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Principles of Tumor Suppression

Review

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Molecular genetic studies of familial cancer syndromes identified and defined the recessive nature of tumor suppressor genes and resolved the paradox of why tumors arising in such families exhibited an autosomally dominant pattern of inheritance. Subsequent characterization of tumor suppressor proteins revealed their widespread involvement in sporadic cancers and pinpointed key mechanisms that protect animals against tumor development. We now recognize that tumor suppressor genes regulate diverse cellular activities, including cell cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell specification, differentiation and migration, and tumor angiogenesis. Their study has become a centerpiece of contemporary cancer research.

It has long been recognized that cancers arise as a result of somatic mutations, a concept dramatically reinforced by the demonstration that cellular "protooncogenes," when mutationally deregulated or abnormally overexpressed, contribute to tumor formation (Stehelin et al., 1976). Our understanding that many such genes encode proteins that govern processes of cell proliferation, differentiation, and development, and that mutations affecting their functions constitutively deregulate particular signaling pathways provided us with some of the clearest mechanistic insights about how and why cancer cells misbehave (Bishop and Varmus, 1985). The discovery of genetically dominant, "activated" oncogenes also fueled the idea that a distinct class of "antioncogenes" might oppose their effects and block tumor development. Indeed, experiments involving somatic cell fusion and chromosome segregation had pointed to the existence of genes that could suppress tumorigenicity (Harris et al., 1969; Stanbridge, 1976). Over the last 15 years, many such tumor suppressor genes have been identified (Table 1). Because their cancer-preventive effects usually require the presence of only a single functional gene, prototypic tumor suppressor genes are recessive, requiring "two-hit" inactivation of both alleles (Knudson, 1971, 1973; Comings, 1973). Thus, the earliest attempts to identify them relied on genetic approaches that fingered instances of biallelic gene inactivation, typically in a setting in which one mutated allele was inherited through the germ line and the other was lost somatically. In retrospect, these features define three cardinal properties of "classic" tumor suppressor genes. First, they are recessive and undergo biallellic inactivation in tumors. Second, inheritance of a single mutant allele accelerates tumor susceptibility, because only one additional mutation is required for complete loss of gene function. Hence, a germline mutation can be the underlying cause of a familial cancer syndrome that exhibits an autosomally dominant pattern of inheritance. Third, the same gene is frequently inactivated in sporadic cancers. Beyond this, strict constructionists might argue that a tumor suppressor is a gene that, when restored to activity, can reverse the tumorigenic properties of a cell, a requirement that rarely has been met experimentally. Indeed, given that tumor suppressors can prevent the acquisition of additional deleterious mutations that might otherwise provide cancer cells with a further selective advantage, tumor suppressor gene inactivation might well allow further genetic changes that could confer resistance to their restoration at a later time.

Because many others have reviewed the properties of individual tumor suppressor genes in detail, I focus here on selected members in order to exemplify the scope of their biologic activities. I then touch briefly on the concepts of haploinsufficiency ("one-hit" inactivation) and combinatorial interactions between genes that more subtly provide tumor resistance. Due to limitations of space, I have chosen to emphasize the roles of representative tumor suppressor genes in human cancer, making less frequent references to mouse model systems.

The Checkered History of p53

The path to discovery of the p53 tumor suppressor—the starting point for this review (Finlay et al., 1989)—was filled with more than a few twists and turns. Linzer and Levine (1979) and Lane and Crawford (1979) first detected p53 in complexes with SV40 T antigen. Oren and Levine (1983) cloned p53 cDNA from SV40-transformed cells and reported, as did Robert Weinberg's laboratory, that p53 could collaborate with mutant Ras to transform primary rat embryo fibroblasts (Eliyahu et al., 1984; Parada et al., 1984). On this basis, it was reasonably concluded in the parlance of the time that p53 was an "immortalizing oncogene," a protein that, like Myc or adenovirus E1A, could collaborate with Ras to transform primary rodent cells.

