinogenesis is also supported by the finding that ACHN cells, a clear cell, renal carcinoma cell line retaining wt VHL, harbors a frameshift mutation of the fibronectin gene. This mutation results in the production of a truncated fibronectin molecule able to stimulate RCC growth in vitro *(134)*.

In a series of experiments described in Section 6, pVHL has been linked to tumor neoangiogenesis, through its ability to downregulate the production of angiogenic peptides. Moreover, rearrangement of the extracellular matrix is a critical biologic process implicated in tumor neoangiogenesis and metastasis. The effect of pVHL on

fibronectin matrix formation may be an additional mechanism by which pVHL regulates neoangiogenesis.

How does pVHL affect extracellular fibronectin deposition? Western blot analysis and pulse-chase experiments suggest that VHL-defective cells are able to synthesize and secrete fibronectin, but the fibronectin fails to oligomerize properly, once secreted. pVHL itself does not appear to be secreted, but, as described above, can be found in association with the ER. Several reports showed that appropriate protein-folding and maturation is surveyed within the ER *(135,136).* Misfolded/misprocessed proteins are actively transported to the cytoplasmic surface of the ER, and degraded by proteasomes following ubiquitination *(137,138).* If, indeed, VEC complexes are involved in protein degradation, as described above, missfolded fibronectin may provide an example of one of the substrates specifically recognized and targeted for destruction by the complex. This remains to be tested.

### *5.4. Sp1 and PKC*

VHL–/– cells have inappropriately high levels of VEGF mRNA, under conditions of normal ambient oxygen tension (*see* Subheading 6.). A number of stimuli upregulate VEGF expression (e.g., hypoxia, certain cytokines, and activated oncogenes such as *ras* and *src*) through either transcriptional or posttranscriptional mechanisms (or both). Mukhopadhyay et al. *(139)* showed that pVHL moderately repressed the activity of a transfected reporter plasmid containing the VEGF promoter. The minimal area of VEGF promoter necessary for this suppression contained multiple Sp1-binding sites. Furthermore, those investigators showed that pVHL binds to Sp1 in vitro, raising the possibility that the abovementioned suppression of the VEGF promoter was linked to complex formation between pVHL and Sp1.

Following this observation, Pal et al. *(140)* showed that pVHL specifically coimmunoprecipitates, in vivo, with the δ and ζ isoforms of protein kinase (PKC). Furthermore, they showed that pVHL inhibits the phosphorylation of Sp1 by PKC ζ, in vitro *(141).* Taken together, these results indicate that pVHL may inhibit certain signal transduction pathways that involve activation of PKC isoforms and Sp1. Experiments addressing the biological relevance of these observations are needed.

## 6. DOWNSTREAM TARGETS OF PVHL

### *6.1. Negative Regulation of Hypoxia-inducible Genes and Implications for VHL Disease*

The initial observation that pVHL binds to elongins C/B, two factors thought to be involved in transcriptional elongation, led to the hypothesis that pVHL regulated the transcription of certain target genes. Two observations helped to identify such genes: subset of patients with renal carcinoma, hemangioblastoma, or pheochromocytoma (sporadic or VHL-associated) manifest paraneoplastic erythrocytosis caused by inappropriate secretion of erythropoetin from the tumor cells *(147);* renal carcinoma and hemangioblastoma are highly vascular tumors, shown by immunohistochemical studies to overexpress the angiogenic peptide, VEGF *(143–145).* Both erythropoetin and VEGF are normally induced by hypoxia. These observations led to the testable hypothesis that pVHL plays a role in a biochemical pathway that senses and/or responds to changes in ambient oxygen tension.

With this in mind, several groups examined the effect of pVHL on hypoxia-inducible genes, which are normally repressed when cells are exposed to well-oxygenated (normoxic) conditions (21% $O_2$ for cells grown in vitro). In contrast, cells lacking pVHL produce high levels of hypoxia-inducible mRNAs, such as the mRNAs encoding VEGF, platelet-derived growth factor-β, transforming growth factor (TGF-α), and the glucose transporter, Glut-1, under both oxygen-poor and oxygen-rich conditions *(96,145,148).* Reintroduction of wt, but not mutant, pVHL restores the normal response of VHL-defective cells to hypoxia. These differences in mRNA levels translate into overproduction of the corresponding proteins *(96,146).*

Given the putative link between pVHL and transcriptional elongation, the effect of pVHL on hypoxia-inducible mRNAs was found to be largely posttranscriptional *(96,146,147).* Specifically, cells lacking pVHL fail to degrade hypoxia-inducible mRNAs following shift to well-oxygenated conditions. It was shown previously that changes in mRNA stability play an important role in the changes in the abundance of these mRNAs that accompany changes in ambient oxygen conditions.

The ability to rapidly alter mRNA stability, and hence the production of specific proteins, in response to various stimuli is important to cellular homeostatesis. Considerable progress has been made in understanding how mRNA decay is regulated in yeast and mammalian cells *(149,150).* mRNA bound to ribonucleoproteins is actively exported from the nucleus through an as-yet-unidentified receptor in mammalian cells *(151,152).* Ribosomal-bound mRNA targeted for decay undergoes polyA tail-shortening, followed by uncapping and exo- and endoribonuclease digestion. mRNA sequences implicated in regulating mRNA stability have been mapped in 3'UTR, coding and 5'UTR regions. Cellular proteins bound to these regions, as well as to the polyA tail, are suspected of being involved in regulating the rate of mRNA decay *(150).*

Regulation of VEGF mRNA involves both transcriptional and posttranscriptional mechanisms *(153–155).* The former involves promoter-binding sites for hypoxia-inducible factors *(156).* The latter depends on a 3'UTR element rich in AUUUA repeats (ARE) *(157,158).* This ARE forms in vivo complexes, with multiple cellular proteins believed to confer mRNA stability *(159,160).* One of them, HuR, was recently cloned and shown to upregulate VEGF expression when transfected into cells *(161,162).* Levy et al. *(163)* showed that ARE–protein complexes are attenuated in VHL+/+ cells growing in normoxic conditions, compared to VHL–/– cells. It is therefore possible to hypothesize, in light of the recent notion that VEC complexes are involved in protein destruction, that RNA-binding proteins involved in mRNA stabilization are degraded by pVHL. Support for this hypothesis is provided by the observation that binding to elongins and Cul-2 is necessary for pVHL to downregulate hypoxia-inducible genes *(109).*

### *6.2. TGF-β*

Renal carcinoma cells overproduce TGF-β, a growth factor with proliferative, antiproliferative, and proangiogenic functions, depending upon the cells examined *(164).* Ananth et al. examined the regulation of TGF-β by pVHL, and showed that VHL–/– cells express high levels of TGF-β mRNA and protein *(165).* As is the case with the hypoxia-inducible genes, pVHL appears to regulate TGF-β mRNA at the post-transcriptional level *(165).* Reintroduction of wt pVHL into these cells suppressed TGF-β production *(165).* The cells examined by those investigators lack TGF-β recep-

tor II, and their growth was not altered by TGF-β in vitro. In contrast, administration of anti-TGF-—neutralizing antibodies inhibited the growth of VHL–/– cells in vivo, presumably by antagonizing some paracrine function of TGF-β.

### *6.3. Carbonic Anhydrase*

Large-scale differential display analysis revealed that wt pVHL downregulates the expression of the transmembrane proteins, carbonic anhydrase 2 (CA12) and 9 (CA9) *(166)*. In contrast to CA12, which is expressed in several normal and tumor tissues, including normal adult kidney, CA9 is normally expressed only in adult gastric mucosa *(167)*. CA9 may, therefore, be viewed as a tumorassociated antigen, when present in kidney cancer. Its specific detection in clear cell renal carcinoma may prove useful for diagnostic purposes.

CA9 and CA12 belong to the larger family of CAs *(168)*. Both CA9 and CA12 isoforms are catalytically active, promoting the extracellular hydration of $CO_2$, and the unstable formation of carbonic acid, which subsequently decomposes into $HCO_3^-$ and $H_3O^+$. This activity is expected to affect the function of several ionic channels that shift protons and $HCO_3^-$ across the cellular membrane *(161)*. The net result of CA9 and CA12 should therefore be to acidify the tumor microenvironment. Acidification of the extracellular environment was reported to enhance invasive behavior of tumor cells in vitro, and may also affect cell growth *(169)*.

The mechanism by which pVHL inhibits the CA12 and CA9 mRNAs is currently unknown. The elongin-binding domain of pVHL appears necessary for regulation of both isoenzymes. An additional domain, spanning the boundary of exon 1 and 2, appears to contribute to the regulation of CA12 by pVHL *(159)*.

## 7. CLINICAL IMPLICATIONS

### *7.1. Diagnostic Criteria and Surveillance Guidelines*

The cloning of *VHL* gene has significantly advanced understanding of VHL disease at both the clinical and molecular level. Prior to the cloning of the *VHL* gene, the diagnosis of VHL disease was based purely on clinical criteria. It is now possible, using semiquantitative Southern blot analysis, to detect VHL mutations in ~100% of patients who meet the clinical criteria for VHL disease. Relatives of affected individuals and patients without family history with VHL-associated tumors (such as hemangioblastoma), who do not yet fulfill clinical criteria for VHL disease (see Subheading 2.) are considered individuals at risk. Such individuals can now be accurately genotyped and entered into appropriate surveillance programs.

There is mounting evidence that specific VHL mutations are associated with specific VHL disease phenotypes (see Subheading 3.2.). The establishment of an international database for VHL mutations, along with progress in understanding of the biochemical functions of pVHL, may ultimately allow the accurate prediction of the expected disease phenotype for a given mutation. This may lead to the development of risk-oriented screening and preventive strategies. For now, a uniform program of surveillance is proposed, based on current understanding of the disease, and the availability of modern, noninterventional, imaging technology. Summary of this program is provided in Table 3.

**Table 3**
**Screening Guidelines for VHL Patients**

| | |
|---|---|
| From conception: | Inform obstetrician of VHL family history. |
| From birth: | Physical examination and neurological assessment |
| | Fundoscopic examination |
| Ages 2–10 | |
| Annual: | Physical examination and neurological assessment |
| | Fundoscopic examination |
| | Blood and 24-h urine catecholamine levels |
| Ages 11–19 | |
| Every 6 mo: | Fundoscopic examination |
| Annual: | Physical examination and neurological assessment |
| | Blood and 24-h urine catecholamine levels |
| | Abdominal US (If abnormal, MRI or CT of abdomen, except in pregnancy) |
| Every 2 yr: | Brain and spine MRI with gadolinium. |
| | Annually at onset of puberty, or before and after pregnancy (not during pregnancy, except in medical emergencies.) |
| Age 20 yr and beyond | |
| Annual: | Physical examination and neurological assessment |
| | Blood and 24-h urine catecholamine levels |
| | Abdominal US. |
| Every 2 yr: | Abdominal CT with contrast (or US for females in reproductive age) |
| | Brain and spine MRI with gadolinium. |
| | Audiometric examination (MRI of the internal auditory canal, in case of signs or symptoms of auditory abnormalities) |

These guidelines are based on consensus conferences organized by the VHL Family Alliance Foundation, and are published in the information booklet of the Alliance (http://www.vhl.org). These general guidelines should be individualized according to the clinical judgment of the caring physician, and they are expected to change with time, as new medical knowledge is generated.

### *7.2. Molecular Targets for Therapeutic Interventions*

The ultimate goal of VHL research is the development of effective therapies for VHL disease, based on the functions of pVHL. Rational approaches to therapy can now be envisioned, based on admittedly limited understanding of how pVHL suppresses tumor growth. Examples of such strategies are provided below:

1. pVHL negatively regulates the expression of hypoxia-inducible polypeptides. This family of proteins includes growth and angiogenic factors likely to be critical in tumor establishment and progression. It is reasonable to hypothesize that VHL patients will benefit from treatment with antiangiogenic drugs. Drugs that inhibit VEGF signaling will be of particular interest.
2. The VEC complex may target specific substrates for degradation through the ubiquitin–proteosme pathway. There is strong evidence that such substrates regulate hypoxia-inducible mRNAs and their corresponding polypeptides. Elucidation of the molecules involved in this specific recognition and degradation process may lead to the identification of novel drug targets. In short, drugs that affected the stability of pVHL substrates may substitute for pVHL function.

3. Upregulation of growth and angiogenic factors in VHL–/– tumors may correlate with concommitant upregulation of their corresponding receptors, thus creating a positive feedback loop promoting tumor development. Identification of specific receptor antagonists may provide an additional strategy for specific treatment of VHL–/– tumors.
4. Further understanding of pVHL biochemical functions may, in time, facilitate the design of effective and specific gene transfer strategies.

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# 13 Wilms' Tumor

## *A Developmental Anomaly*

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## INTRODUCTION

Wilms' tumor (WT), or nephroblastoma, is a pediatric kidney malignancy that was first described by the surgeon Max Wilms, in 1899. WT accounts for 8% of all childhood cancers, and is the fifth most common pediatric malignancy after central nervous system tumors, lymphomas, neuroblastomas, and soft tissue carcinomas. The random risk of developing WT is estimated to be 1/10,000 live births, and 90% of all WT patients are younger than 7 yr of age *(1)*. 93% of WT patients develop a tumor in just one of the kidneys; 7% develop tumors in both kidneys *(2)*. In the case of unilateral disease, the median age at diagnosis is 41.5 mo for males and 46.9 mo for females. Bilateral disease is noted to occur earlier, with a median age of 29.5 mo for males and 32.6 mo for females *(3)*.

As a result of the low incidence of the disease, etiologic epidemiological studies of WT have been difficult to carry out *(4)*. Geographical variations in incidence rates, however, have been reported. The highest incidence rate of WT has been found in the Delaware Valley, in the United States, with 13.7 cases per million children (0–14 yrs) per year. The lowest incidence rate in Shanghai, China, with 0.5 cases per million children (0–14 yrs) per year. Within the Delaware Valley, the incidence rate was appreciably higher among blacks (11.9) than among whites (6.2). Overall, the incidence rate of WT is the highest among blacks in the United States and in Nigeria, suggesting that, apart from environmental factors, genetic predisposition may be an important factor in

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the etiology of WT. Several studies have been undertaken to identify risk factors before conception, during pregnancy, and after birth. A number of putative risk factors have been identified, such as exposure to X-radiation, pesticides, hydrocarbons, lead, and aromatic azo compounds. Other risk factors include the intake of oral contraceptives during pregnancy, antibiotics, alcohol, and coffee. The strongest risk factor identified has been exposure to pesticides. Children can be exposed to pesticides in many ways: insect extermination at home, contact with contaminated clothing of the parents, or via their food. Exposure may already have taken place *in utero*. The fetus may be exposed to substances to which the mother was exposed even before conception. A study of parental age in relation to WT development showed a relative risk of 1.4 for children born of mothers over 40 yr of age, compared to mothers younger than 20 yr of age. In addition, mothers of children with bilateral WT are older than the mothers of children with unilateral WT *(5)*. Whether the increased risk with age is the result of accumulation of harmful substances in the mother remains to be elucidated.

WT is treated with surgery, radiation, and chemotherapy (CT), depending on the stage of the disease at diagnosis. The first nephrectomy was performed by Jessop in 1877, and, although the patient died several months after the operation, his treatment laid the foundation for surgical management of WTs. In 1898, a first attempt to treat renal tumors pharmacologically was described by William Coley, and, in 1916, Alfred Friedlander reported a partial response to radiation therapy. Despite these developments, still no more than 10% of WT patients survived the disease in the 1920s. In 1956, the first specific anti-WT agent, dactinomycin, was described, followed by vincristine in 1963. In the late 1960s, patients were treated with a combination of surgery, radiation, and CT. Randomized clinical trials conducted by multidisciplinary groups, such as the National Wilms' Tumor Study (NWTS) and the International Society of Pediatric Oncology, further improved the treatment regimes, establishing the current survival rate for WT at over 80% *(6–8)*.

The treatment of WT is a success story. However, with the improved survival rate, the side effects of treatment have also become more apparent. Depending on the intensity of the CT and radiation treatment, the patient may develop a whole range of abnormalities (Table 1;*9*). Current studies are therefore focused on simplifying, shortening, and refining the therapy given to WT patients. To fine-tune the treatment regimes, it will be important to refine the staging of the disease. The identification of prognostic factors will be one step in the right direction. The development of other treatments more specific than CT and radiation will be another step. For both steps, it will be important to unravel the underlying mechanism of the disease, which will hopefully lead to a 100% cure rate.

## 2. WT: A CASE OF DISRUPTED DEVELOPMENT

WT is of embryonic origin, and is believed to be derived from metanephric blastemal tissue of the developing kidney that failed to undergo the normal maturation process *(10)*. Therefore, to understand the development of the tumor, it is important to understand the complex process of normal kidney formation *(11–13)*. The kidney is formed through the reciprocal interaction of two tissues: the metanephric mesenchyme and the ureteric bud epithelium. Similar mesenchyme–epithelium interactions have been described during the development of many other organs *(14)*.

Table 1
Late Effects of WT Treatment

| *Treatment* | *Affected tissue* | *Effect* | *Years*[a] |
|---|---|---|---|
| Radiation/chemotherapy | Heart | Cardiotoxicity | |
| Radiation/chemotherapy | Gonads | Delayed pubertal development | |
| Radiation | Thyroid gland | Thyroid carcinoma | 6 |
| Radiation/chemotherapy | Liver | Hepatomegaly | |
| | | Ascites | |
| | | Jaundice | |
| | | Hepatoma | 0–13 |
| Radiation | Intestine | Bowel obstruction | |
| | | Colon carcinoma | 11–26 |
| Radiation | Lung | Reduced lung volume | |
| | | Mesothelioma | 16 |
| Radiation | Skeletal system | Asymmetry vertebral bodies | |
| | | Vertebral end plate irregularities | |
| | | Scoliosis | |
| | | Kyphosis | |
| | | Platyspondyly | |
| | | Hypoplasia ileum | |
| | | Osteochondroma | 5–16 |
| Chemotherapy | Nervous system | Loss of reflexes | |
| | | Muscle weakness | |
| | | Gait disturbances | |
| | | Sensory loss | |
| | | Jaw pain | |
| | | Neuropsychological abnormalities? | |
| Radiation | | Neurofibrosarcoma | 16 |
| Radiation | Kidney | Nephritis | |
| | | Renal cell carcinoma | 21 |
| Radiation | Hematopoietic system | Acute myeloid leukemia | 3–17 |
| Radiation | | Acute lymphoid leukemia | 4–5 |
| | | Chronic granulocytic leukemia | 9 |
| Radiation | | Breast carcinoma | 16 |

[a] Indicates the number of years between WT detection and the development of the second malignancy.

The mammalian kidney is derived from the intermediate mesoderm, which is first distinguishable around E18 in humans and E7.5 in mice. The first event in the differentiation of the intermediate mesoderm is the formation of the wolffian duct (or nephric duct) in a rostral–caudal direction (Fig. 1). The duct runs parallel to a longitudinal band of mesoderm, called the nephrogenic cord, which itself arises from the intermediate mesoderm. As the duct elongates, it induces a series of tubules in the nephrogenic cord. At first the pronephric tubules are formed around E22 in humans and E8 in mice. The pronephroi are transitory, nonfunctional, and analogous to the kidneys of primitive fish. Caudal of the pronephros, the mesonephroi are induced by

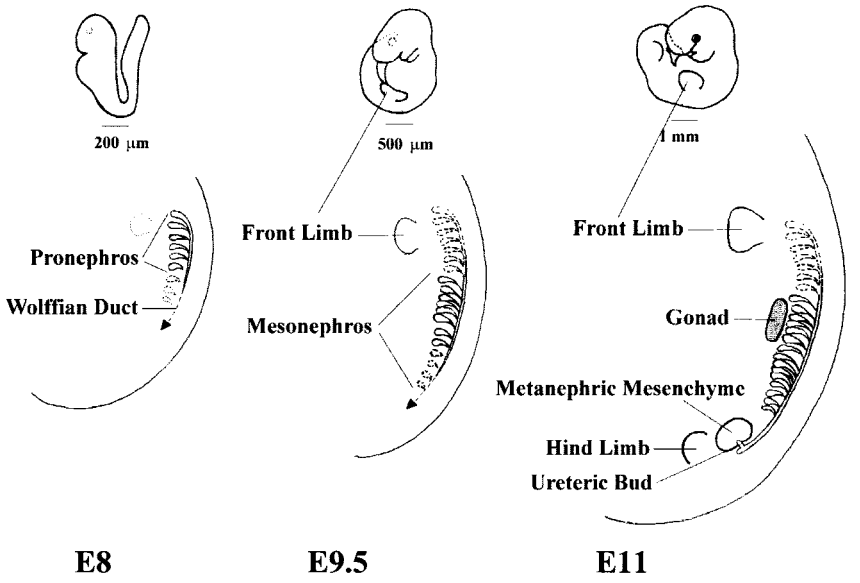



**Fig. 1** An overall scheme of the development of the mouse kidney *(11,12).*

the wolffian duct around E24 in humans and E9.5 in mice. The mesonephroi range from simple epithelium, more rostrally, to longer, convoluted tubules, complete with glomeruli, more caudally. The mesonephros is the permanent kidney in amphibians, and may function transiently in mammals. The human mesonephros disappears in females in the third month of development. In males, some caudal tubules and the wolffian duct persist, and contribute to the male genitals (efferent ductules and vasa deferentia).

Around E35 in humans and E11 in mice, the metanephric mesenchyme induces the wolffian duct, and the ureteric bud evaginates from the caudal end of the wolffian duct, and invades the metanephric mesenchyme and branches (Fig. 2A). In turn, the mesenchymal cells aggregate, undergo a burst of proliferation, form the blastema around the ureteric bud, and undergo an epithelial conversion (Fig. 2B). These early epithelial cells form a spherical cyst, called the renal vesicle. The renal vesicle matures further, via the comma- and s-shaped bodies, into a functional nephron, which consists of the glomerulus, the proximal convoluted tubule, the loop of Henle, and the distal convoluted tubule. Mesenchymal cells, which form a ring of cells around the induced metanephric mesenchyme, fail to generate epithelium, and are thought to give rise to stromal cells, or to undergo programmed cell death *(15).*

### *2.1. Nephrogenic Rests and WT*

In humans, normal kidney development is usually complete by 36 wk of gestation. Groups of renal blastema cells that persist after this period are known as nephrogenic rests, which are considered to be potential precursor lesions of WT. Nephrogenic rests are found to be associated with up to 40% of unilateral WTs and nearly 100% of bilateral WT *(16).* In comparison, nephrogenic rests are found in only 0.2–0.95% of routine

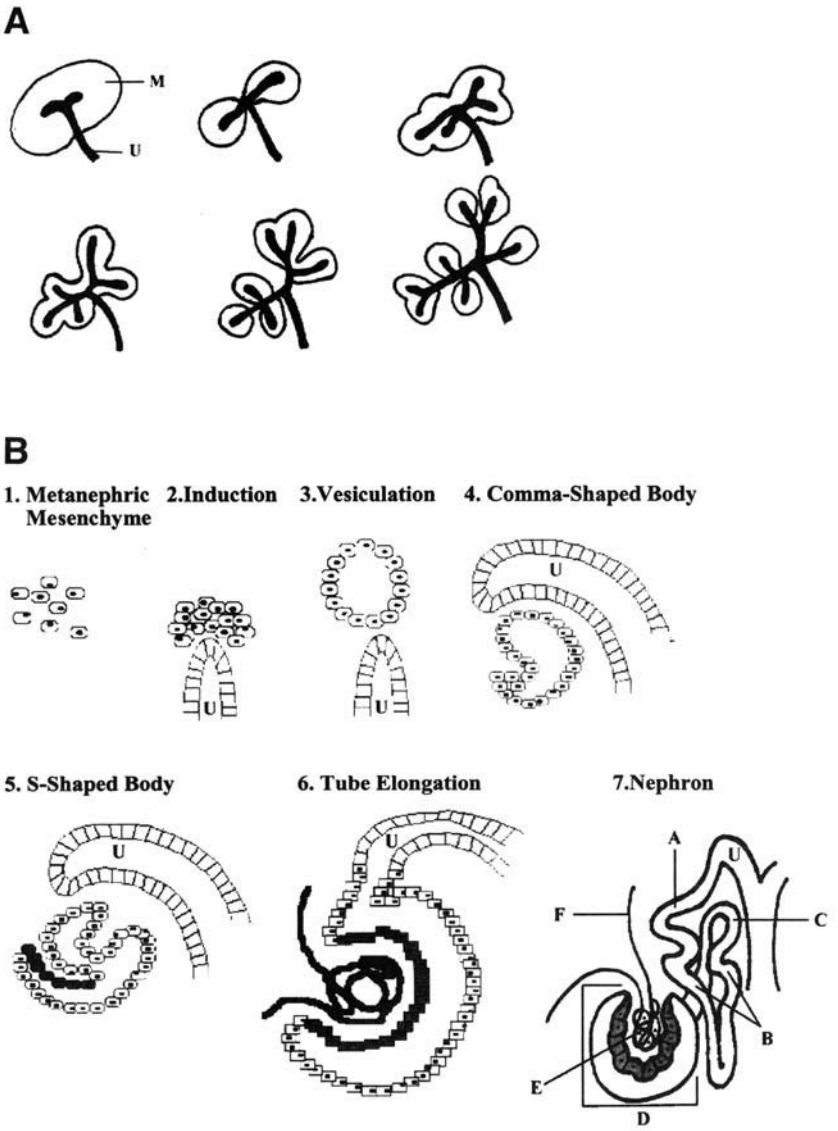



**Fig. 2** Schematic diagrams representing stages of nephrogenesis. **(A)** Branching of the ureteric bud and mesenchymal condensation with its subsequent splitting into secondary and teriary condensates *(11)*. U = Ureter; M = Metanephric mesenchyme. **(B)** Upon induction by the ureteric bud *(1)*, the metanephric mesenchyme cells aggregate, ndergo a burst of proliferation and dorm the blastema around the uterteric bud *(2)*. These early epithelial cells develop into the renal vesicle *(3)* which further matures via the comma-*(4)* and s-shaped bodies *(5)* into a functional nephron *(6,7)*. The nephron consist of the distal convoluted tube (A), the loop of Henle (B), the proximal convoluted tube (C), and the glomerulus (D). Capillaries and megangial cells (E). Blood vessels, vas efferens, and vas efferens (F). The gray cells develop into podocytes.

pediatric autopsies *(17)*. Nephrogenic rests can be divided in two major subgroups: perilobar nehrogenic rests (or multifocal superficial nephroblastomatosis), which are the most common reported, and intralobar rests (or multifocal deep cortical nephroblastomatosis) *(10,16)*.

Perilobar rests are discontinuous collections of metanephric blastemal cells and differentiated derivatives in the form of renal tubules and stroma. They are located at the periphery of the lobules, that is, in the subcapsular region and in the columns of Bertin. There are several histological subtypes of perilobar rests that often co-exist in the same kidney:

1. Nodular renal blastema, which consists of nonmitotic nodules (diameter up to 300 μm) that can only be detected by microscopic examination. These are encapsulated groups of blastemal cells that sometimes show a mild epithelial differentiation.
2. Metanephric hamartomas, which are usually larger lesions. These are always differentiated, most commonly by the formation of epithelial tubules, but sometimes there are differentiated stromal elements. They show little or no mitotic activity.
3. Wilms' tumorlets, which are neoplastic nodules, as described under[2], that have reached a diameter of 1 cm or more. These nodules are monomorphous, consisting of primitive epithelium, in a rosette pattern or with some evidence of tubular differentiation. They do not have a malignant stromal pattern. The tumorlets are well circumscribed, and do not appear to infiltrate surrounding tissues.

Intralobar nephrogenic rests are lesions distributed at or near the corticomedullary junction, and may more readily be mistaken for WT proper. The nodules are of various sizes, and may be confluent. The lesions are ill-defined, often appearing to infiltrate into adjacent renal tissue. The lesions show a high mitotic rate. In addition, they have a significant stromal component, and may mimic triphasic WT, which contains primitive blastemal cells, more differentiated epithelial cells, and stromal cells.

WTs can be subdivided according to their association with nephrogenic rests. WTs, associated with residual intralobar nephrogenic rests, can be composed of tissues mimicking the whole range of nephrogenesis. They have a prominent stromal component, and sometimes contain heterologous stromal elements, such as striated muscle, cartilage, and bone. WTs associated with perilobar nephrogenic rests are mostly composed of blastemal and epithelial elements, mimicking later stages of nephrogenesis.

WTs may also be subdivided according to their histology: favorable or unfavorable. The latter subset is noted to have a poorer prognosis, and can be further divided into two categories: sarcomatous and anaplastic. Sarcomatous tumors include rhabdoid tumors and clear cell carcinomas, but, today, these tumors are classified separately from WT *(6)*. Anaplastic tumors comprise about 4.8% of all WTs *(18),* and are characterized by the presence of hyperchromatic nuclei that are 3× larger than those of nonneoplastic cells, and have multiple mitotic figures.

## 3. GENETICS OF WT

WT has been the subject of intense clinical and basic scientific research, because it represents a model for cancer treatment, a model for the relationship between development and cancer, and it illustrates the impact of genetic alterations on development and tumorigenesis. As a genetic model, WT exhibits many of the same complexities as adult-onset tumors, such as genetic heterogeneity, and incomplete penetrance of predisposing mutations. However, unlike most adult tumors, WTs are in general euploid, and display only a very low (< 5%) frequency of loss of heterozygosity (LOH) throughout the genome *(19–21)*. This low frequency of somatic alterations suggests that the few alterations that are observed are likely to be significant in tumorigenesis.

WT develops in otherwise healthy children, but, in about 10% of the cases, the tumor occurs in individuals with recognized syndromes *(22,23).* The presence of WTs in these syndromes provided a starting point for investigating the genetics of WT. They can be classified into overgrowth syndromes and nonovergrowth syndromes *(6).* Overgrowth syndromes are the result of excessive prenatal and postnatal somatic growth, and result in macroglossia, nephromegaly, and hemihypertrophy. The two most common overgrowth disorders associated with WT are the Beckwith-Wiedemann syndrome (BWS) and isolated hemihypertrophy *(22).* Others include the Simpson-Golabi-Behemel syndrome, a fetal overgrowth disorder that results from mutations in the glypican 3 gene at Xq26 *(24);* the Perlman syndrome, a rare disorder of renal dysplasia, fetal gigantism, and multiple congenital abnormalities *(25);* and the Sotos syndrome *(22).*

Nonovergrowth syndromes associated with WT development include isolated aniridia (malformation or absence iris); aniridia in combination with urogenital abnormalities and mental retardation; urogenital malformations; the Denys Drash syndrome (DDS) *(22,26,27);* trisomy 18 *(28);* neurofibromatosis type 1, which is caused by mutations in the *Nf1* gene at 17q11 *(29),* the Li-Fraumeni syndrome, which is attributed to mutations in the *p53* gene at 17p13 in some families *(30);* the Bloom syndrome, a disorder of DNA repair caused by defects in the *BLM* gene at 15q26 *(31,32);* the hereditary hyperparathyroid–jaw tumor syndrome, which maps to 1q21–q31 *(33);* and the breast–ovarian cancer syndrome, which is caused by mutations in *BRCA1* in 17q21 in many families *(34).* These observations imply that the genetic alterations observed in these syndromes may also play a role in the development of WT.

### *3.1. Wilms' Tumor 1 (WT1) Gene*

In 1964, Miller et al. reported for the first time the association between aniridia and WT. Aniridia occurs in 1/70,000 children; WT arises in 1/10,000 children *(26).* Despite the rarity of these two conditions, aniridia is detected in 1/70 children with WT and WT is detected in 1/3 children with aniridia.

Aniridia is often accompanied by varying degrees of mental retardation and genitourinary abnormalities. The developmental abnormalities of the genitourinary tract range from common conditions, such as hypospadias and cryptorchidism, to more extreme abnormalities, such as renal hypoplasia or agenesis, horseshoe kidney, ureteral atresia, or bifid ureters *(2,35).* In cases in which the patient also develops WT, the syndrome is described as the WAGR-syndrome (WT, aniridia, genitourinary abnormalities, mental retardation). Cytogenetic analyses of these patients showed a common congenital deletion of chromosome (chr) band 11p13 *(36,37).* Several studies indicated that the locus involved in aniridia was different from the locus involved in WT. A translocation, disrupting 11p13, was found in two families with hereditary aniridia *(38,39).* The translocated chr segregated with aniridia, but the affected individuals did not develop WT. In addition, Turleau et al. *(40)* described a boy with a proximal 11p13 deletion, WT, and genitourinary abnormalities, but without aniridia or mental retardation. Molecular analysis of two other patients (one with WT and one with aniridia) showed that the loci for aniridia and WT were indeed distinct *(41).*

The discovery of a sporadic WT, with two nested deletions within 11p13 *(42),* made it possible to identify a genomic fragment of 350 kb that was homozygously deleted in this tumor *(43).* Using a library of human genomic DNA derived from a somatic cell

hybrid, clones were identified that were homozygously deleted in this tumor, and which showed cross-species hybridization. These genomic clones were then used to isolate a series of cDNAs, encoding a gene now called the Wilms' tumor 1 *(WT1)* gene *(44)*. Simultaneously, using a chr-jumping cloning technique, the same gene was isolated by another group *(45)*.

Several lines of evidence suggest that the *WT1* gene plays an important role in the etiology of WT. First, *WT1* is expressed in the developing human, rat, and mouse fetal kidney. The spatial and temporal expression of *WT1* is in agreement with the hypothesis that disruption of its function leads to tumorigenesis *(46–48)*. Second, *WT1* is found to be mutated in 94% of all DDS patients *(49)*. The DDS is a rare disease that encompasses three abnormalities: WT, nephropathy involving mesangial sclerosis, and genital/intersex anomalies. DDS patients were shown to be constitutionally heterozygous for *WT1* mutations *(50),* with the mutation reaching homozygosity/hemizygosity in the WTs of almost all cases *(51)*. Further evidence for the involvement of *WT1* in WT development came from whole-animal experiments. Rats, injected with the alkylating agent, N-nitroso-N-methyl-urea (NMU), developed renal tumors that resemble WT, and *WT1* was found to be mutated in almost 50% of these tumors *(52)*. In another study, mice were generated in which one *WT1* allele was targeted to make a specific DDS mutation *(53)*. As a result, both heterozygous and chimeric adult mice showed mesangial sclerosis, male genital defects, and one mouse developed WT. In this tumor, the transcript of the nontargeted allele showed an exon 9-skipping event, implying a causal link between *WT1* dysfunction and Wilms' tumorigenesis.

In humans, however, approx 90% of all WTs are sporadic, and only 5% of these show intragenic *WT1* mutations. Cytogenetic, comparative genomic hybridization, and LOH studies suggest that, apart from *WT1,* several other genes may be involved in the development of WT.

### *3.2. WT 2,3,4,5. … Gene?*

Patients with the BWS have an increased predisposition to develop cancer. About 4% of these patients develop WT; about 3.5% develop different types of childhood tumors, including adrenocortical carcinoma, hepatoblastoma, rhabdomyosarcoma, and neuroblastoma. Genetic evidence shows that defects in chr region 11p15.5 are associated with the development of both BWS and WT *(54,55)*. Structural change, involving the short arm of chr 11, is one of the most common abnormalities found in WT. 11p variations have been reported for approx 30% of all WTs studied *(20,56,57),* and LOH is limited to 11p15 in 50% of the informative cases *(56)*. The preferential loss of maternal 11p15 alleles in sporadic WT suggests that genomic imprinting may be involved in the development of the disease. The 11p15.5 region contains several genes that are imprinted, including *IGF-II, H19 (58–62), IPL (63), KVLQT1 (64),* and *ORCTL (65)*. *IGF-II* is normally expressed from the paternal-derived allele, and acts as a mitogenic factor in a wide range of cell types *(66)*. *H19* is expressed from the maternally expressed gene, and is believed to play a role in growth suppression, directly or indirectly, by altering the expression of *IGF-II (67–70)*. The cyclin-dependent kinase inhibitor (CDKI), $p57^{KIP2}$, is preferentially expressed from the maternally derived allele, and can induce cell cycle arrest in G1 *(62,71)*. Loss of imprinting (LOI) and overexpression of *IGF-II* have been found in many WTs *(72–74)*. In contrast, expression of $p57^{KIP2}$ and *H19* is repressed in a majority of WTs *(60,75)*. Of all the genetic

subclasses of the BWS, patients with paternal uniparental disomy, affecting 11p15, have the greatest incidence of tumors *(76).* These patients express two copies of all paternally expressed imprinted genes (e.g., *IGF-II*); the maternally expressed genes (e.g., *H19* and $p57^{KIP2}$) are lost. This may result in aberrant proliferation. Mice have been generated that lack either *H19* or $p57^{KIP2}$, or which overexpress *IGF-II (68, 77–79).* These mice exhibit most phenotypes of the BWS, but do not develop WTs. It is possible that the combination of *H19* and $p57^{KIP2}$ underexpression and *IGF-II* overexpression is necessary in order to see WT development.

Alternatively, other genes, like *IPL, KVLQT1, ORCTL,* or *NAP2 (80),* play a more important role in WT development than *H19,* $p57^{KIP2}$, and *IGF-II.* Recently, using constitutional and tumor DNA from 38 WT patients, the location of a putative WT gene within 11p15 has been refined to a region of about 800 kb *(81).* Both *NAP2* and *ORCTL* map to this region. In addition, a novel locus at 11p15 was identified, which spans a distance of approx 336 kb. Integrin-linked kinase $p59^{ILK}$ maps to this region. This gene has previously been shown to induce anchorage-independent growth and a tumorigenic phenotype in rodents *(82).*

Genetic alterations of genes in 11p15 may not result in development of WT *per se.* The alterations may, however, predispose to the development of WT, e.g., overexpression of growth-promoting *IGF-II* and underexpression of the growth inhibitors *H19* and $p57^{KIP2}$ may lead to overgrowth, which increases the chances of disruption of normal kidney development, leading in some cases to the development of WTs. The observation that *H19* expression is lost in nephrogenic rests supports this hypothesis *(83).* The fact that WT can develop in overgrowth syndromes other than BWS, which are not linked to 11p15, also supports this hypothesis. In addition, other tumor types also display 11p15 LOH and LOI, such as rhabdomyosarcoma *(84,85),* breast cancer *(81,86),* ovarian carcinoma *(87),* stomach carcinoma *(88),* hepatoblastoma *(89),* and glioma *(90).* This suggests that altered expression of 11p15 genes plays a more widespread role in tumorigenesis.

Analyses of more than 500 WTs indicate that, apart from 11p13 and 11p15, several other chr regions may be involved in the development of WT *(20,56,57,91–93).* In WT, chr gains are more common than chr loss. The most common gains involve chr 1q (~18%), 6 (~15%), 8 (~21%), 12 (~39%), 13 (~14%), 17 (~13%), 18 (~12%), and 20 (~14%). Losses are mostly detected in 1p (~10%), 4q (~5%), 11q (~10%), 17p (~4%), 16q (~14%), and 22q (2–14%). Because these changes are found in many other tumors, they could well be secondary events associated with tumor progression.

The LOH of 16q may be such a secondary event. Patients with tumor-specific LOH for chr 16q had a statistically poorer survival than those without LOH in this region *(57,93).* Loss of 1p was also associated with a worse outcome, although this difference did not reach statistical significance *(57).* The prognostic value of alterations in both 1p and 16q is currently being analyzed in the NWTS 5 *(94).* A potential candidate gene at 16q is the uvomorulin gene, which maps to 16q22.2, and lies within the region of loss in WT *(57).* Adhesion molecules, such as uvomorulin, may function as tumor suppressors. Concerning LOH in 1p, different translocation breakpoints in 1p35 have been detected in WT cells (Mannens, M, personal communications). The WT breakpoint has currently been located within a region of 1 CM.

Cytogenetic abnormalities involving chr 7p have been reported in about 13% of WTs *(93,96,97).* Although the numbers are very small, there may be an association

between LOH of markers on 7p with earlier onset and more advanced staging of WT *(93)*.