As a harbinger of what was to come, others found that the cellular *p53* gene was rearranged and inactivated in mouse erythroleukemia cells by insertion of the Friend murine leukemia virus into the locus (Mowat et al., 1985). These changes were observed to occur in vivo during the natural course of virus-induced disease, although the precise nature of the selective advantage conferred by *p53* disruption remained unclear. Things began to further unravel when a murine p53 cDNA derived from F9 embryonal carcinoma cells failed to collaborate with Ras in the cotransformation assay but formed foci of transformed cells when mutated (Hinds et al., 1987). Ensuing investigations "call(ed) into question what the correct *p53* wild-type sequence is and whether a wild-

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Table 1. Representative Tumor Suppressor Genes

Gene	Function	Familial Cancer Association	Other Major Tumor Types
p53	Transcription factor	Li-Fraumeni syndrome	>50% of cancers
RB	Transcriptional corepression	Retinoblastoma	Many
INK4a (p16)	Cdk inhibitor (RB activation)	Melanoma	Many
ARF	Mdm2 antagonist (p53 activation)	Melanoma	Many
APC	Wnt/Wingless signaling	Familial adenomatous polyposis	Colorectal cancer
PTCH	Hedgehog signaling (receptor)	Basal cell nevus (Gorlin) syndrome	Medulloblastoma, basal cell carcinoma, rhabdomyosarcoma
SMAD4/DPC4	TGF-β signaling (Transcription factor)	Juvenile polyposis (hamartomas)	Pancreatic and colon cancer
PTEN	Lipid phosphatase (phosphoinositide metabolism)	Cowden syndrome	Glioblastoma, endometrial, thyroid, and prostate cancers
TSC1,2	GTPase activating protein complex (mTOR inhibition)	Tuberous sclerosis (hamartomas)	Renal cell carcinoma (rare), angiofibromas
NF1	GTPase activating protein for Ras	Neurofibromatosis	Sarcomas, gliomas
WT1	Transcription factor	Wilm's tumor	
MSH2 and MLH1	DNA mismatch repair	Hereditary nonpolyposis colorectal cancer (Lynch syndrome)	Endometrial, gastric, ovarian, bladder cancer
ATM	DNA damage sensor (protein kinase)	Ataxia telangiectasia (T-cell lymphoma)	Lymphoreticular malignancies
NBS1	DNA repair, S phase checkpoint control	Nijmegen breakage syndrome (T cell lymphoma)	Lymphoreticular malignancies
CHK2	Protein kinase (G1 checkpoint control)	Li-Fraumeni syndrome	
BRCA1, BRCA2	DNA repair	Familial breast and ovarian cancer	
FA genes	DNA repair, S phase checkpoint	Fanconi Anemia	Acute myelogenous leukemia
VHL	E3 ligase recognition factor for HIFα	Von Hippel-Lindau syndrome	Renal cell carcinoma, cerebellar hemangiosarcoma

type *p53* gene can transform cells in culture" (Finlay et al., 1988). Moreover, the formation of oligomeric complexes between the wild-type and mutant p53 proteins raised the possibility that the mutationally inactivated forms might act in a transdominant manner to inhibit the function of the wild-type protein (as they can!) (Eliyahu et al., 1988; Hinds et al., 1989). Finlay and coworkers (1989) concluded this chapter with their landmark paper demonstrating that p53 can act as a suppressor of transformation.

Recognizing that *p53* was deleted in human colorectal cancers, analysis of the second *p53* allele in tumor cells showed that it had sustained mutations, implicating *p53* loss as a driving force (Baker et al., 1990). Mutations of *p53* were soon documented in many other forms of sporadic cancer and were revealed to be a causative genetic factor in patients with the familial Li-Fraumeni cancer susceptibility syndrome (Malkin et al., 1990). Intriguingly, the demonstration that p53 is a sequence-specific DNA binding protein was made only later (Kern et al., 1991). The wild-type protein was soon revealed to be induced by DNA damage and to cause G1 phase arrest, suggesting that p53 performs a cell cycle "checkpoint" function that guards cells against genotoxic insult (Kastan et al., 1991). We now appreciate that p53 is a homotetrameric transcription factor that is activated in response to many forms of cellular stress, including irradiation, hypoxia, drug-induced genotoxic damage, and even oncogene activation (Prives, 1998) (Figure 1). In turn, p53 orchestrates a global transcriptional response that either counters cell proliferation or, more dramatically, induces apoptosis. Its reputation as a tumor suppressor is secure, as p53 is now recognized to be the singly most frequently inactivated gene in human cancers (Olivier et al., 2002).