Loss of 17p has been detected in approx 4% of the WTs analyzed. The *p53* gene maps to region 17p13, and is frequently found to be mutated in anaplastic WTs *(98,99)*. Anaplasia is a feature of WT that is associated with resistance to CT *(17)*. Anaplastic WT represents 4.8% of WT cases *(18)*, a percentage similar to the LOH in 17p. Anaplastic changes are confined to specific regions of the primary tumor. Screening for LOH of 17p or expression of *p53* may help the pathologist to access anaplasia, and in this way determine the intensity of the treatment. Patients with WTs with diffuse anaplasia have been shown to benefit from more aggressive CT *(100)*.

### 3.3. Familial WT Genes

Although WT is usually sporadic, approx 1% occurs in families in which susceptibility appears to be inherited as an autosomal dominant trait with incomplete penetrance *(101)*. Utilizing comparative genomic hybridization, eight samples of familial WT showed several chr abnormalities *(102)*. As previously reported for sporadic WTs, gains were most frequently found in chr 6, 8, and 12 *(56,72)*. Most frequent sites of loss were found in chr 3, 4, 9, 16, and 20.

Linkage analyses have been performed in several WT families (Table 2). The analysis of some families excluded the possibility of a predisposing mutation in the previously described regions: 11p13, 11p15, and 16q *(103–105)*. However, three other families have been described in which the father, carrying a germ-line *WT1* mutation, transmitted the mutation to an offspring who developed WT *(107–109)*. The tumor showed loss of the wild-type (WT) allele, in agreement with the classical two-hit model by Knudson and Strong *(110)*. Two of the fathers were unaffected; the third had been successfully treated for WT. In one family, WT was linked to chr region 17q12–q21 *(105)*. A putative candidate gene in this region is the insulin-like growth factor binding protein 4 gene *(IGFBP4)*. IGFBP4 is a major binding protein for *IGF-II (111)*. Since deregulation of *IGF-II* expression is implicated in WT, IGFBP4 may play an important role in the development of the disease.

In five other families, WT was linked to chr region 19q13.3–q13.4 *(112)*, which harbors several potentially relevant genes, such as the apoptosis regulator *bax (113,114)*, the DNA repair genes, *Ercc1* and *Ercc2 (115)*, and the G0/G1 switch regulator, *Fos-B (116)*.

### 3.4. Working Model of WT Development

From the above paragraphs, it is clear that the genetics of WT is complicated and involves many different genes. Although many of these genes have not yet been cloned, they can be divided into three classes:

1. Genes whose disruption increases the chance of developing any type of tumor, including WT. This group encompasses genes that are involved in WT associated nonovergrowth syndromes and overgrowth syndromes. For example, disruption of the *BLM* gene, in the nonovergrowth Bloom syndrome, results in impaired DNA repair *(31,32)*, and loss of 11p15 in the overgrowth BWS may result in a general growth advantage. In agreement with the latter, analyses of both tumors and associated nephrogenic rests suggest that loss of 11p15 may be an early event in the pathogenesis of WT *(91,117)*.

Table 2
Linkage Analyses of WT Families

| *Family* | *16q* | *19q* | *11p13* | *11p15* | *17q* | *Refs.* |
|---|---|---|---|---|---|---|
| WTX502 | NL | – | NL | NL | – | *104,306* |
| WTX524 | NL | L | NL | – | – | *103,112* |
| WTX637 | NL | L | – | – | – | *306,112* |
| WTX149 | ? | NL | – | – | – | *306,112* |
| WTX593 | NL | L | – | – | – | *306,112* |
| WTX917 | – | L | – | – | – | *112* |
| WTX614 | – | L | – | – | – | *112* |
| WTX668 | – | NL | – | – | – | *112* |
| WTX480 | NL | – | NL | NL | L | *105* |
| K1104 | – | – | – | NL | NL | *106* |

L, linkage; NL, No linkage.

2. Genes whose disruption plays a role in tumor progression, e.g., loss of genes on 16q and 1p appear to have an adverse effect on the relapse-free period and overall survival of the WT, patient *(57,93).* In agreement, analysis of WTs and associated nephrogenic rests suggest that LOH of 16q and 1p are late events in the pathogenesis of WT *(91,117).*
3. Genes that play a direct role in the disruption of normal nephrogenesis leading to WT. It appears that different chr abnormalities may lead to a similar phenotype, e.g., trisomy 12 has been reported as the only cytogenetic abnormality in one WT, and so have trisomy 6 and trisomy 18 *(56).* In order to explain this phenomenon, one must assume that all chr abnormalities disrupt the same molecular pathway. Concerning the molecular pathway, only one gene has been cloned that plays a direct role in the etiology of WT: the *WT1* gene. The authors therefore hypothesize that many of the observed chr abnormalities will affect genes that are part of the *WT1* pathway. Understanding the regulation and the function of the *WT1* gene will help to unravel the *WT1* pathway, and to identify the genes involved.

## 4. WT1 GENE AND ITS PRODUCTS

The *WT1* gene is localized at human chr 11p13. It contains 10 exons and spans about 50 kb (*44,45;* Fig. 3). Comparison of partial nucleotide sequences of the *WT1* gene, from chicken, alligator, *Xenopus laevis,* zebrafish, and pufferfish, reveals extensive conservation, suggesting an important role of the gene throughout evolution *(118,119).* The gene mainly encodes a transcript of about 3 kb, but transcripts of 2.5 and 1.8 kb have been reported *(47,48,120).* Translation of the *WT1* transcript reveals a protein that contains four zinc fingers of the Kruppel C2-H2 class in the carboxy (C)-terminus, and which has a proline/glutamine-rich amino (N)-terminus, features that are commonly found in bona fide transcription factors (TFs), such as Egr-1, Sp1, and CTF/NF1 *(121,122).*

As a result of alternative RNA splicing, each transcript can encode for four different proteins *(123)* with mol wt of 52–54 kDa *(124).* One alternative splicing event results in either inclusion or exclusion of exon 5, which encodes a stretch of 17 amino acids (±17 aa) just N-terminal of the four Zn fingers. The other event involves a selection of

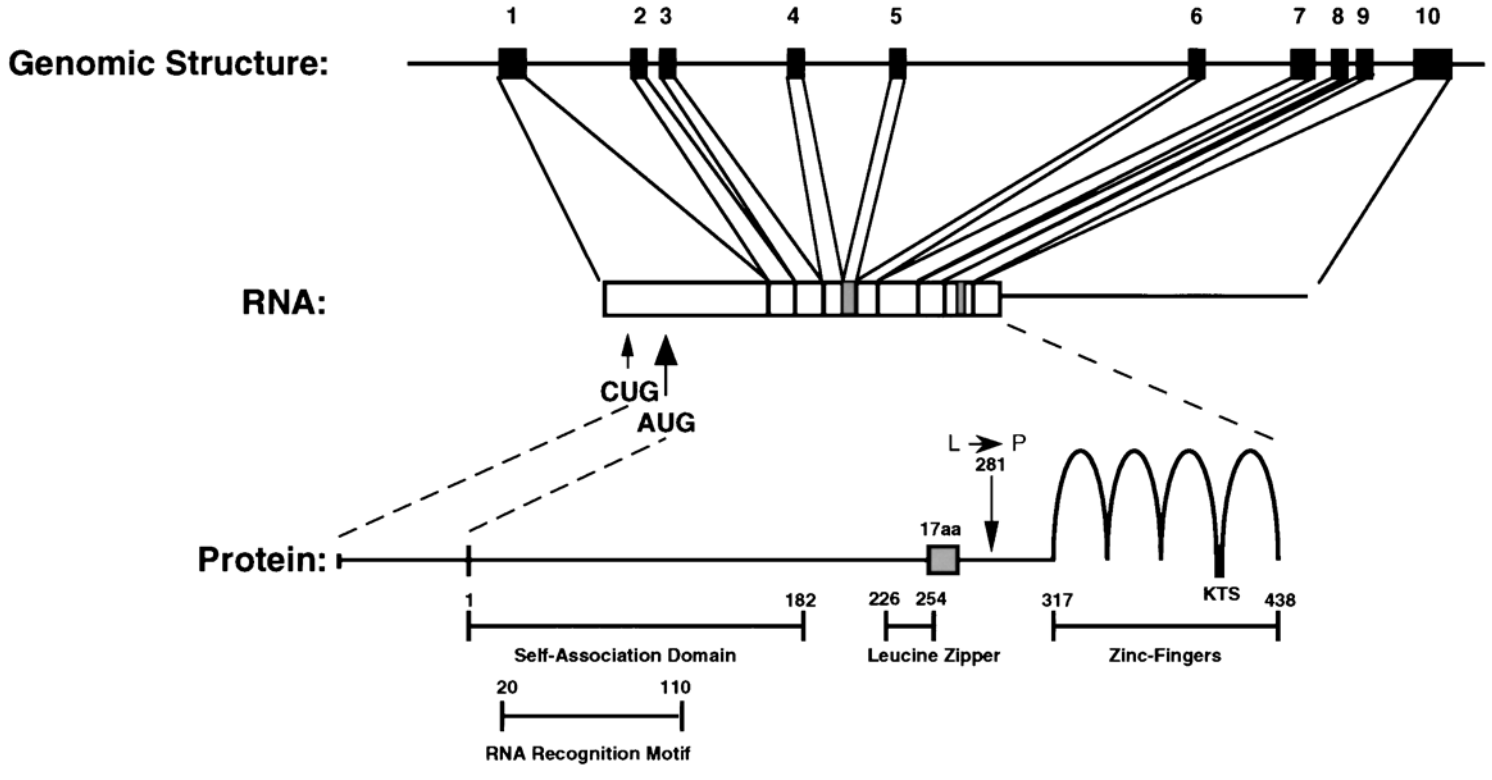



**Fig. 3** Schemetic structure of the *WTI* gene, mRNA, and protein products.

alternative splice-acceptor sites in exon 9, resulting in the presence or absence of a 3-aa insert (lysine, threonine, serine [±KTS]) between Zn finger three and four. The presence of the KTS insert in chicken, alligator, and marsupial, and the conservation of the genomic sequence in the genomic DNA of the zebrafish, underlines the importance of this alternative splice form in vertebrate development *(118).* The 17-aa insertion, however, is only present in mammals. Recently, an alternative non-AUG translational initiation site (CUG) was identified upstream of the major AUG translational initiation site, resulting in WT1 proteins with a higher mol wt *(125).* Downstream of the major AUG translational initiation site, there may be yet another translation initiation site, resulting in WT1 proteins with a lower mol wt (Scharnhorst V, personal communication). Together with the observed RNA editing, leading to the replacement of a leucine by a proline at position 281 *(126),* there could be as many as 24 different WT1 isoforms.

Little or no research has yet been done on the function of the larger or smaller WT1 proteins derived from the alternative translation initiation sites. The WT1 proteins discussed in this review are therefore solely derived from the major AUG translation initiation site. The authors refer to the WT1 splice variant, which lacks both inserts, as the WT1–/– isoform; the splice variant that only contains the 17-aa insert, as the WT1+/– isoform; the splice variant that only contains the KTS insert, as the WT1–/+ isoform, and the splice variant that contains both inserts, as the WT1+/+ isoform. Occasionally, the authors refer to the splice variants lacking or containing the KTS insert as the WT1(–KTS) or the WT1(+KTS) isoforms, respectively. Similarly, the authors refer to the splice variants lacking or containing the 17-aa insert isoforms as the WT1(–17aa) and the WT1(+17aa) isoforms, respectively.

## 5. BIOCHEMICAL FUNCTIONS OF WT1 PROTEINS

### *5.1. WT1 Proteins May Function as TFs*

Because the predicted protein sequence suggested the WT1 isoforms to be transcription factors, several studies focused on identifying their target genes. Binding site selection with oligonucleotides, whole genomic polymerase chain reaction, and DNase I footprint analysis (127–131) revealed several WT1 DNA-binding sites. Extrapolation of these data, toward known promoter sequences, led to the identification of a number of putative WT1-target genes (Table 3). In support of the possible transcriptional regulation of these genes by WT1, it was found that WT1 could affect their promoter activity in transient transfection assays. However, whether these data reflect the normal physiological situation remains to be elucidated. As has been pointed out by Hastie *(132),* crystallographic and biochemical studies predict that WT1 should interact with a 12-nucleotide G-rich stretch of the form GNGNGGGNGNGN *(133).* These G-rich binding sequences are found in promoter regions of the many genes that have CpG islands. These include housekeeping genes, and it seems unlikely that WT1 regulates all these genes. Furthermore, it has been shown that the choice of the cell system *(134),* the type of expression vector *(135),* or the exact topology of the reporter construct *(136)* can influence the transcription-regulating activity of the WT1 proteins.

What role the different splice variants play in the regulation of gene expression is currently unknown. Initially, it was found that only the splice variants lacking the KTS insertion could bind to Egr-1-like consensus sequences *(127),* and repress the transcription of reporter constructs containing these sequences in their promoter region *(137).*

This suggested that the WT1 (–KTS) and the WT1(+KTS) isoforms may regulate different target genes. Later on, DNA sequences were identified to which both the WT1(+KTS) and the WT1(–KTS) forms could bind, albeit with different affinities *(128).* It was concluded that both splice variants may differentially regulate the same target genes. The latter hypothesis was supported by studies showing that both the WT1(+KTS) and the WT1(–KTS) proteins could bind overlapping DNA sequences in the promoters of the *IGF-II* gene *(138),* the gene for the Platelet-derived growth factor, a chain (PDGFA) *(136),* the *WT1* gene *(139),* and the *PAX-2* gene *(140).* The presence or absence of the 17-aa stretch does not seem to affect the DNA-binding activity. Instead, this insert seems to have a transcriptional repressor function in addition to more N-terminally located sequences *(136,137,141).* The WT1+/+ protein suppresses the activity of the WT1 promoter about 25-fold better than the WT1–/+ protein, in transient transfection assays *(139).* It has even been observed that the WT1+/+ protein represses transcription of a certain modified PDGF-A promoter construct, but the WT1–/+ protein activates transcription *(136).* Altogether, these data suggest that the WT1 splice variants may function as TFs with different transactivating activities on (partly) different target genes.

### *5.2. WT1 Products May Function as Co-factors*

The WT1 isoforms may not function as TFs *per se,* but may exert their effect on transcription by binding to other proteins. Using the two-hybrid system and in vitro association, WT1–/– was found to bind steroid factor 1 (SF1) *(142).* Transient transfection assays, using an artificial mullerian inhibiting substance (MIS)-promoter/Luc reporter construct, showed that WT1(–KTS) isoforms function as co-factors when co-transfected with SF1. The transcriptional activation was increased by more than 20-fold, compared to SF1 alone; WT1(–KTS) itself had no effect, and did not bind to the MIS promoter.

In another study *(143),* WT1 was found to bind to the hsp70 protein, and expression of WT1 in U2OS cells led to the induction of hsp70. The (HSE) site in the hsp70 promoter was identified as being required for this induction. Transcriptional activation of hsp70 was observed following transfection of wild type WT1 as, well as the C-terminal deletion mutant WT1 delZ, demonstrating that this effect was independent of WT1's DNA-binding capacity. In addition, gel retardation assays showed no binding of WT1 to the HSE consensus sequence. These observations were consistent with current models of HSE-dependent hsp70 induction, suggesting that binding of WT1 to hsp70 induces the release of HSF from its complex with hsp70, resulting in HSF-mediated induction of hsp70 expression.

### *5.3. WT1 Proteins May Function as Posttranscriptional Regulators*

In addition to their role as TFs, the WT1 proteins may regulate gene expression at the posttranscriptional level. It appears that Zn finger motifs, as are present in the WT1 protein, may not simply be DNA-binding modules, but may behave in a more complex multifunctional manner *(144),* e.g., the *X. laevis* TFIIA protein contains nine Zn fingers, which are involved in transcriptional activation of the 5S RNA promoter, but also bind specifically to 5 SRNA *(145),* implying a role in both gene transcription and RNA metabolism. The human and mouse MOK2 proteins have 10 and 7 Zn fingers, respectively, and show a similar ability to bind to both DNA and RNA *(146).* It has been shown that the expression

of reporter gene constructs, containing promoter 2 and exon 2 sequences of *IGF-II,* is downregulated when WT1-binding sites are present within 5′ untranslated leader sequences *(147).* Nuclear run-on assays indicate that this downregulation is mediated by posttranscriptional events. In agreement with such a function, both the WT1(+KTS) and the WT1(–KTS) proteins have been shown to bind to *IGF-II* exon 2 RNA *(148).* Whether this binding indeed downregulates the expression remains to be elucidated.

WT1 may also play a role in RNA splicing. Using M15 cells (WT1-expressing mouse mesonephros cells), Larsson et al. *(149)* observed that the subnuclear localization of WT1 is very similar to the clusters of interchromatin granules that contain components of spliceosomes *(150–153).* Subsequent immunofluorescence showed that WT1 and snRNPs indeed co-localize; disruption of the spliceosomes resulted in the increased concentration of both snRNPs and WT1 in similar enlarged foci. Nuclear staining of COS7 cells, transfected with four different WT1 splice variants, revealed that the WT1(+KTS) proteins localize in the nucleus in a more speckled pattern, compared to the diffuse pattern of the WT1(–KTS) isoforms. The WT1(+KTS) proteins co-localized with proteins of the splicing machinery, suggesting a role for these WT1 isoforms in splicing, whereas the WT1(–KTS) isoforms co-localized with TFs such as Pax-6 and Sp1. These differences in subnuclear localization may not always be observed. In adenovirus-transformed baby rat kidney cells *(154)* or hepatoma cells *(155),* the WT1(+KTS) isoforms were not present in clusters, and showed the same localization as the WT1(–KTS) isoforms. In osteosarcoma cells, the WT1(+KTS) isoforms did localize in clusters, but the pattern looked different from that in COS7 cells *(156).* In the latter study, the WT1(+KTS) isoforms did not co-localize with SC35, a spliceosome assembly factor that is required for the initial step of pre-mRNA splicing. WT1 mutants with a disrupted DNA-binding domain localized to the same clusters as the WT1(+KTS) isoform, and the authors therefore concluded that the observed subnuclear clusters may represent storage sites for WT1 isoforms, with reduced DNA-binding activity.

However, in support of a role for WT1 in splicing, an evolutionary conserved N-terminal RNA recognition motif has been identified in all known WT1 isoforms *(157).* In addition, Davies et al. *(158)* recently showed, with two-hybrid analysis, in vitro binding assays, and in vivo immunoprecipitation, that WT1 interacts with an essential splicing factor, U2AF65, and gel-filtration experiments indicate that the WT1 protein is present in spliceosomes. Furthermore, Ladomery et al. (in preparation) found that WT1 is enriched by oligo(dT) chromatography, as is U2AF65, suggesting again a role for WT1 in RNA processing. In order to prove that WT1 is indeed involved in RNA splicing, a functional assay must be developed. This may prove to be rather difficult, since WT1 may only affect the splicing of specific target RNAs, and no target RNAs have been identified thus far. Recently, Bardeesy and Pelletier *(159)* have tried to identify RNA-binding sites by systematic evolution of ligands by exponential enrichment. Using bacterially produced, truncated WT1 (–KTS) protein, several sequences were identified. The sequences were found in a number of genes, but their physiological significance remains to be established.

## 6. BIOLOGICAL ACTIVITIES OF WT1 PROTEINS

WT1 appears to function at three different stages of kidney development. The onset of nephrogenesis, the progression of nephrogenesis, and the maintenance of normal

podocyte function. As described in subheading 2, the kidney is formed through the reciprocal interaction of two tissues: the metanephric mesenchyme and the epithelium of the ureteric bud (Fig. 2). Upon induction by the ureteric bud, the mesenchymal cells undergo a burst of proliferation, aggregate, and form the blastema around the ureteric bud. The blastema develops into the renal vesicle, and matures further, via the comma-and S-shaped bodies, into epithelial cells that form the proximal tubules, the distal tubules, and the glomerulus of the nephron. *WT1* expression can be detected in metanephric mesenchymal cells *(160)* at the onset of nephrogenesis. *WT1* expression is low in the developing blastema, and increases in the comma- and S-shaped bodies, as nephrogenesis progresses. Upon further differentiation, *WT1* expression is downregulated, except in the podocyte cells of the mature glomerulus, where it is maintained into adulthood.

In WT1-null mice, the ureteric bud is absent, indicating that WT1 is essential for the development of this structure *(161)*. Because *WT1* is not expressed in the ureteric ECs *(162),* the lack of growth may be the result of the absence of growth factor expression in the metanephric mesenchymal cells. Organ culture experiments have demonstrated that mesenchymal factors are the major driving force for the development of epithelia *(14)*. Some of these mesenchymal factors have been identified, such as scatter factor/hepatic growth factor, and neuregulin/NDF. These factors can induce motility, growth, and morphogenesis of epithelial cells in culture, but whether these factors play a role in the outgrowth of the ureteric bud remains to be elucidated.

A factor that is involved in the development of the ureteric bud is giant cell line-derived neutrophilic factor (GDNF), a c-ret-binding ligand. GDNF is a member of the transforming growth factor-β (TGF-β) superfamily *(163),* and is expressed in the metanephric mesenchyme *(164)*. The c-Ret protein, a member of the receptor tyrosine kinase family, is expressed in cells on the tip of the ureteric bud, and mice that lack the c-*ret* gene show ureter defects and severe renal agenesis *(165)*. In mice that lack the *GDNF* gene, the metanephric mesenchyme cells undergo apoptosis, with complete disappearance by E13.5 *(164)*. In the WT1-null mice, the metanephric mesenchyme cells also undergo apoptosis *(161)*. Since *TGF-β* is a putative target gene of WT1, it is tempting to speculate about the regulation of *GDNF* by WT1.

Induction of the mutant blastema by embryonic spinal cord tissue, the strongest inducer of metanephric mesenchyme to form kidney tubules *(11),* did not result in the differentiation of the WT1-null mesenchyme *(161)*. This experiment indicated that the failure of the WT1-null blastema cells to differentiate is not only the result of the absence of the ureteric duct, but is also a cell-autonomous defect. During normal development, WT1 may exert its effect by rendering the metanephric mesenchymal cells sensitive to induction by the ureteric duct. In this connection, Wnt-1-expressing NIH3T3 cells can induce kidney mesenchyme to differentiate into epithelial tubules *(166)*. Expression of *WT1* may, therefore, upregulate the expression of receptors that recognize members of the Wnt family. Alternatively, *WT1*-expression may be essential for the early steps of differentiation, immediately after the induction by the ureteric bud. The *Wnt-4* gene and the *Pax-2* gene may be involved in this process. During normal nephrogenesis, both genes are expressed in condensing kidney mesenchymal cells, shortly after induction by the ureteric bud, and expression persists in the comma- and s-shaped bodies before being downregulated *(167,168)*. In Wnt-4-null mice, there appears to be a developmental arrest of the metanephric mesenchyme, so that no tubular epithelium is ever formed *(167)*. In homozygous Pax-2-mutant mice, there is a com-

plete lack of kidneys, ureters, and genital tracts *(169).* It has been shown that WT1 can regulate the expression of *PAX-2 (140).* In turn, PAX-2 is also capable of regulating the expression of the *WT1* gene *(170),* suggesting the presence of a feedback loop. Whether the presence of WT1 is essential for the proper regulation of the *Wnt-4* and the *PAX-2* gene during nephrogenesis remains to be elucidated.

The expression pattern of *WT1* suggests that the gene is also involved in the further progression of nephrogenesis. In support of this, Moore et al. *(171)* showed that the kidney phenotype of the WT1-null mouse could be partially rescued by introducing a Yeastartificial chromosome (YAC) containing the genomic human *WT1* sequence. Analysis of 43 embryos showed that, in 15 embryos, the ureter had repeatedly branched, and condensation of the metanephric mesenchyme was taking place. In 12 embryos, nephrogenesis had taken place, until the stage of the comma-shaped bodies. Formation of s-shaped bodies was rare, and glomeruli were never seen.

In the adult kidney, *WT1* continues to be expressed in the podocytes *(46,48,162).* The podocytes, which line the blood vessels in the glomerulus, are involved in a variety of glomerular functions *(172).* The podocytes can synthesize the glomerular basement membrane, and may also play a role in its degradation, since they show abundant endocytic activity. In DDS patients, in which *WT1* is heterozygously mutated, the podocytes are often underdeveloped *(173).* Glomerular nephropathy is the most consistent finding in these patients, who suffer from hypertension, following the collapse of the arteries in the glomerulus. This is caused by the production of fibrotic material by the so-called mesangial cells. Regarding the role that podocytes may play in the degradation of the glomerular basement membrane, the authors hypothesize that proper expression of *WT1* may be essential for the normal development and maintenance of this membrane. The podocytes also provide structural support to the glomerular tuft, and may influence the filtration rate in the glomerulus. So WT1 appears to play an important role throughout nephrogenesis and the maintenance of normal podocyte function. But what does WT1 do at the cellular level?

### *6.1. Proliferation*

Kudoh et al. *(174)* showed that microinjection of the WT1(+17aa) isoforms into synchronized NIH3T3 cells significantly blocked serum-induced cell cycle progression. Similar results were obtained when CV-1, Cos-7, F9, or P19 were injected with the WT1+/+ isoform. The inhibitory activity of the WT1-isoforms was abrogated by the overexpression of either cyclin E/CDK2 or cyclin D1/CDK4.

Hewitt and Saunders *(175)* found that expression of both the WT(+17aa) and the WT1(–17aa) isoforms in a rat renal carcinoma cell line (IFC), suppressed proliferation. In contrast to the observations by Kudoh et al. *(174),* the WT1(–17aa) isoforms displayed a stronger growth-suppressive effect than the WT1(+17aa) isoforms. Accordingly, FACS analysis showed that a higher percentage of WT1(–17aa)-expressing cells was found in the G0/G1 phase. In agreement with these findings, Englert et al. *(176)* found that expression of a WT1(–KTS) isoform in Saos-2 cells resulted in a 25% increase of these cells in the G1 fraction. In addition, they found that the CDKI p21, was induced upon the expression of WT1(–KTS) in Saos-2, U2OS, and baby rat kidney cells. Because CDKIs are potent inducers of cell cycle arrest, the increase in p21 levels may well account for the observed prolongation in the G1 phase. Northern analysis and transient transfection assays suggest that p21 may be a direct target gene of WT1. In

order to induce p21 in Saos-2 cells, binding of WT1 to hsp70 appears to be necessary *(143)*. Another gene may account for the observed suppression of proliferation upon WT1 expression. Stable expression of the WT1–/– isoform in 293 cells reduces the proliferation rate of these cells by a factor of 2 *(177)*. Using suppression-subtractive hybridization polymerase chain reaction, a WT1 target gene was isolated that was upregulated about 15-fold in cells expressing WT1. The gene was identified as the retinoblastoma suppressor-associated protein 46 (RbAp46), and expression of this gene in 293 cells reduced the proliferation rate again by about twofold. This observation suggested that RbAp46 may be a downstream mediator of WT1.

The above results suggest that WT1 only plays a role in growth suppression. However, Yamagami et al. *(178),* showed recently that suppression of endogenous WT1 expression induced G2–M arrest in K562 cells. WT1 antisense oligomers exhibited significant inhibitory effects on the cell growth of K562 cells, in association with significant reductions in WT1 protein levels *(179)*. FACS analysis revealed that these cells were arrested in G2–M. WT1 may therefore play a role in the control of two cell cycle checkpoints. WT1 overexpression induces G1/S arrest; WT1 suppression induces G2–M arrest.

### *6.2. Differentiation*

During nephrogenesis, the induced metanephric mesenchyme cells differentiate from mesenchymal to ECs *(180)*. Similar transitions have been observed in other tissues expressing WT1, suggesting that WT1 may play a role in this process *(46,50,161,181,182)*. Consistent with a prominent role for WT1 in the mesenchymal–epithelial transition, Hosono et al. *(183)* recently showed that expression of the WT1–/– isoform in NIH3T3 cells initiates features of epithelial differentiation.

Most WTs comprise a mixture of undifferentiated blastemal cells, differentiated epithelial cells, and mesenchymal stromal cells. However, ectopic components, not normally found in nephrogenesis (particularly skeletal muscle), are observed in 5–10% of WTs *(184)*. Metanephric-mesenchymal stem cells may have the capacity to differentiate into skeletal muscle cells, as well as ECs, and complete loss of WT1 function may lead to the activation of the myogenic program. High levels of myogenic gene expression were observed in 5/7 WTs in which a homozygous WT1 mutation was documented. In addition, expression of the wild type WT1 isoforms in C2 myoblasts, suppressed myogenesis upon dexamethasone treatment, but a mutant form did not.

The individual WT1-isoforms may have different effects on differentiation. Rat renal cells expressing the WT1(–17aa) isoforms grow widely scattered, lacking close cell–cell contact. In addition, they are smaller and have fewer processes, compared to nonexpressing cells. WT1(+17aa)-expressing renal rat cells have a greater cytoplasmic volume than nonexpressing cells, and display close cell–cell contacts *(175)*.

Other cell systems also imply a role for WT1 in differentiation. Myoblastic leukemic M1 cells treated with leukemia inhibitory factor (LIF) can be induced to undergo terminal macrophage differentiation, coupled to growth arrest and apoptosis of mature cells *(185)*. WT1 cannot be detected in M1 cells. However, 24–168 h after adding LIF, WT1 expression is high. To study the role of WT1 during differentiation of M1 cells, stable transfectants were made. No transfectant could be obtained expressing the WT1(–KTS) isoforms. Upon expression of the WT1(+KTS) isoforms, the M1 cells differentiated to various stages along the monocytic differentiation pathway. The cells,

however, did not undergo terminal differentiation, suggesting that expression of the WT1(+KTS) isoforms are important for only part of the differentiation program. In contrast, Svedberg et al. *(186)* showed that expression of either the WT1–/– or the WT1–/+ isoform partially blocked the differentiation program of monoblastic U937 cells upon incubation with either retinoic acid (RA) or vitamin $D_3$.

Treatment of embryonic carcinoma cells (P19) and embryonic stem cells with RA resulted in differentiation of these cells, and the activation of WT1 expression. Immunohistochemical analysis showed that *WT1* is expressed in endodermal, glial, and epithelial cells, suggesting that WT1 plays a role in the differentiation of these cell types *(187).*

In contrast, several other cell systems show that differentiation correlates with the downregulation of *WT1* expression. HL60 cells differentiate into granulocytes upon treatment with dimethyl sulfoxide or RA *(188).* At the same time, expression levels of WT1 drop. When treated with TPA or vitamin $D_3$, HL60 cells differentiate into mature macrophages and monocytes, respectively, and again WT1 levels decrease. A similar downregulation of *WT1* was observed when chronic myelogenous K562 leukemia cells were induced by sodium butyrate or TPA to differentiate into erythroid cells or megakaryocytic cells, respectively *(189).* Experiments with antisense WT1 indicated that downregulation of *WT1* is not sufficient for the observed differentiation of K562 cells *(190).* The K562 cells, treated with antisense WT1, stop growing and subsequently undergo apoptosis, suggesting that WT1 plays an important role in proliferation and survival.

### *6.3. Apoptosis*

During development of the rat kidney, 3% of the cells within nephrogenic areas are apoptotic at any given time, implying that large-scale apoptosis takes place during renal development *(191).* In WT1-null mice, mesenchymal cells fail to differentiate, and degenerate via apoptosis, indicating that WT1 expression is required for their survival. WT1 may therefore function as a survival factor. In agreement with this hypothesis is the observation that WT1 is able to suppress p53-induced apoptosis *(192).* In addition, when the WT1-expressing leukemia cell line, K562, is treated with antisense WT1, the cells stop growing, and subsequently undergo apoptosis *(190).* However, WT1 is also able to induce apoptosis. Expression of each of the four splice variants in both Saos-2 and U2OS cells results in apoptosis *(193);* expression of only the WT1(–KTS) isoforms induces apoptosis in HepG2 and Hep3B cells *(155).*

Whether a cell will go into apoptosis is determined by a variety of signals of both extracellular and internal origin *(194–197),* and WT1 may play an important role in the regulation of these signals (Fig. 4). As to the internal signals, it has been shown that WT1 can regulate the expression of *Bcl-2, c-myc,* and *c-myb* (Table 2). Bcl-2 can promote cell survival, and inhibits apoptosis in certain cell types *(198–202);* overexpression of c-myc or c-myb can induce apoptosis *(197,201,203–206).* WT1 may regulate the expression of *TGF-β*, which has been shown to induce apoptosis in Hep3B cells *(207,208)* and rat hepatocytes *(209).* WT1 may also regulate the expression of *IGF-II,* which may function as a survival factor by binding to the insulin-like growth factor-1 receptor *(210).* Cell-type-dependent transcriptional activity of the WT1 isoforms may explain the opposite effects of WT1 on apoptosis. In one cell type, e.g., *WT1* expres-

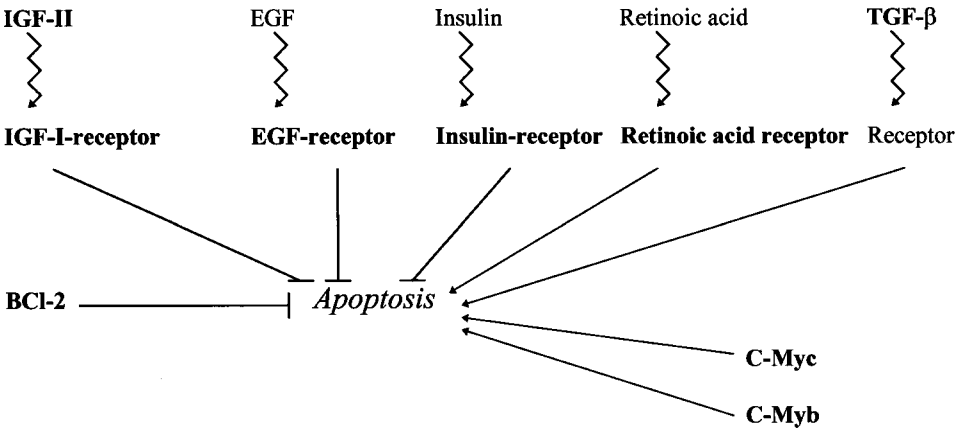



**Fig. 4** Proposed roles of WT1 in apoptosis. Genes for IGF-II, IGF-I-receptor, EGF-receptor, Insulin-receptor, Bcl-2, Retinoic acid receptor, TGF-β, C-Myb are all putative target genes of WT1. Differential regulation of these genes may determine whether a cell will go into apoptosis.

Table 3
Potential Target Genes of *WT1* Gene Products

| *Gene* | *Ref* |
|---|---|
| *WT1* | (*139,247,307*) |
| *Egr-1* | (*127,137,234*) |
| *IGF-II* | (*308,265*) |
| *IGF-I* receptor | (*309,310,287,288*) |
| *PDGF-A* | (*136,281,311,312*) |
| *CSF-1* | (*313*) |
| *TGF-β* | (*314*) |
| *PAX-2* | (*140*) |
| *Nov-H* | (*315*) |
| *RAR-a* | (*316*) |
| *Inhibin-a* | (*317*) |
| *C-myb* | (*286*) |
| *ODC* | (*284*) |
| G-protein ai-2 | (*283*) |
| *bcl-2* | (*318*) |
| *c-Myc* | (*131,318*) |
| *EGF-receptor* | (*131,155,194*) |
| *Ki-ras* | (*131*) |
| Insulin-receptor | (*131,155,319*) |
| Syndecan-1 | (*320*) |
| Midkine | (*321*) |
| *p75* neurotrophin-R | (*322*) |
| MIS | (*323*) |
| SRY | (*323*) |
| Androgen | (*323*) |
| *RbAp46* | (*177*) |
| *p21* | (*176*) |
| *Dax-1* | (*324*) |

sion may result in the downregulation of survival factors; in another cell type, WT1 may boost their expression. To what extent this will affect cell survival is dependent on the crosstalk between the cell and its cellular environment. WT1 may also play an important role in this crosstalk. Extracellular signals can be provided by growth factors, the extracellular matrix *(211),* and direct cell–cell contact *(212).* Concerning the first group, WT1 may regulate the expression of several growth factor receptors that play a role in the onset of apoptosis, such as the RA receptor-α, the IGF-I receptor *(IGF-IR),* the EGF receptor, and the insulin receptor (Table 3; Fig. 4). Overexpression of WT1 in F9 embryonic carcinoma cells induces only apoptotic cell death in the presence of RA *(213).* Downregulation of the IGF-IR correlates with increased apoptosis in C6 glioblastoma cells *(210);* overexpression of the EGF receptor rescues both U2OS *(193)* and Hep3B cells *(155)* from WT1-induced apoptosis. Overexpression of the insulin receptor also rescues Hep3B cells from WT1-induced apoptosis *(155).* The mechanisms by which WT1 is involved in apoptosis may be complex, as is clear from the onset of atresia in granulosa cells *(134),* in which the absence of WT1 may eventually lead to the abrogation of trophic support.

The above data indicate that WT1 plays a role in proliferation, differentiation, and apoptosis, three processes whose balance is important for normal development. Inactivation of several putative WT1-target genes, such as *IGF-II (214), IGF-IR (214,215),* Gαi-2 *(216), Pax-2 (217),* or the *Bcl-2 (218)* results in reduced and aberrant nephrogenesis. The balance between the expression levels of these target genes may determine whether a cell will proliferate, differentiate, or undergo apoptosis. Disruption of any of these three processes may result in tumor formation.

## 7. DISRUPTION OF WT1 FUNCTION AND TUMORIGENESIS

WT1 has been proven to be essential throughout the whole process of nephrogenesis. The involvement of WT1 in proliferation, differentiation, and apoptosis, and its temporal and spatial expression pattern, makes it a prime suspect in tumorigenesis. Disruption of the function of WT1 may explain the development of many WTs.

### *7.1. Homozygous Mutation/Deletion of WT1*

A WT1-null mutation in mice results in failure of kidney development *(161).* The metanephric mesenchyme cells fail to differentiate and undergo apoptosis. But what will happen if the expression of WT1 is switched later in development? In this case, the metanephric cells may already have been induced by the ureteric bud. The absence of WT1 prohibits the proliferating metanephric mesenchyme cells from differentiating into epithelial nephrons. Instead, the metanephric mesenchyme cells may develop into stromal cells or into heterologous elements, such as muscle tissue. Several studies support this hypothesis by showing that stromal cells or heterologous tissues in WTs do not express WT1 *(162,219–221).* In a study by Schumacher et al. *(222),* 67% of stromal predominant tumors with germ-line WT1-mutations, showed LOH, while in one WT a different somatic mutation was found in addition to the germ-line mutation. These data indicate that WTs, associated with intralobar nephrogenic rest (prominent stromal component), may represent a subset of WTs in which the classical two-hit inactivation model, with loss of functional WT1 protein, is the underlying cause of tumor development.

### 7.2. Heterozygous Mutation of WT1

WT1 is found to be homozygously mutated/deleted in only a few WTs *(223–229),* and, since the *WT1* gene is not subjected to imprinting in the kidney *(230),* the mutation of one allele will not be enough to silence expression. However, tumors may develop as a result of reduced WT1 expression, because suppression of tumorigenicity may be dose-dependent *(231).* Contradicting this possibility is the observation that mice heterozygous for the WT1-null mutant, appear normal, and develop no tumors *(161).*

However, heterozygous mutations of WT1 may in some cases be enough to disrupt normal development. Heterozygous point mutations in the *WT1* gene, as have been found in DDS patients, lead to a much more severe phenotype than the heterozygous deletions inherited by children with WAGR syndrome. Heterozygous deletions of the *WT1* gene can lead to WT and mild developmental defects of gonads and kidneys *(41,50).* Children with DDS develop WTs and usually die of severe glomerular nephropathy *(232,233).* The mutant WT1 proteins in DDS may therefore act in a dominant-negative way, abrogating the function of the wild type proteins. Transient cotransfection experiments demonstrate that mutant WT1 can affect the transcriptional activity and localization of the wild type protein, possibly by dimerization *(156,234).*

The mutated protein may have a completely different spectrum of target genes, compared to the wild type protein. Alternatively, the mutated protein may compete with the wt protein for the same DNA-binding sites, but have a different transactivating activity. Considering the latter, a point mutation in exon 5 (codon 266) decreased the transcription-repressing activity of WT1 by 2–3-fold in transient transfection assays *(235).* A point mutation in exon 3 (codon 201) or exon 6 (codon 273) resulted in a much more dramatic effect, converting the wild type protein from a repressor into an activator of transcription *(181,236).*

Based on the tumors analyzed so far, it has been estimated that 5–25% of the WTs contain homozygous or heterozygous *WT1* mutations *(132,237–241).* In fact, *WT1* expression can still be detected in most WTs, and has even been found to be highly expressed in a high percentage of these tumors *(46,219,221,242–244).* Therefore, most WTs cannot be explained by inactivating intragenic mutations or deletions. However, there may be other mechanisms by which the function of the gene can be disrupted.