RB, the First "Classic" Tumor Suppressor

Backing up a bit, the cardinal features of tumor suppression were first exemplified in studies of retinoblastoma and Wilm's tumor before p53 was identified. Alfred Knudson's perspective as a pediatrician and cancer geneticist sparked his interests in childhood malignancies in which hereditary features were manifest. He articulated the idea that retinoblastoma might be caused by two mutations, one of which might be inherited through the germ line (Knudson, 1971, 1973). Families transmitting such mutations would manifest a pattern of dominant inheritance, so that affected children would develop disease early in life that frequently affected both eyes. In nonhereditary cases requiring two de novo mutations, the disease would be rare, develop later, and be unilateral almost without exception. In 1976, several groups, Knudson's among them, used banding techniques to demonstrate interstitial deletions of chromosome 13q14 in retinoblastoma, leading to speculation that "the *RB* gene" might reside at this locus (Knudson

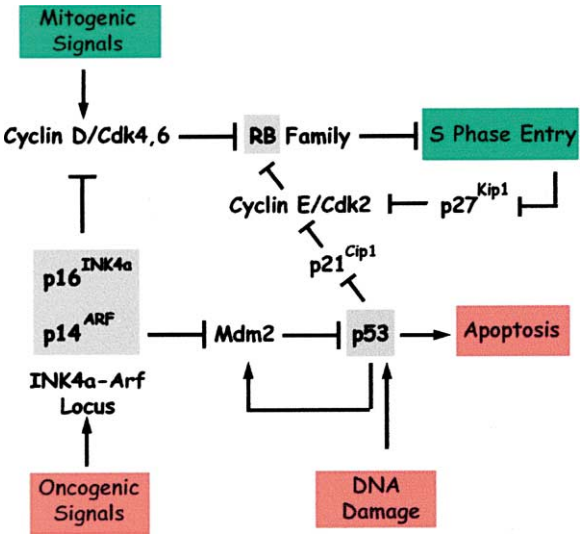

Figure 1. RB and p53 Regulate Cell Cycle Checkpoint Controls

Mitogenic signals activate cyclin D-dependent kinases, which phosphorylate RB and RB family proteins (p107 and p130) to facilitate entry into S phase (top). The Cdk2 inhibitor, $p27^{Kip1}$, expressed at high levels in quiescent cells, is phosphorylated by cyclin E-Cdk2 in late G1 phase and degraded as cells enter S phase. Constitutive oncogenic signals can activate the *INK4a/ARF* locus. By antagonizing the activity of cyclin D-dependent kinases, $p16^{INK4a}$ activates RB and prevents entry into S phase. Mdm2 is a p53-inducible gene that normally acts to terminate the p53 response. The $p14^{ARF}$ protein inhibits Mdm2 to induce p53, leading either to p53-dependent apoptosis or to induction of the Cdk2 inhibitor $p21^{Cip1}$, inhibition of cyclin E/Cdk2, and RB-dependent cell cycle arrest. As cells exit the division cycle, $p27^{Kip1}$ is stabilized and reaccumulates. DNA damage signals activate p53 via ARF-independent pathways.

et al., 1976; Francke and Kung, 1976; Noel et al., 1976). Patients with familial tumors who carried constitutional chromosome 13q14 deletions were observed to have a 50% reduction of esterase D activity in their normal cells but no remaining activity in their tumor cells, indicating that *esterase D* and *RB* were closely linked. One such patient had no detectable deletion of chromosome 13q14 in her normal cells but had a missing chromosome 13 in her tumor (Benedict et al., 1983). Hence, surrogate marker analysis had detected a submicroscopic first hit, and subsequent loss of chromosome 13 had likely inactivated the second *RB* allele. Using restriction fragment length polymorphisms, Cavenee et al. (1983) localized the affected region much more precisely and, importantly, were able to formally conclude that inherited and sporadic cases of retinoblastoma lost the same critical allelic sequences. Using probes from the region, the *RB* gene was soon cloned (Friend et al., 1986).

We now recognize that *RB* is part of a gene family that includes two other members, *p107* and *p130*, which collectively corepress genes that regulate programs governing cell cycle progression, apoptosis, and differentiation. Like p53, the RB family proteins exert much of their growth suppressive control during the G1 phase of the cell division cycle. RB family proteins physically interact with transcription factors, the best characterized of which are the E2Fs. These play key roles in coordinately regulating many genes required for DNA metabolism and replication and whose expression is required to enable cells to enter the DNA synthetic (S) phase of the cell cycle (Nevins, 2001; Trimarchi and Lees, 2002). When bound at E2F-responsive promoters, RB family proteins help to repress gene expression by recruiting histone deacetylases and chromatin-remodeling factors to these loci (Harbour and Dean, 2000). By contrast, phosphorylation of RB family proteins by mitogen-activated, cyclin-dependent kinases cancels RB-mediated repression. Importantly, this provides a signaling pathway linking extracellular cues to the molecular apparatus that controls the initiation of DNA replication in mammalian cells (Weinberg, 1995) (Figure 1). Loss of *RB* weakens these controls, dissociating the cell cycle machinery from extracellular signals, dampening the ability of proliferating cells to exit the division cycle, and compromising the execution of RB-dependent differentiation programs in certain tissues.