### 7.3. Disruption of Spatial and Temporal Expression

The *WT1* gene is expressed during normal development in a strict temporal and spatial pattern *(46,118,160,245).* Extrapolation of the cell culture data to the in vivo situation predicts that spatial or temporal expression failures may have devastating effects, e.g., a cell may proliferate when it should go into apoptosis. Thus, proper regulation of *WT1* expression is essential. Several potential TF recognition sites have been identified in the *WT1* promoter (Table 4). Transient transfection assays show that WT1 *(246),* SP1 *(247,248),* nuclear-factor-κB *(249),* Pax-2 and Pax-8 *(170,250)* may indeed regulate the promoter activity of the *WT1* gene. Disruption of their function may lead to loss or aberrant *WT1* expression. In addition to the TF binding sites, a silencer region has been identified in intron 3 *(251),* and an enhancer region was localized 50 kb downstream of the *WT1* transcription start site *(252).* The silencer region does not seem to function in cells of renal origin *(251),* and the enhancer only appears to work in cells

Table 4
Potential TF Binding Sites in *WT1* Promoter and in the 3′ Enhancer Region

| *WT1 promoter* | *3′ Enhancer region* |
|---|---|
| CTCF | CTCF |
| E2A | E2A |
| NF-IL6 | NF-IL6 |
| PEA3 | PEA3 |
| TCF-1 | TCF-1 |
| WT1 | AP-1 |
| Egr-1 | CF-1 |
| Sp1 | HINF-A |
| Sp2 | TBP |
| Sp3 | GATA |
| Pax-2 | |
| Pax-8 | |
| AP-2 | |
| AP-4 | |
| Ets-1 | |
| F-ACT1 | |
| GCF | |
| H4TF1 | |
| PuF | |
| TCF-2a | |
| TEF-2 | |
| NF-κB | |

that express the hematopoietic transcription factor, GATA-1 *(253).* Disruption of these regulatory sequences may lead to abnormal *WT1* expression, and subsequent tumorigenesis. No mutations have been detected in the *WT1*-promoter in WTs *(254),* but only a very limited region has been analyzed.

The synthesis of the WT1 products may not only be regulated at the transcriptional level. Transcripts from the antisense strand of the *WT1* gene have been detected in 293 cells *(255),* and recently an antisense *WT1* promoter has been identified in intron 1 of the *WT1* gene, which was activated by the expression of *WT1 (256).* Expression of the anti-sense RNA in 293 cells resulted in a 50% decrease of WT1 at the protein level. Although Grubb et al. *(162)* showed that *WT1* transcripts and proteins are co-ordinately expressed, these results suggest that the antisense RNAs may affect the translation of WT1 mRNA into protein.

### *7.4. Disruption of Ratio Between Splice Variants*

Besides disruption of spatial or temporal expression of *WT1,* changes in the ratio between the splice variants could also lead to tumor formation. Transient transfections indicate that each splice variant may have a different transcriptional activity on (partly) different target genes *(127,128,136–139).* Tumorigenicity assays also show that the isoforms can have different effects *(154,257,258).* Expression of the WT1–/– isoform,

in adenovirus-transformed baby rat kidney cells, increased the tumorigenic potential of the cells; expression of the WT1–/+ isoform suppressed the tumorigenicity. These data indicate that disruption of the ratio between the splice variants may result in tumor formation. In support of this, Simms et al. *(259)* found that 7/10 WTs show an increase in the ratio between the WT1(–17aa) and the WT1(+17aa) transcripts, relative to the ratio in normal kidney tissue. Recently, another group showed a similar increase in the ratio between the WT1(–17aa) and the WT1(+17aa) transcripts in 4/7 sporadic unilateral WTs *(260).* If this increased ratio reflects higher expression levels of the WT1–/– isoform, it could explain the abundant expression of the putative target gene, *IGF-II,* in WTs *(261–264).* The WT1–/– isoform has been shown to induce the expression of the endogenous *IGF-II* gene in both RM1 cells *(265)* and NIH3T3 cells *(183).* In turn, these findings could explain the observation that expression of the WT1–/– isoform increases the tumor growth rat-of adenovirus-transformed baby rat kidney cells *(154).*

In some tumors, the splice donor/acceptor sites may be mutated; in other tumors, the splicing machinery may be disrupted. Which splicing factors are involved in the regulation of the ratio between the WT1 isoforms remains to be elucidated. It has been suggested that alternate splicing of certain genes may be regulated by the antagonistic activities of the serine arginine family of splicing factors and the heterogeneous nuclear ribonucleoprotein particle A1 (hnRNPA). Tissue-specific differences in the activities of these proteins may determine the splicing pattern *(266).*

### *7.5. Posttranslational Modification*

Phosphorylation can regulate the activity of several TFs by affecting the activity of the transactivation domain, the DNA-binding activity, or the nuclear translocation *(267–270).* The activity of the WT1 protein may also be modulated by phosphorylation. The C-terminal domain of WT1 contains a number of potential phosphorylation sites for protein kinase A (PKA) and PKC *(271).* Recently, it has been shown that the in vitro phosphorylation of WT1 by PKA or PKC inhibited DNA binding *(271,272;* Semba, K, personal communication). In support of this, transient transfection assays in NIH3T3 cells indicated that activation of PKA inhibits WT1's transcriptional repressor activity. Based on the crystal structure of the Egr-1 protein *(133),* the Ser365 and the Ser393 residues in Zn fingers 2 and 3, respectively, are involved in making contacts with the guanine residues of the DNA-binding sites. One could envisage that phosphorylation of these sites could perturb DNA binding. In NMU-induced rat embryonic tumors, which resemble WTs, Ser365 was converted into phenylalanine (Phe) *(52),* and electrophoretic mobility assays showed that this mutation severely impaired the DNA-binding activity of the WT1 protein *(128).* Phosphorylation of both residue Ser365 and Ser393 has been observed in transient transfection assays *(272).* Phosphorylation by PKA of full-length WT1, in WT1-transfected COS cells, abolished its DNA-binding activity. In vitro phosphorylation of WT1 also abolished its DNA-binding activity, which was restored upon alkaline phosphatase treatment. In CV-1 cells, the Ser365-Phe mutant still retained DNA-binding activity, even when PKA was expressed, indicating that this site is important in the regulation of DNA binding by phosphorylation. In agreement with the findings by Ye et al. *(271),* PKA treatment of WT1-expressing CV-1 cells inhibited the transcriptional-repressing activity of the WT1 proteins. The transcriptional activity of the WT1 mutants, in which the Ser365 and/or the Ser393 had been converted, was not affected by PKA treatment.

In in vitro binding assays with the WT1 Zn-finger domain, however, phosphorylation still abolished DNA binding in a double mutant in which both Ser residues had been mutated. The latter result suggests that other phosphorylation sites may be of additional importance for the regulation of the DNA-binding activity of WT1. Concerning this, TPA-induced WT1 phosphorylation occurs at an as-yet-unknown site (Semba, K, et al., personal communication), e.g., whether the 17-aa insert may be a site of phosphorylation, important in the regulation of DNA binding activity remains to be elucidated. Deletion of four Ser residues in this aa stretch abolishes the repressor activity of this insert in transient transfection assays *(136).* Phosphorylation of WT1 may also inhibit its transcriptional repressor activity by affecting the subcellular localization. Treatment of glomerular visceral ECs with forskolin (a strong PKA activator) resulted in a shift of WT1 from a nuclear localization to both nuclear and cytosolic localization *(271).*

### *7.6. Proteins that Modify WT1 Function*

Recent data suggest the existence of an interactive nuclear component in NIH3T3 cells that is required for the transcriptional repressor function of the WT1 protein *(273).* Gel filtration assays show that the WT1 protein may be present in complexes ranging from 100 to 669 kDa *(274).* Apart from the association of the WT1(+KTS) isoforms with factors of the splicing machinery *(149,158),* several other proteins have been found to interact with WT1. Immunoprecipitation and gel filtration assays indicate that WT1 physically interacts with p53, and transient transfection assays show that p53 can affect the transcriptional activity of WT1 *(274).* WT1(–KTS) acts as a transcriptional repressor in NIH3T3 cells, but functions as a transcriptional activator in Saos-2 cells, which lack the p53 protein *(274;* Table 3). Moreover, co-transfection of wild type p53 suppresses WT1-mediated transcriptional activation in this cell line, but mutant p53 fails to do so. However, transient transfection assays show that WT1–/– stimulates the Egr-1 promoter activity in both the p53-negative Saos-2 cells *(274)* and the p53-positive U2OS cells *(193),* indicating that the effects of WT1 may be determined by other factors, in addition to p53.

Three additional WT1-binding proteins have been identified; human par-4 *(275),* UBC9 *(276),* and Ciao 1 *(277).* Transient transfection assays show that par-4 reduces WT1-induced promoter activity in 293 cells. At the same time, it enhances the ability of WT1 to repress transcription. Because par-4 does not affect the DNA-binding activity of WT1, it has been suggested that par-4 may function as a repressor by binding to WT1, and in this way bringing an additional repression domain to the promoter. Ciao 1 reduces the transcriptional activity mediated by WT1. The function of UBC9 binding to WT1 is less clear. In yeast, UBC9 is involved in cell cycle progression, and is required for viability and the degradation of S- and M-phase cyclins *(278).* In this context, it is important to note that WT1 can block cell cycle progression, and this block is relieved by expression of either cyclinE/CDK2 or cyclinD1/CDK4 *(174).*

The function of the WT1 isoforms may also be affected indirectly by several other proteins. The Egr-1 protein belongs to the group of immediate-early transcription factors *(279)* and, like WT1, Egr-1 is a DNA-binding protein with highly conserved Zn finger structures of the cysteine 2–histidine 2 subclass toward the C-terminal end. The Zn fingers 2, 3, and 4 of the WT1 protein exhibit 61% aa sequence homology with Zn fingers 1, 2, and 3 of the Egr-1 protein *(280).* Both proteins can bind to similar DNA

sequences, suggesting that they may regulate the same target genes. In transient transfection assays, it has been shown that Egr-1 activates several promoters; WT1 suppresses the activity of these promoters *(137,140,281–284).* It is conceivable that the expression of Egr-1 modulates the effect of WT1 by upregulating the expression of the same genes that are downregulated by WT1. Reciprocal expression of Egr-1 and WT1 has been observed during differentiation of LLC-PK1 cells *(282,283).* Maximum Egr-1 expression coincides with maximum activation of the *Gαi-2* promoter, which contains an Egr-1 consensus sequence; maximum WT1 expression coincides with suppression of this promoter activity.

These data argue that the Egr-1 and the WT1 protein may compete for the same target genes, but have different transcription modulating activities. The same may be true for the Sp1 protein, another transcription factor of the Zn finger family. Recently, a 9-bp CTC repeat was identified in the 5′-flanking sequence of *WT1*, which accounted for approx 80% of WT1 transcription in stable transfected cells *(248).* Enhancer activity of the element correlated completely with its ability to form a DNA–protein complex in gel shifts. Subsequent studies indicated that the CTC-binding factor is the transcriptional activator, Sp1. WT1 binds to a similar CTC repeat sequence, which has been found in the promoter region of the *Ki-ras*, the *EGF-R*, the *insulin-receptor*, the *c-myc*, and the *TGF-β* gene *(131).* Whether the CTC repeat is a potential site for SP1–WT1 competition, as has been reported elsewhere for SP1 and Egr-1 *(285),* remains to be elucidated. Sp1 recognizes the same DNA sequences as WT1 in the *ODC*, the *c-Myb*, and the *IGF-IR* promoter *(284,286,287),* and functions as a strong activator of *IGF-IR* expression, whereas WT1 functions as a suppressor *(288).* p53 may also compete for the same target genes as WT1. For example, p53 has been shown to activate the *EGF-R* promoter *(289);* WT1 suppresses the activity of this promoter *(193).* The TFs mentioned above may also act in concert with WT1, and different expression levels of both proteins may affect the phenotype of the cell. For example, WT1 and Egr-1 may act in concert during the differentiation of HL60 cells into macrophages or granulocytes. During this process, the expression of *WT1* is downregulated *(188),* but only the cells that express *Egr-1* differentiate into macrophages. The other cells differentiate into granulocytes *(290).* In further support of the importance of Egr-1, it has been shown that the addition of antisense *Egr-1* prevents the HL60 cells from differentiating into macrophages; the HL60 cells can no longer differentiate into granulocytes when *Egr-1* is overexpressed.

## 8. CONCLUSIONS

WT is a pediatric kidney malignancy that affects about 1/10,000 children. More than 80% of all cases can be successfully treated, but the treatment is traumatic for the patient, and, with improved survival rates, various side effects have become more apparent. To reduce these side effects, current studies are focused on shortening, simplifying, and refining the treatment strategies. Unraveling the underlying mechanism of the disease will be helpful in this process. Cytogenetic and molecular studies indicate that several chr regions are involved in the development of WT, but so far only one gene has been cloned and proven to play a role in the etiology of this type of tumor: the *WT1* gene. However, if this gene is so important, why are *WT1* mutations only found in about 5% of sporadic WT? And why are there only a few familial WT cases in which *WT1* mutations appear to play a role? This discrepancy

could be explained by the fact that *WT1* does not only play an important role in kidney development, but is also essential for proper development of the genitalia *(134). WT1*-null mice do not develop gonads *(161)* and WAGR patients may have genital abnormalities like cryptorchidism (undescended testis) and hypospadias (defect in the wall of male urethra or female vagina, resulting in an opening on the underside of the penis or in the vagina). In addition, DDS patients show genital/intersex anomalies that can be extremely variable *(132).* At the most severe end of the spectrum, there may only be rudimentary streak gonads. At the mild end of the spectrum, XY individuals may have mild pseudohermaphroditism, such as that seen in boys with the WAGR syndrome.

*WT1* is not only essential for proper gonad development, but expression studies indicate that it may also play a role in conception. *WT1* is expressed in the Sertoli and granulosa cells, which play an important role in spermatogenesis and maturation of the follicles, respectively *(134). WT1* expression can also be detected in the antimesometrial region of the uterus, which undergoes decidualization upon implantation of the oocyte *(182).* So disruption of the function of *WT1* could preclude reproduction, and hence the transmission of most germinal WT1 mutations, but there may be other contributory mechanisms. For example, several lines of evidence suggest that *WT1* plays a role in blood cell development *(134).* One could envisage that disruption of its function in the hematopoietic system may lead to embryonic death, as well. Altogether, the above data can explain the low incidence of WT1 mutations found in WTs and the few familial WT cases with WT1 mutations. Only less disruptive alterations of the WT1 function will be transmitted, which may include mild mutations of WT1, but also other disruptions, occurring either upstream or downstream of WT1.

The previous subheading discussed several mechanisms by which the function of WT1 could be impaired, leading to the development of WT. However, nothing or relatively little is known about the molecular pathways involved. It will therefore be important to identify the gene products that regulate the transcription of WT1, the factors that determine the splicing of the WT1 transcripts, the proteins that modulate the activity of the WT1 isoforms, and the targets of the WT1 isoforms. Further analysis of the chromosomal regions implicated in the development of WT (subheading 2), may well reveal the identity of these factors. This could become a very heterogeneous group, encompassing TFs, splicing factors, phosphatases, kinases, interacting proteins, and, in addition perhaps, numerous target genes.

Experiments with cell culture systems have been the major source of information about the function of the *WT1* gene. *WT1* has been shown to play a role in differentiation, proliferation, and apoptosis. Disruption of any of these processes may result in the formation of WT. Whether WT1 is involved in only one or all of these processes in vivo remains to be elucidated. Notwithstanding the usefulness of cell culture systems, they have their limitations. Many of the functions attributed to WT1 appear to be cell-type dependent, e.g., expression of the WT1–/– isoform induces apoptosis in hepatoma cells *(155),* differentiation of NIH3T3 cells *(183),* and stimulates in vivo growth rate of adenovirus-transformed baby rat kidney cells *(154).* In addition, in all ectopic expression studies, only one WT1 isoform is expressed at a time; studies of endogenous expression suggest that the expression of all isoforms is essential for normal functioning of the *WT1* gene *(123).* Furthermore, the effect of *WT1* on a cell is determined by the environment of the cell, e.g., ectopic expression

of *WT1* in F9 embryonic carcinoma cells only induces apoptotic cell death in the presence of RA *(213)*.

The above illustrates that, in order to get additional insight into the physiological function of WT1, analysis will have to be carried out in animal models. Only few animal models have been described so far. *WT1* knockout mice have been generated, but, since these mice die early in development, it is not possible to investigate the function of the *WT1* gene at later stages of development. The creation of conditional knockouts could circumvent this problem. Use may be made of the doxycycline-mediated, quantitative, and tissue-specific control of gene expression in transgenic mice *(291)*. With this system, it would be possible to switch *WT1*-expression on and off at different stages of development by adding doxycycline to the drinking water. In this way, the function of *WT1* during different stages of kidney development could be examined. At the same time, it will provide a system to test the validity of all the putative target genes identified so far. The system can also be used to boost the expression of just one isoform at different time-points, in order to examine how the ratio between the isoforms affects normal development. In the same way, one could induce the expression of mutated forms of *WT1*.

Further information about the function of *WT1* may come from experiments in which *WT1* is ectopically expressed. CMV-driven expression of *WT1* has been shown to be embryonically lethal *(292),* but the use of tissue-specific promoters may circumvent this problem. Ectopic expression of *WT1* may prove or disprove certain hypotheses concerning the function of *WT1*. For example, *WT1* is believed to play a role in the suppression of muscle differentation *(184)*. Will mice develop muscles if *WT1* is ectopically expressed in these tissues?

### *8.1. Diagnostics and Therapy*

Knowledge about the biological function of WT1 in Wilms' tumorigenesis is limited, but there may be a few applications in terms of diagnosis and therapy. In acute leukemia, a clear correlation has been observed between the relative expression levels of WT1 and prognosis *(293)*. Leukemia patients with relatively low expression levels had significantly higher rates of complete remission, disease-free survival, and overall survival, compared to patients with high expression levels. No such correlation has been investigated in WT, to the authors' knowledge. Since WT1 has been shown to inhibit p53-mediated apoptosis *(192),* the expression levels of WT1 may well have implications for CT and radiation therapy of WTs. WT1 stabilizes p53, and the relative expression levels of p53 in WTs appear to correlate very well with *WT1* expression levels *(192)*. In addition, immunostaining showed that p53 expression follows that of *WT1*. Strongest immunoreactivity is seen in the epithelial tubular components of the tumor; the stromal elements were usually negative *(294)*.

There is a trend that *WT1* mutations in acute myeloid leukemias make these tumors more resistant to treatment. No such correlation has been investigated for WT. Because mutant *WT1* does not rescue cells from p53-induced apoptosis *(192),* certain *WT1* mutations may be associated with increased sensitivity to treatment.

Male patients with pseudohermaphroditism should be screened for mutations in *WT1*. In cases in which the *WT1* sequence harbors one of the DDS mutations (Fig. 5), the patients are likely to develop WT. If a mutation in *WT1* disrupts alternative splicing at the exon 9 splice donor site, no WT1(+KTS) isoforms will be synthesized, and the

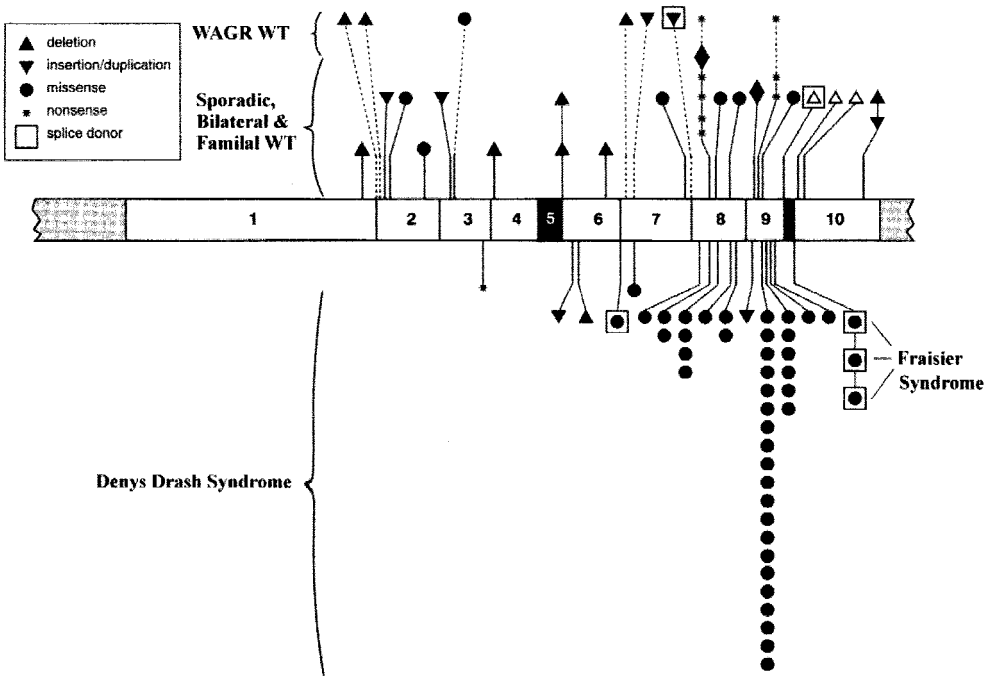


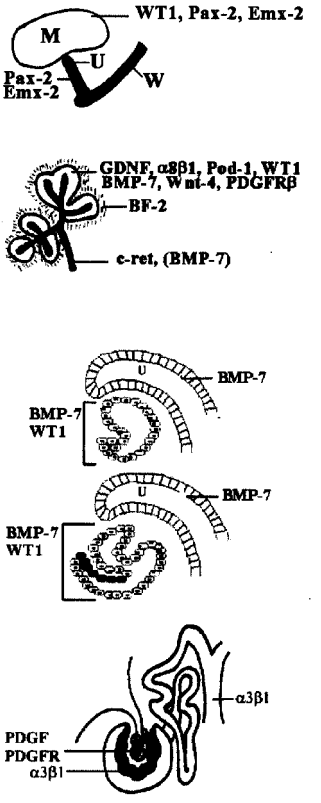

**Fig. 5** Intragenic WT1 mutations (Williamson and Van Heyningen) *(134)*.

patient is likely to develop gonadoblastoma. These patients suffer from Frasier syndrome, characterized by focal glomerular sclerosis, delayed kidney failure, and complete gonadal dysgenesis *(295)*.

This review has focused on the role of *WT1* in Wilms' tumorigenesis, but *WT1* may also be involved in the development of several other tumors like Leukemia *(293,296–298)*, granulosa and Leydig cell tumors *(299)*, ovarian tumors *(300)*, leukemia's *(293,296,301)*, melanomas *(302)*, mesotheliomas *(303)*, breast cancer *(304)*, and desmoplastic small round cell tumors *(305)*. The authors hypothesize that the *WT1* gene allows a cell to respond appropriately to signals from its environment, and the many *WT1* isoforms allow the cell to fine-tune this response. Disruption of the function of *WT1* may result in a failure of the cell to differentiate or undergo apoptosis, and may lead to aberrant proliferation. Concerning the communication of a cell and its environment, it must be kept in mind that the kidney is formed through the reciprocal interaction of the metanephric mesenchyme and the ureteric bud epithelium. It is conceivable that WT is not the result of a cell-autonomous defect alone, but may also be the result of a communication breakdown between these tissues. Therefore, defects in cells that normally do not express *WT1*, may, in some cases, also cause WT formation. More than 200 genes/proteins are listed in the Kidney Development Database *(13)*. Expression studies, in combination with the analyses of knockout mice, have confirmed the importance of several of these genes in kidney development as a whole, and the signaling cascade between the metanephric mesenchyme and the ureteric bud

Table 5
Crucial Genes in Kidney Development

| *Gene* | *Kidney phenotype in null mice* |
|---|---|
| *WT1* | No outgrowth ureteric bud *(161).* |
| *Pax-2* | No outgrowth ureteric bud *(169).* |
| *Emx-2* | No branching of ureteric bud, followed by degeneration *(325).* |
| *GDNF* | No ureteric bud or reduced branching *(164,326,327).* |
| *Lim-1* | No kidneys *(328).* |
| c-*ret* | No ureteric bud or reduced branching *(165).* |
| α8β1 integrin *(329).* | Reduced growth/branching of ureter |
| *Pod-1* | Reduced branching *(330).* |
| *Wnt-4* | Undifferentiated mesenchyme, interspersed with branches of the ureter *(167).* |
| *BF-2* | Inhibition of mesenchyme differentiation into comma and s-shaped bodies *(15).* |
| *BMP-7* | Inhibition of differentiation of comma- and s-shaped bodies into glomeruli *(331,332).* |
| *PDGF-B* | No mesangial cells or glomerular capillary tufts *(333).* |
| *PDGF-B-R* | No mesangial cells or glomerular capillary tufts *(334).* |
| α3β1 integrin | Abnormal podocyes and reduced number of collecting ducts *(335).* |




The genes are listed in the order in which their absence has an effect on kidney development. The expression pattern of each gene is visualized on the right. M, metanephric mesenchyme; U, ureter; W, wolffian duct.

epithelium (Table 5). Further analysis of this signaling cascade will help understanding of Wilms' tumorigenesis.

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# 14 Fanconi Anemia Pathway and Cancer Susceptibility

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***Contents***



## 1. INTRODUCTION

Fanconi anemia (FA) is a rare autosomal recessive disease characterized by multiple congenital abnormalities, bone marrow failure (BMF), and cancer susceptibility. The mean age of onset of anemia is 8 years, and mean survival is 16 years. Death in FA usually results from complications of BMF. Considerable progress in the field of FA research has resulted from the recent identification and cloning of three FA genes. The purpose of this chapter is to describe the clinical and diagnostic features of FA, review the cellular phenotype of FA, review the structure and putative function of the cloned FA genes, and discuss the possible function of the FA genes as tumor suppressors.

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## 2. CLINICAL COURSE OF FA

The congenital abnormalities and clinical course of FA have been extensively reviewed *(1,2)*. Patients with FA have growth retardation and abnormalities of the skin, upper extremities (frequently with defects in the thumbs or forearms), kidneys, and gastrointestinal system. The large range of organ systems affected in FA implicates the FA genes in a general developmental process required during normal human embryogenesis.

The hematological complications of FA have also been extensively reviewed *(3,4)*. FA patients develop macrocytosis and pancytopenia, typically during the first decade of life. Deficiencies in platelets or red cells usually precede white blood cell abnormalities. The patients have fetal-like erythropoiesis, with increased i antigen and hemoglobin F, and generally have high serum erythropoietin levels. The progression to pancytopenia is highly varied among FA patients, and some patients develop hematological malignancies even before anemia is clinically evident.

At least 20% of patients with FA develop cancers *(3)*. Of the FA patients with cancer, approx one-half develop acute myeloblastic leukemia; however, cancers of several organ systems, including skin, gastrointestinal, and gynecological systems, have been described. There appears to be an increased incidence of head and neck carcinomas and esophageal carcinomas in FA patients. Cancer tends to be a disease of older FA patients, with an average age of 15 yr for leukemia, 16 yr for liver tumors, and 23 yr for other tumors *(5)*.

## 3. DIAGNOSIS OF FA

The diagnosis of FA exploits the sensitivity of FA cells to bifunctional alkylating agents, diepoxybutane (DEB) or mitomycin C (MMC). FA cells have increased spontaneous chromosome (chr) breakage that is amplified by the addition of these crosslinking agents. Similar, spontaneous, but not DEB-induced, chr changes are observed in other inherited chr instability syndromes, such as Bloom syndrome (BS) and ataxia telangiectasia (AT). The DEB test is a highly sensitive and specific test for FA *(6)*, and has been used successfully in prenatal diagnosis of FA.

Other diagnostic tests for FA are also available. In one flow cytometric technique, cells that have been fluorescently prelabeled are treated with alkylating agents. The percentage of cells arrested in the G2–M phase of the cell cycle is measured. In this way, FA cells are indistinguishable from normal cells by their increased accumulation in the G2–M phase of the cell cycle. In some cases, FA carrier cells can also be distinguished from normal cells *(7)*.

New diagnostic approaches have resulted from the cloning of the FA genes. For instance, specific antisera for the FANCA, FANCC, and FANCG proteins have been described, which are useful for diagnostic immunoblotting of FA cell lines. Also, retroviruses, which transduce the FANCA, FANCC, and FANCG cDNAs, have been generated. Transduction of FA cell lines with these retroviral supernatants allows efficient subtyping of FA patients *(8)*. Finally, FA can be diagnosed on the basis of direct mutational analysis of the three cloned FA genes.

The diagnosis of FA has recently been confounded by the observation that some patients may have significant mosaicism. Patients with mosaicism have two populations of cells exhibiting either a normal or an FA phenotype. Such mosaicism may gen-

erate a false-negative DEB chr breakage assay, if the percentage of cells with a normal phenotype is sufficiently high. Approximately 25% of patients with FA have spontaneously occurring mosaicism.

The molecular basis of mosaicism in FA has not yet been completely determined. In some cases *(9),* a somatic mutation in a mutant FA gene allele results in correction of the mutant allele and subsequent expression of a functional FA protein. For instance, the somatic mutation of a mutant (frameshifted) FANCC allele could theoretically result in the correction of the mutant allele and the expression of a functional protein product. A hematopoietic progenitor (FA) cell could theoretically be corrected by such a somatic reversion, giving rise to a clone of hematopoietic cells with a normal (non-FA) phenotype. Such a normal cell may have a selective advantage, and may be more likely to proliferate faster than other FA cells of the host. The comparatively-high frequency of hematopoietic cell mosaicism in FA may result from a combination of the increased mutational (reversion) frequency in FA, and the selective advantage of functionally corrected cells. Mosaicism has already been described in other diseases, such as Bloom Sydrome (BS) and adenosine deaminase deficiency *(10,11).*

## 4. TREATMENT OF FA

The treatment of FA is similar, but not identical, to the treatment of other forms of acquired aplastic anemia. Patients are treated with supportive care (i.e., blood transfusions) for their BMF. The use of the cytokines, granuloate colony-stimulating factor, erythropoietic, and granuloate-macrophage colony-stimulating factor has also been efficacious for some FA patients *(12–14).* The treatment of choice for FA is allogenic BM transplant with a histocompatible sibling donor. Umbilical cord blood offers a potential source of hematopoietic stem cells for FA patients without sibling matches. Gene therapy (GT) also offers an alternative strategy for FA treatment, but this approach requires prior knowledge of the patient's complementation group *(15–17).* A more detailed discussion of GT for FA is provided in Sections.

## 5. CELLULAR PHENOTYPE OF FA CELLS

Because of the cellular sensitivity to crosslinking agents, FA is often compared to other syndromes of drug sensitivity and genomic instability, including AT, xeroderma pigmentosum (XP), Cockayne syndrome, BS, and hereditary nonpolyposis colorectal cancer *(2).* The chr instability of these syndromes may result from a cellular defect in any one of several processes, including DNA repair, cell cycle regulation, or DNA replication.

In addition to crosslinking agent sensitivity, FA cells have several other phenotypic abnormalities. FA cells have cell cycle abnormalities, as demonstrated by the prolongation of the G2 phase of the cell cycle *(7,18).* FA cells also have pronounced hypersensitivity to oxygen and reactive oxygen species *(19–22),* and a G2 phase specific hypersensitivity to ionizing radiation *(23).* Extracts of FA cells have been shown to have direct defects in DNA repair *(24–28).* FA cells have been shown to have increased apoptosis *(29,30)* and abnormal p53 responsiveness *(30,31).* FA patients have also been shown to have an intrinsic hematopoietic *(32,33)* and gonadal *(34)* stem cell defect. More recently, FA cells were shown to be hypersensitive to the effects of interferon (IFN-$\gamma$) and tumor necrosis factors (TNF-$\alpha$) (see below), accounting, at least in part,

for the aplastic anemia in FA patients *(34,35).* Many of these abnormal phenotypic characteristics are also evident in primary cells derived from mice homozygous for a disrupted *FANCC* gene. How the absence of the FA proteins leads to these cellular abnormalities remains unknown.

## 6. MOLECULAR BIOLOGY OF FA GENES

### *6.1. Cloning of FA Genes*

The complementation analysis of FA cells, using somatic cell fusion studies, has allowed the identification of at least eight complementation groups *(36–40;* Table 1*). At least four of the eight groups (A, C, D, and G) map to discrete chr loci* (41–44). FA is therefore a genetically heterogenous disorder, unlike the syndromes, AT and BS, which arise from mutations in single genes. The three cloned FA genes (for A,C, and G) encode orphan proteins, with no sequence similarity to each other or to other proteins in GenBank.

### *6.2. Molecular Biology of FANCC*

FANCC was cloned by functional complementation of an Epstein-Barr virus (EBV)-immortalized type C FA cell line *(45).* As predicted by the complementation test, the FANCC cDNA corrects the MMC sensitivity and DEB sensitivity of FA-C cell lines, but does not correct the MMC sensitivity of FA cells derived from other FA groups. Cells derived from FA-C patients have mutations in both alleles of the *FANCC* gene, consistent with the autosomal recessive inheritance pattern of the FA syndrome. The human *FANCC* gene is composed of 14 exons *(46),* spans approx 120 kb, maps to human chr 9q22.3 *(46),* and encodes a 558-amino-acid (aa) polypeptide (63 kDa) (Table 2). The murine, rat, and bovine FANCC cDNA homologs have also been cloned *(47,48).* The murine FANCC protein is 78% identical to the human FANCC protein. When murine FANCC is expressed in human FA-C cells, functional complementation is established *(47).*

Mutational analysis of the *FANCC* gene has revealed a relatively small number of characteristic mutations, represented in specific ancestral backgrounds (Fig. 1). The IVS4+4 A > T mutation is found in patients of Ashkenazi-Jewish ancestry, and accounts for >80% of FA in this population *(49–50).* Patients homozygous for this mutation have severe FA, with multiple congenital abnormalities and early onset of hematological disease *(51).* Whether this mutant *FANCC* allele corresponds to a severe FA phenotype in other (non-Ashkenazi-Jewish) genetic backgrounds remains unknown. The 322delG mutation is found in patients of Northern European ancestry, particularly from Holland. Patients homozygous for this mutation have comparatively mild FA, with fewer congenital abnormalities and later onset of hematological disease *(51).* Based on the relative prevalence of mutations in exon 14 and the high conservation in this exon across species, the carboxy-terminal region of FANCC is likely to contain a critical functional domain. Consistent with this hypothesis, recent studies suggest that the C-terminal region of FANCC may be required for interaction with FANCA *(52).*

Analysis of the FANCC mRNA and protein have provided some insight into the cellular function of the *FANCC* gene. The FANCC mRNA is expressed in multiple cell types and organ systems, consistent with a general function of FANCC in organism

**Table 1**
**Complementation Groups of FA**

| *Subtype* | *Estimated percentage of FA patients* | *Chromosome location* | *Protein product* |
|---|---|---|---|
| A | 66 | 16q24.3 | 163 kDa |
| B | 4 | ? | ? |
| C | 12 | 9q22.3 | 63 kDa |
| D | 4 | 3p22–26 | ? |
| E | 12 | ? | ? |
| F | Rare | ? | ? |
| G (XRCC9) | Rare | 9p13 | 68 kDa |
| H | Rare | ? | ? |

**Table 2**
**Structural Features of FA Genes**

| *Gene* | *Cloned cDNA (kb)* | *Full length gene (kb)* | *Structure (no. exons)* | *mRNA transcripts (kb)* | *Expression* | *Comments* |
|---|---|---|---|---|---|---|
| *FANCC* | 4.6 | 120 | 14 | 2.3<br>3.2<br>4.6 | Ubiquitous | 5′UTR has two alternatively spliced exons |
| *FANCA* | 5.5 | 80 | 43 | 4.7 (major)<br>7.5<br>3.0<br>2.0 | Ubiquitous | – |
| *FANCG* | 2.5 | 6 | 14 | 2.2<br>2.5 | Ubiquitous | – |

development. Increased expression of the FANCC mRNA has been observed in the skeletal system, suggesting a more specialized function of FANCC in bone development *(53).*

The FANCC protein is primarily a soluble cytoplasmic protein *(54,55),* although a nuclear complex of FANCA, FANCC, and FANCG proteins has also been detected *(52,56);* (*see* below). The function of the FANCC protein remains mostly unknown, but it has recently been shown to physically interact with other cellular proteins, including GRP94 *(57),* Cdc2 *(58),* and NADPH reductase *(59).*

### *6.3. Molecular Biology of FANCA*

FANCA was cloned by two independent strategies. One group cloned FANCA by functional complementation of an EBV-immortalized FA-A cell line *(60).* The second group cloned the *FANCA* gene by a positional strategy *(61).* As predicted by the complementation test, the FANCA cDNA corrects the MMC and DEB sensitivity of FA-A

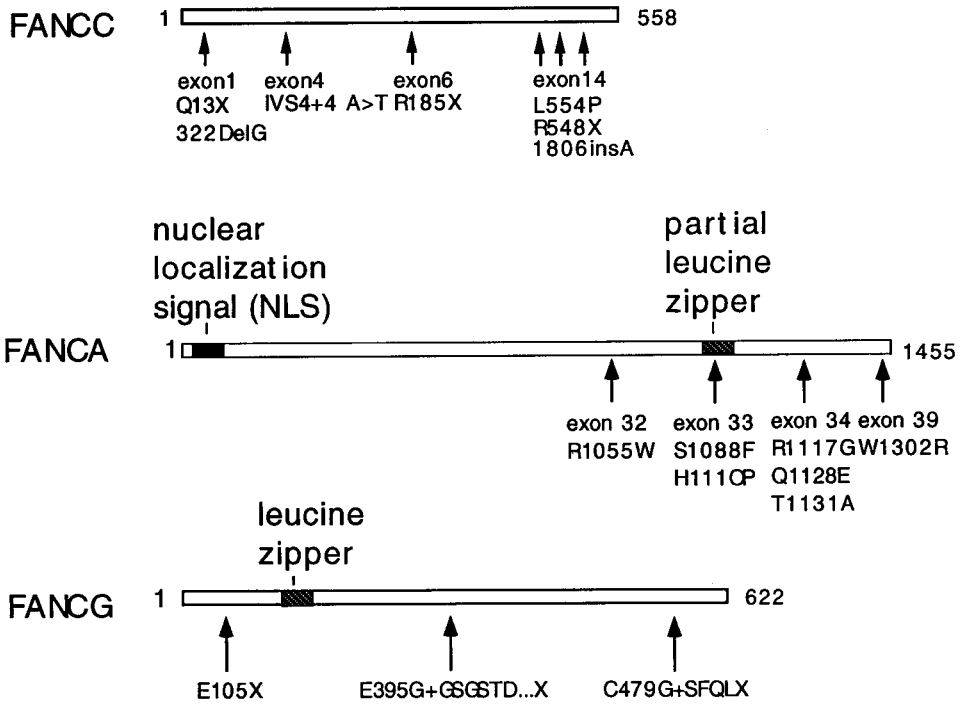


**Fig. 1.** Patient-derived mutations of the FANCC, FANCA, and FANCG proteins. The wt FANCC protein (558 aa) is shown schematically at the top. The IVS4+4 A > T mutant allele encodes a protein with an in-frame deletion of 37 aa *(49,55)* The 322delG has a frameshift mutation in exon 1 *(45)*. The IVS4 + 4 A > T mutation and 322delG account for approx 75% of FANCC mutations. Less common mutant alleles of FANCC are also shown. The relative frequency of these various FANCC alleles has recently been described *(51)*. The wt FANCA protein (1455 aa) is also shown. The protein contains a bipartite nuclear localization signal at its N-terminus and a partial Leu zipper. Mutation analysis has demonstrated that the *FANCA* gene is highly polymorphic. The region of the FANCA protein from aa 1046 to 1320, encoded by exons 32–39, appears to be critical for FANCA function, based on the prevalence of missense mutations in this region. The wt FANCG protein (622 aa) is also shown. The protein contains a Leu zipper between aa 135 and 163, but contains no other recognizable protein motifs. Based on published analysis of FA-G patients, only a few mutations are known. The mutant *FANCG* allele, 313G > T, is predicted to encode a truncated FANCG protein, E105X. The mutant *FANCG* allele, 1184–1194 del, encodes FANCG (E395G+GSGSTDPGRQSPRCCDSMX). The mutant allele IVS11+1G>C encodes FANCG (C479G+SFQLX). Whether these mutant *FANCG* alleles encode mutant FANCG proteins remains unknown.

cells, but does not correct the drug sensitivity of FA cell derived from FA groups B–H. The *FANCA* gene is composed of 43 exons, spans approx 80 kb, maps to human chr 16q23.4, and encodes an orphan protein of 1455 amino acids (163 kDa) (*see* Table 2).