INK4a-ARF: Regulating RB and p53

Given the roles of RB and p53 in tumor suppression, it would be expected that other gene products that act epistatically to regulate their expression or functions might also be frequent targets of deregulation in cancer cells. Prominent among these is the INK4 family of Cdk inhibitors, which block the ability of the cyclin D-dependent kinases, Cdk4 and Cdk6, to phosphorylate and thereby inactivate RB's growth suppressive functions. The founding member, $p16^{INK4a}$ (Serrano et al., 1993) is inactivated in cases of familial melanoma (Kamb et al., 1994) and has since been found to be disabled in many tumor types (Ruas and Peters, 1998). Intriguingly, the *INK4a* locus encodes a second, structurally and functionally unrelated protein that is also a potent tumor suppressor. Two alternative transcripts, initiated at separate promoters and incorporating sequences from distinct first exons (designated 1α and 1β), are each spliced to common downstream exon sequences that are translated in alternative reading frames. Whereas the transcript that contains exon-1α sequences specifies $p16^{INK4a}$, the mRNA incorporating exon-1β sequences encodes the *a*lternative *r*eading *f*rame (ARF) protein, designated $p14^{ARF}$ in humans and $p19^{Arf}$ in the mouse (Quelle et al., 1995). Equally surprising, the ARF protein activates p53 by binding directly to the p53 negative regulator Mdm2 and protecting p53 from Mdm2-mediated degradation (Sharpless and DePinho, 1999; Sherr, 2001) (Figure 1). Thus, one locus encodes two proteins, $p16^{INK4a}$ and $p14^{ARF}$, that functionally interface with RB and p53, respectively. Despite their intimate linkage, the two genes are independently regulated, targeted differentially by various signals, and separately silenced and mutated in various forms of cancer. We still do not understand what purported selective advantage might have led to their economy of gene organization during evolution, particularly since deletions involving *INK4a-ARF* simultaneously compromise the functions of both RB and p53.

The *INK4a-ARF* promoters respond to sustained hyperproliferative signals. As a singular example, constitutive and simultaneous activation of multiple signaling pathways by oncogenic Ras induces both *Ink4a* and *Arf*, thereby activating both Rb and p53 and arresting cell proliferation (Serrano et al., 1997). In contrast, the

loss of *Ink4a-Arf* extends the replicative capacity of cells in culture, contributes to their establishment as continuously proliferating cell lines, and sensitizes them to transformation by oncogenic Ras (Serrano et al., 1996; Kamijo et al., 1997). In short, RB, p53, $p16^{INK4a}$, and $p14^{ARF}$ form part of a signaling network that monitors mitogenic signaling and restrains aberrant growth-promoting signals from driving cell cycle progression inappropriately (Figure 1). Inactivation of this signaling network occurs in most, if not all, forms of human cancer.

Ligand-Dependent Signaling and Tumor Suppression

A series of genes known to affect positional identity, tissue patterning, and proliferation during embryonic development are also targets of mutations in cancer cells. Included in this group are genes such as *APC*, *PTCH*, *SMAD4/DPC4*, *PTEN*, *TSC1,2*, *NF1*, and *WT1* (Table 1). Many such proteins mediate the flow of information from ligand-dependent cell surface receptors to families of nuclear transcription factors that govern both developmental and proliferative programs. Misregulation of these genes might affect a cell's progression toward a terminally differentiated, nonproliferating state, thereby allowing additional mutations to accumulate until a fully tumorigenic phenotype emerges.

Cell specification within certain tissues is a continuous process that occurs throughout the life of an organism. For example, the processes governing the steady formation of blood cells and the rapid renewal of epithelial cells in the intestine and skin rely on functions of tissue stem cells that can either self-renew or differentiate (Weissman, 2000). Although it has been argued that between four and seven mutations must occur to transform a normal cell into a tumor cell (Hanahan and Weinberg, 2000), the proliferation and terminal differentiation of renewing cell populations in blood, intestine, skin, and other organs, occur on a temporal scale that may be too rapid to accommodate the multiple mutations required for tumorigenesis. Hence, others have speculated that cancers arise from mutations in longer resident, tissue stem cell populations, thereby shifting the balance between self-renewal and differentiation and misspecifying cell fates and numbers within a target organ (Taipale and Beachy, 2001; Reya et al., 2001).