The *FANCA* gene generates multiple FANCA mRNA transcripts, including a 4.7-kb transcript (major) and transcripts of 7.5, 3.0, or 2.0 kb. The mRNAs for FANCA and FANCC are ubiquitously expressed, although considerable variation is observed in specific tissues. In human adult tissues, the highest level of expression of both genes was observed in testis, thymus, and BM. In general, transcription levels were elevated in fetal tissues and in actively proliferating cells. Discordant expression pat-

terns were observed for FANCA and FANCC. The FANCC transcripts were found in similar abundance in all tissues; the FANCA transcripts demonstrated more tissue-specific variation, with high levels in liver and kidney. Taken together, these data suggest alternative transcriptional regulatory mechanisms for the two genes. The 5′ *cis*-acting regulatory (promoter) regions of the *FANCC* and *FANCA* genes contain highly GC-rich regulatory sequences, a feature of several known housekeeping genes *(62,63).*

The FANCA protein contains a nuclear localization signal (NLS) at its N-terminus *(64),* and a partial leucine (Leu) zipper motif between aa 1069 and 1090. The importance of this Leu zipper region remains unclear. Some, but not all, mutations in this region of FANCA result in loss of FANCA function *(65).* Although the primary aa sequence of FANCA provides little insight into its biochemical function, missense mutations derived from FA-A patients may help identify critical functional domains of the FANCA protein.

Based on mutational screens published to data *(66–68)* the region of the FANCA protein from aa 1046 to 1320, encoded by exons 32–39 *(62),* appears to be critical for FANCA function. Multiple patient-derived missense mutations have been identified in this region (Fig. 2; *66,67*). The FANCA1263delF (exon 34), encoded by the mutant *FANCA* allele, 3788–3790del, was found in 30 FA-A patients, and appears to account for 5% of known *FANCA* mutations *(66).* In many cases, it is difficult to distinguish between pathogenic point mutations and simple polymorphisms. As for the FANCC protein, the cellular function of the FANCA protein is unknown. Recent studies demonstrate that the FANCA protein forms a complex with FANCC and FANCG (*see* model in Subheading 6.4). FANCA has also been reported to form a complex with the kinase, IKK-2 *(69).* FANCA is phosphorylated on serine residues *(70),* and requires nuclear localization for its function *(64).*

### *6.4. Molecular Biology of FANCG*

The demonstration that the human *FANCG* gene *(71)* is identical to the previously cloned human *XRCC9* gene is recent *(72),* and, accordingly, little is known about the encoded FANCG protein. The human *XRCC9* cDNA was originally expression-cloned by its ability to partially complement the MMC sensitivity of a mutant CHO cell line *(72).* de Winter et al. *(71)* subsequently cloned the same cDNA by expression, based on its ability to complement an FA-G cell line. The FANCG/XRCC-9 protein is yet another orphan protein that contains an internal Leu zipper as its only recognizable motif. The *FANCG* gene is composed of 14 exons, spans approx 6 kb, maps to human chr 9p13 *(44,72),* and encodes a 68 kDsa protein (Table 2). For FANCG, major mRNA bands of 2.2 kb and 2.5 kb were detected in all human tissues tested *(72).* As for FANCA and FANCC, the expression was 10–100-fold higher in testis, compared to other tissues.

Only one study has been published that identifies patient-derived mutations in the *FANCG* gene *(71).* One mutation, found in three patients with German ancestry, was a G-to-T transition at nucleotide 313, which changes codon 105 from glutamic acid to a stop (E105X). A second mutation was an 11-bp deletion in exon 10 and a splice-site mutation (IVS11 + 1 G to C). A third mutation was a splice-site mutation, IVS13 – 1 G to C, found in two affected children (Fig. 1). Whether any of these mutant *FANCG* alleles direct the synthesis of a truncated, nonfunctional FANCG protein remains unknown (Fig. 2). Further identification of relevant functional domains of the FANCG protein

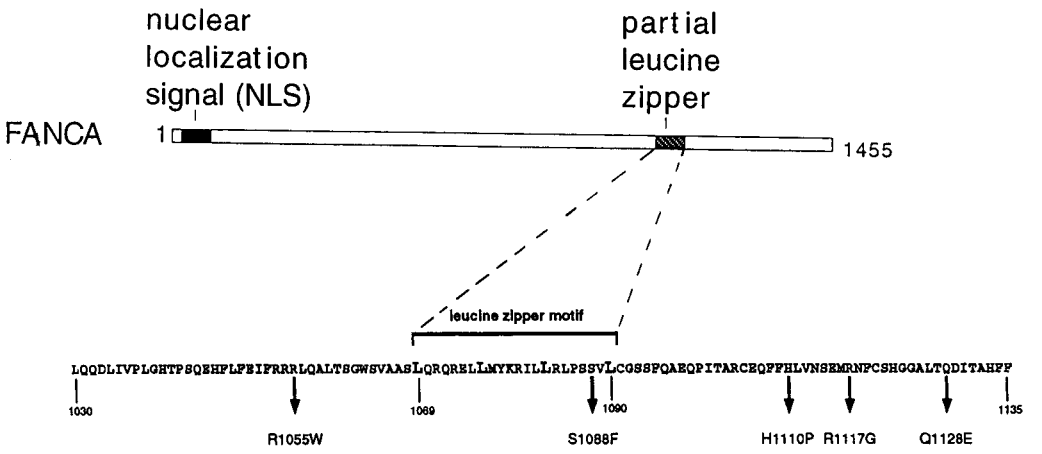


**Fig. 2.** The C-Terminal region of the FANCA protein contains a domain required for functional activity. A schematic representation of the FANCA protein is shown. The region of the protein, from aa 1030 to 1135 is enlarged. This region contains a high incidence of mutations in the FA-A patient population, suggesting that it contains a critical functional domain. Recent data supports the notion that this domain is required for FANCC binding, nuclear accumulation, and functional activity *(117).*

may be gained by a comparison of the human and murine FANCG proteins (Fig. 3). The human and murine proteins have 72.2% identity. One Leu residue of the putative Leu zipper is not conserved in the mouse protein. Whether the murine FANCG cDNA corrects the MMC sensitivity of FA-G cells remains unknown.

## 7. MOUSE MODELS OF FA

Two murine models of FA-C have been developed, using targeted recombination in embryonic stem cells *(34,73).* The phenotype of homozygous FANCC mutant animals was similar in both models. FANCC mutants are viable and show no obvious birth defects of the skeletal system or urinary system. Cells derived from these animals show classic hypersensitivity to bifunctional crosslinking agents. Nonetheless, spontaneous pancytopenia did not develop during the first year of life, and no leukemia or increased cancer susceptibility was observed.

The FANCC–/– mice have a germ cell defect. The germ cell defect in FANCC–/– male mice, resulting in subnormal fertility, appears to be secondary to decreased sperm viability, and not to a defect in meiosis. The cells from the FANCC–/– mice also are hypersensitivity to the apoptotic effects of IFN-γ and TNF-α *(34).* This TNF-α and IFN-γ hypersensitivity is also observed in primary cells and cell lines derived from human FA patients.

FANCC–/– mice do not show any of the baseline hematologic defects found in FA patients, but the mice were found to be highly sensitive to DNA crosslinking agents in vivo *(74).* Specifically, acute MMC exposure caused extreme BM aplasia. Sequential, nonlethal MMC exposure caused a progressive decrease in peripheral blood counts. BMF in these MMC-treated FANCC–/– mice was shown to result from a reduction in the number of early and committed hematopoietic progenitor cells. Taken together,

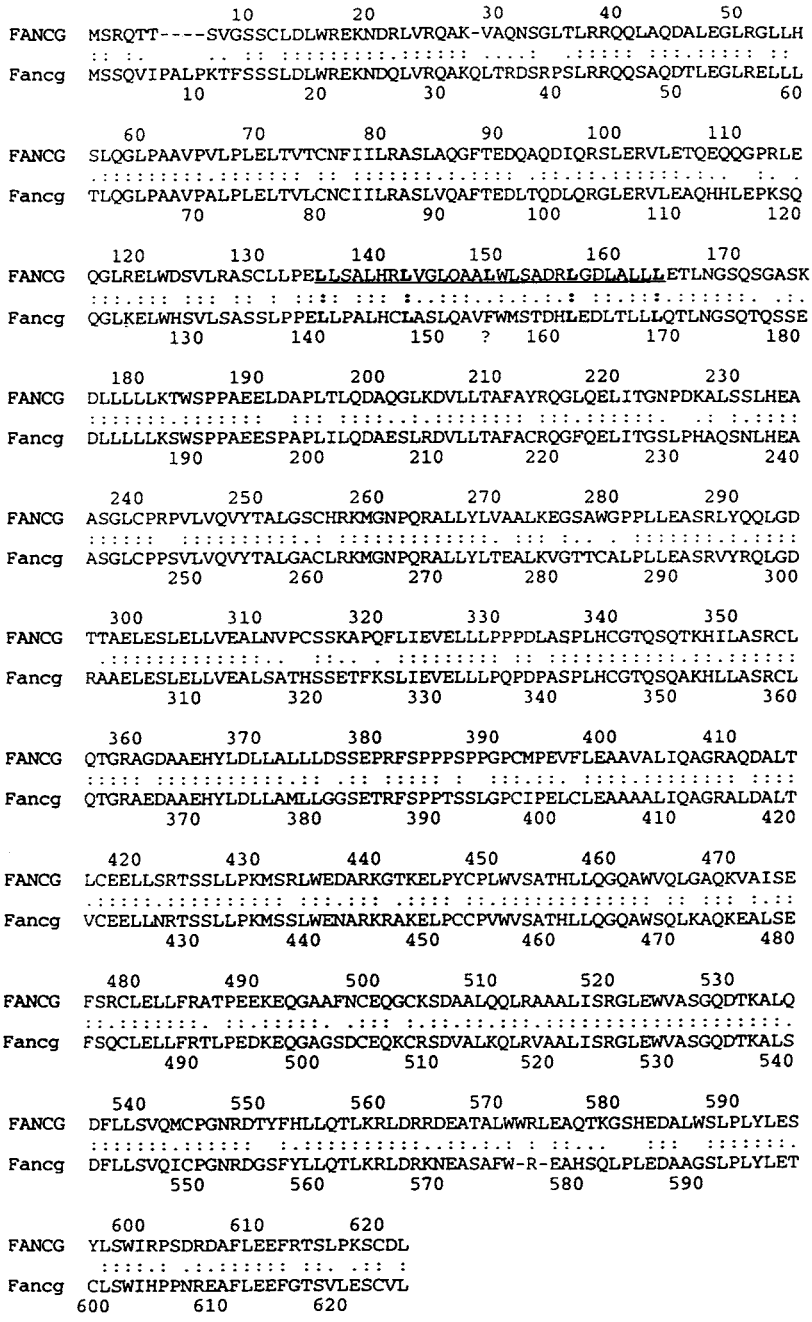


**Fig. 3.** Sequence comparison of human and murine FANCG proteins. The human FANCG/XRCC9 protein sequence is shown, compared to the predicted sequence of the murine homolog (fancg), obtained from Genbank. Overall, the two proteins have 72.2% identity. The Leu zipper region is underlined. One Leu (L150) is not conserved in the murine sequence.

these results are consistent with a function of the FANCC protein product in the viability or differentiation of early hematopoietic progenitor cells or stem cells.

## 8. MODELS OF FA GENE PATHWAY

Little is known about the cellular function of the FA proteins. Multiple studies have been performed in order to identify binding proteins of FANCC and FANCA. To date, it is known that FANCC interacts with several cytoplasmic binding proteins *(75),* as well as cdc2 *(58),* GRP94 *(76),* and NADPH reductase *(59).* More recently, FANCA has been shown to interact with the cytoplasmic kinase, IKK2 *(69).* The physiological relevance of these protein–protein interactions has not yet been determined. A more unifying hypothesis has recently emerged, based on the recognition that the three FA proteins (FANCA, FANCG, and FANCC) interact in a functional nuclear complex *(56).*

### *8.1. Interaction of FA Proteins in a Common Pathway*

Mutation of any of the cloned FA genes leads to a conserved cellular and clinical phenotype, which suggests that the FA genes encode proteins that function in a coordinate manner (Fig. 4). For instance, one can envision the FA proteins functioning in a common, multisubunit complex (protein complex hypothesis) or in a common biochemical pathway (pathway hypothesis). Considerable experimental data now exist to support the model of a multisubunit complex *(52,56).*

The authors have recently determined that FANCA, FANCG, and FANCC form a complex that is localized in both the cytoplasm and the nucleus of normal cells *(56).* Initial studies provided evidence for a FANCA–FANCC interaction. First, for lymphoblasts, primary fibroblasts, and primary BM cells, expressing normal (endogenous) levels of the FANCA and FANCC proteins, this laboratory detected a physical complex of FANCA and FANCC *(52).* The FANCA–FANCC protein complex was detected by reciprocal immunoprecipitation–Western blotting protocols, with either anti-FANCA or anti-FANCC antisera. Second, the FANCA–FANCC complex was detected in protein fractions from the cytoplasm and the nucleus of primary cells. The co-immunoprecipitation was more efficient from nuclear extracts *(64),* suggesting a relative enrichment of the complex in the nucleus. Other studies have also used confocal microscopy to localize FANCC to the nucleus *(77).* Third, the interaction of FANCA and FANCC in a complex is critical to the function of the proteins. For lymphoblast lines derived from FA patients, mutant FANCC proteins fail to bind to FANCA *(52),* and mutant FANCA proteins fail to bind to FANCC *(70).* Functional complementation of these cells rescued FANCA–FANCC binding. Fourth, additional evidence, independent of the use of anti-FANCA antisera, demonstrated that cellular FANCA and FANCC bind in a complex (I. Garcia-Higuera, unpublished observation). For these studies, the authors generated an amino-terminal Flag-tagged FANCA protein, and expressed this protein in an FA-A cell line, GM6914. The Flag-tagged FANCA protein corrected the MMC sensitivity of the transfected cells, and co-fractionated with FANCC from an anti-Flag column. Fifth, the authors have shown that the FANCA protein is a phosphoprotein, and that its phosphorylation correlates with FANCC binding *(70).* FANCA is not phosphorylated, and the FANCA–FANCC complex is not detected in FA cells derived from other FA complementation groups (groups B, E, F, G, and H), suggesting that products of other FA genes regulate the assembly of the nuclear complex *(70).*

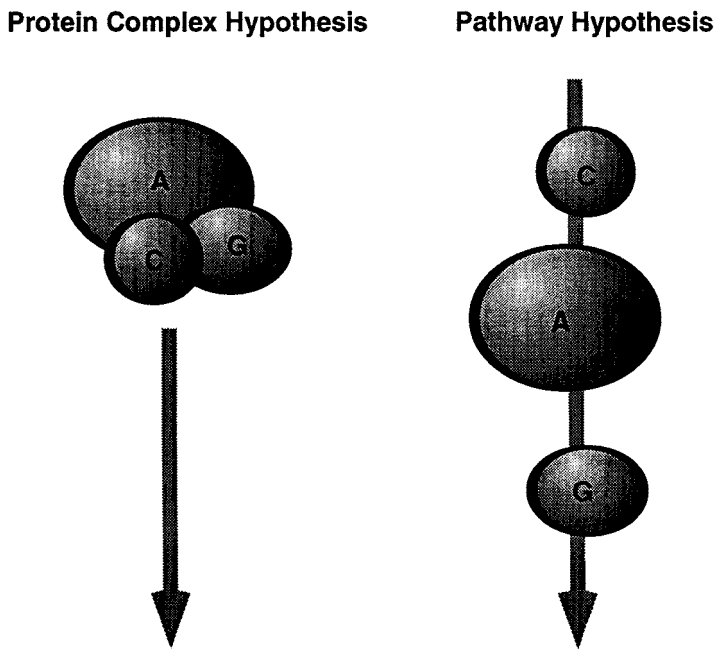


**Fig. 4.** Functional interaction of the FA proteins. Shown are two possible models for the interaction of the FA proteins, FANCA, FANCG, and FANCC. Because loss or mutation of these proteins yields the same specific FA clinical and cellular phenotype, it is assumed that the proteins interact, either directly (protein complex hypothesis) or indirectly (pathway hypothesis). Recent data discussed in the text supports the pathway hypothesis.

More recent data demonstrate that the FANCG protein is also part of the FANCA–FANCC complex *(56).* Expression of the FANCG protein in a FA-G lymphoblast line corrects the MMC sensitivity of the cell line, and restores the binding interaction between FANCA and FANCC. In these FANCG-corrected FA-G cells, an antibody to FANCG co-immunoprecipitates a complex of FANCA, FANCG, and FANCC proteins. The binding of FANCA and FANCG appears to be a constitutive, direct interaction. The two proteins bind to each other when the proteins are translated in vitro and mixed. Also, the two proteins bind to each other in extracts of FA cells from multiple complementation groups. The binding between FANCA and FANCG is therefore different from the binding interaction between FANCA and FANCC. The FANCA–FANCC interaction appears to be a weaker or regulated interaction. The FANCA and FANCC proteins do not bind in vitro, suggesting that their binding requires additional adaptor proteins or posttranslational modifications. For instance, the binding of FANCA to FANCC requires the phosphorylation of FANCA. Finally, the binding of FANCA and FANCC is not observed in cell lines derived from several FA complementation groups, including groups B, E, F, G, and H, suggesting that the products of other FA genes may be required for the binding interaction to occur. A schematic representation of the molecular interactions among the three proteins is shown (Fig. 5). The trimolecular complex of FANCA, FANCC, and FANCG is found in both the cytoplasm and the

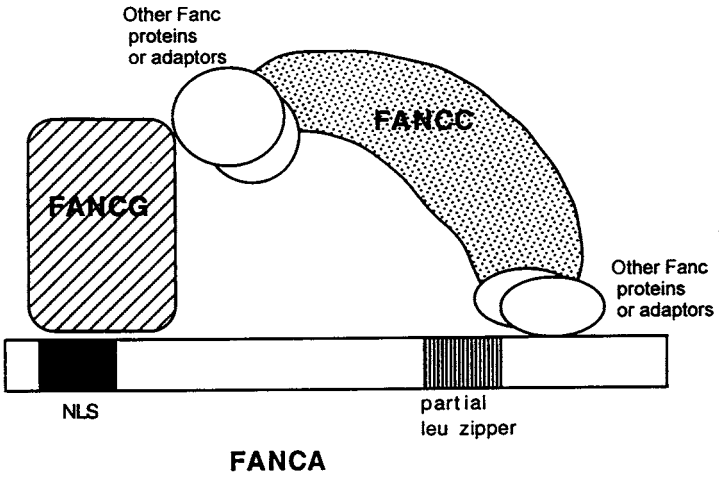


**Fig. 5.** Schematic model of molecular interactions of the FANCA, FANCG, and FANCC proteins. Based on available data, at least two regions of the FANCA polypeptide are required for the FANCA–FANCG–FANCC interaction. First, the amino-terminal region of the FANCA protein, which includes the bipartite NLS region, is required for constitute interaction with the FANCG protein. Second, a region of the C-terminus of FANCA is required for the FANCC interaction. Patient-derived point mutations in this region disrupt the FANCC interaction. The FANCC interaction is a weaker interaction or a regulated interaction, requiring FANCA–FANCG binding and the products of other FA genes *(70)*. The region of FANCG required for FANCA binding is not known. The region of FANCC required for FANCA binding may be the C-terminus of FANCC. The C-terminus of FANCC is highly conserved among human, murine, and bovine FANCC proteins. Also, an FA-C patient-derived point mutation (FANCC-L554P) ablates FANCA binding.

nucleus, suggesting the three proteins act in a coordinate manner to execute a common function, such as DNA repair, transcription, or chr segregation.

Based on this recent evidence of a regulated interaction of FANCA, FANCG, and FANCC, a model of the FA pathway can be devised (Fig. 6). According to this model, monomeric FANCA and FANCG protein bind constitutively in the cytoplasm of the cell. This interaction appears to be stoichiometric: Complexes of FANCA and FANCG are efficiently immunoprecipitated with antisera to either FANCA or FANCG. Upon some cellular stimulus, FANCA phosphorylation occurs and FANCC binds to the FANCA–FANCG protein complex. Products of other FA genes may mediate the phosphorylation of FANCA and the formation of the FA protein complex. For instance, other FA genes may encode the FANCA kinase, or may encode adaptor proteins in the FA protein complex. The trimolecular complex of FANCA–FANCG–FANCC then translocates to the cell nucleus. Defects in the FA pathway result in a failure of the complex to translocate to the nucleus, and thereby result in chr instability, the hallmark of FA. Consistent with this model, the FANCA protein is not phosphorylated, and FANCA–FANCG–FANCC interaction is not detected in cells from other FA subtypes, including groups A B C E F G, and H. FA-D cells are distinct. These cells remain sensitive to MMC, despite FANCA phosphorylation, FANCA–FANCG–FANCC binding, and nuclear accumulation of the complex. Taken together, these results suggest that the FANCD protein may act independently (either upstream or downstream) of the complex.

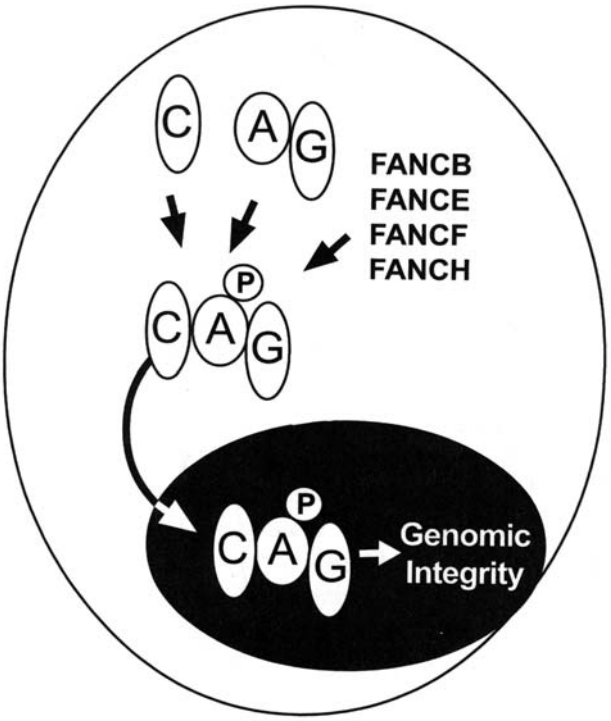


**Fig. 6.** Model for the regulated molecular interactions in the FA pathway. Based on available data, the FANCA and FANCG proteins bind constitutively in the cytoplasm of the normal cell. FANCC protein binds to the FANCA–FANCG complex, but this binding requires FANCA phosphorylation and requires the products of other FA genes. The trimolecular complex subsequently translocates to the cell nucleus. The FANCA–FANCG complex does not accumulate in the nucleus in the absence of FANCC protein binding. Whether FANCC is required for nuclear transport or stabilization of the complex in the nucleus is not known. After nuclear translocation, the complex executes a nuclear function, such as the repair of DNA or the orderly segregation of sister chromatids. Recent evidence suggests that the FA proteins comlex acts upstream of the FANCD protein (Higuera I and D'Andrea AD, unpublished observation).

Although considerable evidence exists for the interaction of the FA proteins in a complex, little is known regarding the biochemical and cellular function of this complex. Recent lines of evidence suggests that the FA protein complex may play a cellular role in the suppression of IFN-α— and TNF-α—induced apoptosis, the modulation of cell cycle events, or the repair of crosslinked DNA. Evidence supporting these models is provided below.

### *8.2. Suppression of IFN-— and TNF-—Induced Apoptosis by FA Pathway*

Recent evidence suggests a direct relationship between the FA pathway and the IFN-γ signaling pathway. First, multiple studies *(34,35)* have demonstrated that FA cells have decreased cellular survival and an increased IFN-α—mediated apoptotic response. FA-C overexpression has been shown to protect Ba/F3 cells from apoptosis, following interleukin-3 deprivation *(78)*. Also, primary BM cells from an FANCC–/–

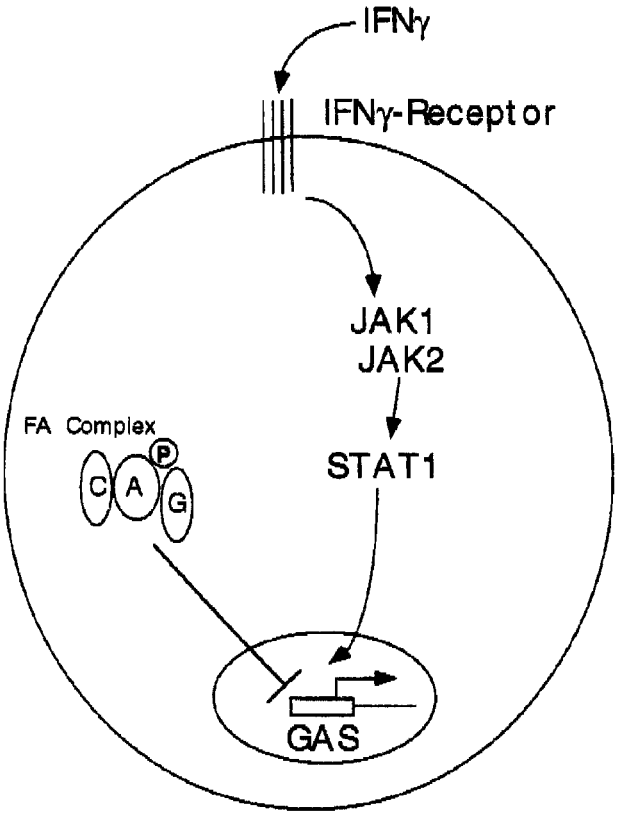


**Fig. 7.** Possible role of the FA pathway in the suppression of IFN-α—mediated apoptotic responses. Stimulation of the IFN-γ receptor results in activation of JAK1 and JAK2 kinases and phosphorylation of the STAT kinase, STAT1. STAT1 phosphorylation leads to the induction of specific genes, some of which mediate cell cycle arrest and apoptosis. According to this model, the FA protein pathway may normally function to downregulate the IFN-γ response. Absence of the FA protein complex would lead to increased IFN-γ signaling and increased apoptosis in response to IFN-γ.

mouse have increased IFN-α—mediated cell death *(34)*. Second, FA cells have been shown to express increased levels of IFN-α—inducible genes. IFN-γ is known to activate the (JAK1)/JAK2/STAT1) pathway, resulting in the induction of immediate-early genes that regulate apoptosis. STAT1-deficient cells are defective in the IFN-α—mediated cell death response *(79)*. FA cells also have increased expression of the IFN-α—inducible genes, *IRF1, fas,* and *MxA (80)*. This may be a selective or partial IFN-γ response, since not all IFN-α—inducible genes are upregulated in FA cells. For instance, HLA class I antigen, IP10, and STAT2 are not upregulated in FA cells. Taken together, these results suggest that the FA pathway may normally function to suppress the apoptotic response to IFN-γ. According to this model, absence of a functional FA pathway, resulting from mutations of an FA gene, may lead to an increased IFN-γ response and increased apoptosis. A model depicting this possible suppressive mechanism of the FA pathway is shown in Fig. 7.

FA cells are also hypersensitive to the apoptotic responses of the cytokine, TNFα *(35)*. This hypersensitivity may result from increased expression of nuclear factor-κB

(NF-κB)-inducible genes. Taken together, these results also suggest that the FA pathway may normally function to suppress the TNF-α—induced NF-κB response. A recent report demonstrates that the TNF-α—activated kinase, IKK2, binds to FANCA *(69).* Whether or not IKK2 phosphorylates or affects the activity of the FA protein complex remains unknown.

### *8.3. Cell cycle and FA pathway*

Several lines of evidence suggest that FA cells have an underlying molecular defect in cell cycle regulation. First, FA cells have a prolonged G2 transit time, which is enhanced by treatment with chemical crosslinking agents *(7,18).* Second, the G2 arrest and reduced proliferation of FA cells can be partially corrected by overexpression of a protein, SPHAR, which is a member of the cyclin family of proteins *(81).* Third, caffeine abrogates the G2 arrest of FA cells *(30).* Consistent with these results, caffeine is known to constitutively activate cdc2 and to override a normal G2 cell cycle checkpoint that may be defective in FA cells. Fourth, other diseases characterized by genomic instability, including Li-Fraumeni syndrome and AT, result from abnormalities in cell cycle regulation, suggesting a possible relationship with FA. Finally, a specific interaction between the FANCC protein and the cyclin dependent kinase, cdc2 *(58),* has been demonstrated, suggesting that FA proteins may be substrates or modifiers of the cyclin B/cdc2 complex. How these cell cycle abnormalities in FA cells result from the absence of a functional FA pathway remains unknown.

### *8.4. DNA repair and FA pathway*

Recent studies suggest that the FA protein complex may have DNA repair activity and/or DNA-binding activity. As stated previously, FA cells have a high level of chromosome breakage, both spontaneous and induced by crosslinking agents. This increased chr instability is reflected by an overproduction of intragenic deletions. For instance, in FA cells, there is a high proportion of deletions at the endogenous HPRT locus *(82).* Recent studies *(83)* have also suggested that FA cells are defective in error-free processing of blunt-end double-strand breaks (DSB). Studies with transfected DNA templates further demonstrate that FA cells are defective in the fidelity of specific blunt-end DNA end joining, *(84).*

Lambert et al. *(25,85)* have isolated a nuclear protein complex with increased binding affinity for psorolen-crosslinked DNA. The complex contains a 230-kDa protein (p230), along with the FANCA and FANCC proteins *(86).* These provocative recent studies suggest that a nuclear complex containing the FA proteins may function to bind and repair DNA that is selectively crosslinked by MMC. To this extent, a nuclear FA complex of proteins is reminiscent of the excision repair complex, composed of XP proteins, and directly involved in the repair of DNA templates containing ultraviolet light-activated thymine dimers *(87).* Further studies are needed to explore the relative DNA-binding and possible endonuclease activities of the nuclear FA protein complex.

Given the selective sensitivity of FA cells to DNA-crosslinking agents, it is possible that FA cells have a specific defect in DNA-crosslink repair. DNA crosslinks represent a unique class of DNA lesions, capable of blocking both DNA replication and RNA transcription. Little is known about the mechanism of crosslink repair in mammalian cells. Crosslink repair has been studied in *Saccharomyces cerevisiae,* and has been shown to require both nucleotide excision repair (NER) and double-strand break repair

(DSBR) by the homologous recombination function *(88).* For the repair of psoralen-crosslinked DNA, the NER pathway initially generates DSBs near the interstrand crosslink. Then Rad51-dependent homologous recombination function subsequently repairs the DSBs in yeast *(89).* In *Escherichia coli,* crosslink repair appears to occur through homologous recombination, without the generation of DSB intermediates *(90).*

Based on the requirement for DSBR in the repair of crosslinks in yeast, the FA protein complex may directly or indirectly interact with the protein complexes involved in this function. In general, DSBR can be executed by two separate, nonoverlapping pathways, the nonhomologous end-joining pathway (NHEJ) and the homologous recombination pathway (HR) (reviewed in refs. [*91* and *92*]. Accordingly, the FA protein complex may interact with the proteins in the NHEJ pathway (Ku, DNA-PK, XRCC4, and DNA ligase IV), or with proteins in the HR pathway (Rad51 family members) *(93,94).* Future studies are needed to determine whether the FA protein complex interacts with proteins in these other repair pathways.

## 9. RELEVANCE OF FA PATHWAY TO OTHER HUMAN DNA REPAIR SYNDROMES AND TUMOR SUPPRESSOR PATHWAYS

The FA pathway is analogous to genetic pathways for other human chr instability syndromes, such as XP and AT. XP is an autosomal recessive disease with seven complementation groups, and mutations in XP genes result in cellular sensitivity to UV light. The protein products of at least five of the XP genes assemble in an excision repair complex that functions to repair UV-induced thymine dimers *(95–97).* The XP protein products have variable binding affinity within the excision repair complex *(98)* and the related transcription complex, TFIIB *(99).* Some proteins are tightly bound, and some are loosely bound to the complex. AT, and the related disorder, Nijmegens breakage syndrome (NBS), are genetic cancer susceptibility syndromes characterized by cellular sensitivity to ionizing radiation. The ATM protein is a protein kinase that regulates a pathway involved in the repair of double-strand DNA breaks, induced by ionizing irradiation. The ATM protein directly phosphorylates the tumor suppressor protein, p53 *(100).* The NBS protein, in contrast, interacts in a complex with at least two other cellular protein (RAD50 and Mre11) *(101–103),* and thereby presumably regulates (DSBR or chr segregation. How the ATM protein interacts with the NBS–RAD50–Mre11 complex remains unknown. In addition to these human syndromes, other gene products are known to play a direct or indirect role in DNA repair. These gene products were originally isolated, in large part, through the characterization of CHO cell mutants (XRCC gene products) *(93,104).*

Based on these other model systems, several themes have emerged regarding these genetic chr instability syndromes. First, DNA repair results from the concerted effort of several gene products. Second, these protein products form protein complexes dedicated to the repair of specific classes of DNA adducts. Third, inducible changes in phosphorylation, assembly, or translocation of these complexes are often observed. The authors hypothesize that the FA proteins may comprise a parallel pathway, dedicated to the repair of DNA crosslinks. How mutations in any of these pathways lead to specific kinds of cancers, such as lymphomas in AT, remains mostly unexplained.

In some cases, proteins involved in DNA repair processes have been shown to co-localize, suggesting that at least some of these proteins participate in coordinated sig-

naling pathways. For example, immunofluorescence studies have demonstrated that human Rad51, XP type G protein, and proliferating cell nuclear antigen relocalize in response to DNA damage, implicating these proteins in the repair of specific classes of DNA damage. More recently, it was shown *(105,106)* that Rad51 co-localizes with BRCA1 and BRCA2 following cellular treatment during S phase, thereby directly implicating BRCA1 in a DNA repair response. Whether the FA proteins co-localize with any of these other proteins, known to be involved in regulating chr integrity, remains to be determined. Future analysis of the FA protein complex, in the absence or presence of cellular MMC treatment, may help to resolve this question.

## 10. GT FOR FA

FA is a particularly attractive candidate disease for GT approaches. The genes associated with three of the major complementation groups have been cloned and sequenced, and expression of these genes in FA mutant cells has been shown to complement the mutant phenotype, and restore resistance to clastogenic agents. The FA proteins need only be expressed at low levels in cells to restore normal phenotype, but can also be expressed at supraphysiological levels without interfering with normal cellular functions *(60,71).* This is similar to adenosine deaminase deficiency *(107–109),* and contrasts with β-globin disorders, such as sickle cell disease and thalassemia, in which expression of the introduced gene must be closely regulated. Finally, corrected FA cells have a selective growth advantage over the corresponding parental cells in vitro and in vivo *(17,110),* and successful correction of even a small proportion of hematopoietic stem cells may lead to substantial reconstitution of host hematopoiesis in vivo.

For successful GT of primary hematopoietic disorders, it is necessary to stably express the introduced gene in hematopoietic stem cells having extensive self-replicative potential *(111–113).* Correction of more mature cells would lead only to transient correction of the defect. The phenotypic identity of these stem cells remains controversial, but increasing evidence based on repopulation of the *nod/scid* mouse-strain with human cells suggests that the stem cells reside in the $CD34^-$ $lin^-$ subpopulation of BM and cord blood *(114,115).*

Retroviral vectors suitable for stably expressing introduced genes in a variety of cell types were initially described nearly 15 years ago. The majority of vectors used are based on the Moloney murine leukemia virus, into which the gene(s) of interest have been introduced by standard recombinant DNA techniques. For the production of infectious virus, the proviral genome of interest is transfected into packaging cell lines, which produce retroviral vectors, without the concomitant production of replication-competent virus. These packaging lines express a helper genome, which encodes viral structural proteins; the provirus contains the gene(s) to be transferred and the *cis*-active elements required for viral encapsidation, replication, and integration. A number of genetic modifications have been made in packaging cell lines, to minimize the likelihood of production of replication-competent virus by recombination between the proviral and helper genomes. Typical packaging cell lines produce approx $10^6$ infectious viral particles per milliliter of supernatant. Infection of human hematopoietic precursors is thought to require minimum titers of $10^7$, and possibly as much as $10^9$ particles/mL, and supernantants must therefore be concentrated by ultrafiltration

and/or ultracentrifugation *(116).* Retroviral vectors are able to infect only actively replicating cells, and $G_0$ cells are therefore refractory to proviral integration.

Vectors incorporating the *FANCA, FANCC,* or *FANCG* cDNAs have been constructed, and shown to successfully correct the sensitivity of FA lymphoblast lines and fibroblasts to clastogenic agents in vitro. These vectors thus provide a simple alternative to conventional complementation assays for determining the complementation group of individual FA patients *(8,16).* Similar vectors have been used to transduce purified human $CD34^+$ BM cells from FA-A and FA-C patients in vitro. In-vitro-derived colonies from these cells were shown to contain the corresponding proviral DNA, using polymerase chain reaction techniques. Limited studies, in which FA-C human marrow cells infected with a FA-C retroviral vector were reintroduced into patients, failed to show engraftment of the transduced cells *(17).* However, this area remains one of active research interest, and a number of groups are currently pursuing preclinical studies of GT in FA.

## 11. FUTURE DIRECTIONS

The cloning of three FA genes *(FANCC, FANCA,* and *FANCG)* has provided an unprecedented opportunity to understand the molecular basis of FA. With these genes, new approaches can be taken to explore the cellular role of the FA proteins. First, the mammalian genes may be used to identify FA gene homologs in yeast or Drosophila. A genetic dissection of the FA gene pathway in one of these organisms may implicate the pathway in a basic cellular function, such as cell growth, cell cycle, or genomic stabilization. Second, the FA genes may be used for the generation of additional mouse models of FA. Cells derived from mice deficient in the *FANCC* gene product exhibit genomic instability, similar to that observed in cell lines derived from FA patients. Similarly, knockouts of the *FANCA* and *FANCG* genes are now possible. Interactions of FA genes could be further analyzed by crossing different knockout strains. Third, the recognition that the three cloned FA proteins interact in a nuclear complex will provide the basis of future biochemical assays. For instance, it will be important to examine the nuclear complex for its ability to bind to chrs or to regulate in vitro DNA repair processes.

The availability of the FA genes will directly impact the diagnosis and therapy of FA. Mutations in FA genes can be directly screened as an adjunct diagnostic procedure to the DEB test. Retroviruses that transduce the FA genes can be used for rapid diagnosis and complementation analysis of primary cells from suspected FA patients. GT studies with autologous peripheral blood CD34 positive stem cells can be initiated for FA patients who do not have sibling-matched histocompatible donors. The observation of somatic reversion in FA provides some reason for optimism regarding the efficacy of GT. Finally, once the molecular basis of the disease is established, more rational therapies or preventive measures may be devised for patients and families with FA.

## ACKNOWLEDGMENTS

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