Mutations inactivating the *APC* gene are responsible for familial adenomatous polyposis (FAP), a disease in which hundreds of adenomatous polyps arise in the colon and rectum of affected individuals, and where colorectal cancer invariably follows relatively early in the lives of untreated patients. The responsible gene was assigned to chromosome 5q21 and identified by positional cloning (Groden et al., 1991; Kinzler et al., 1991). Although germline mutations in *APC* account for the early appearance of colorectal tumors in FAP patients, somatic mutations affecting both *APC* alleles also occur as early events in >80% of sporadic, nonhereditary colorectal cancers as well (Powell et al., 1992).

The APC protein interacts with β-catenin (Rubinfeld et al., 1993; Su et al., 1993), a key component of the Wnt/Wingless signaling pathway (Figure 2A). In the absence of a Wnt signal, β-catenin associates with a protein scaffold complex containing APC and axin, in which the various components undergo phosphorylation by glycogen synthase kinase-3β (GSK-3β). Phosphorylated β-catenin is recognized by an E3 ubiquitin protein ligase that marks it for degradation by the proteasome (Figure 2A, left). Wnt signaling inhibits the enzymatic activity of GSK-3β, stabilizes β-catenin, and enables it to associate with TCF/LEF protein complexes to activate the transcription of target genes, including those like c-*Myc* and cyclin D1 (*CCND1*) that promote proliferation (Figure 2A, right) (Polakis, 1997; Fearnhead et al., 2001). Disruption of the mouse *Tcf7/2* gene, whose product forms transcriptionally active complexes with β-catenin, depletes intestinal epithelial stem cells, highlighting the role of this signaling pathway in normal intestinal development (Korinek et al., 1998).

Mutations that disable *APC* terminate the polypeptide chain prematurely, canceling its ability to negatively regulate β-catenin turnover and constitutively activating this signaling pathway (Morin et al., 1997; Korinek et al., 1997). Mutant forms of β-catenin that are resistant to phosphorylation and proteasomal turnover have also been detected in colorectal cancers (Morin et al., 1997). Apart from its role in transcriptional control, β-catenin associates independently with E-cadherin and aids in cell adhesion. E-cadherin loss has also been observed in epithelial tumors, and its ability to suppress cell transformation may similarly result from limiting the amount of β-catenin available for transcriptional signaling (Gottardi et al., 2001). Indeed, experimental perturbations that prevent β-catenin from entering the nucleus limit the proliferation of colon cancer cells (Shih et al., 2000).

Hedgehog (Hh) proteins (Figure 2B) also play important roles in patterning decisions, acting from early embryogenesis onward to specify the body plan and organ development. In mammals, three Hedgehog ligands [Sonic (S), Desert (D), and Indian (I)] are differentially expressed in various tissues, although Shh is the best characterized and most ubiquitous. Genetic studies of Hh signaling in *Drosophila* first pinpointed the key signaling components (Murone et al., 1999; Taipale and Beachy, 2001), but their exact biochemical functions remain unclear, and mammalian homologs of each of the *Drosophila* proteins have not been identified. The key upstream target of the signaling pathway in flies is the Ci transcription factor, for which three GLI paralogs have been identified in mammals. In the absence of Hh signaling, Ci/GLI is thought to repress transcription of target genes, whereas ligand stimulation reverses this process (Figure 2B). The Hh receptor, Patched, is an upstream negative regulator of the signaling pathway. The *PTCH* gene was identified as a tumor suppressor in Gorlin's syndrome (Gorlin, 1987), where its inactivation is associated with development of basal cell carcinoma (BCC) and medulloblastoma (Johnson et al., 1996; Hahn et al., 1996a). As for FAP, in which the loss of *APC* predisposes to the appearance of multiple adenomatous polyps, persons inheriting a dysfunctional *PTCH* allele develop numerous BCCs. *PTCH* mutations have also been identified in a significant percentage of patients with sporadic BCC and medulloblastoma. Again, the appearance of many differentiated precursor lesions in BCC is consistent with the idea that tissue stem cells may be targeted.

TGF-β signaling (Figure 2C) regulates the expression

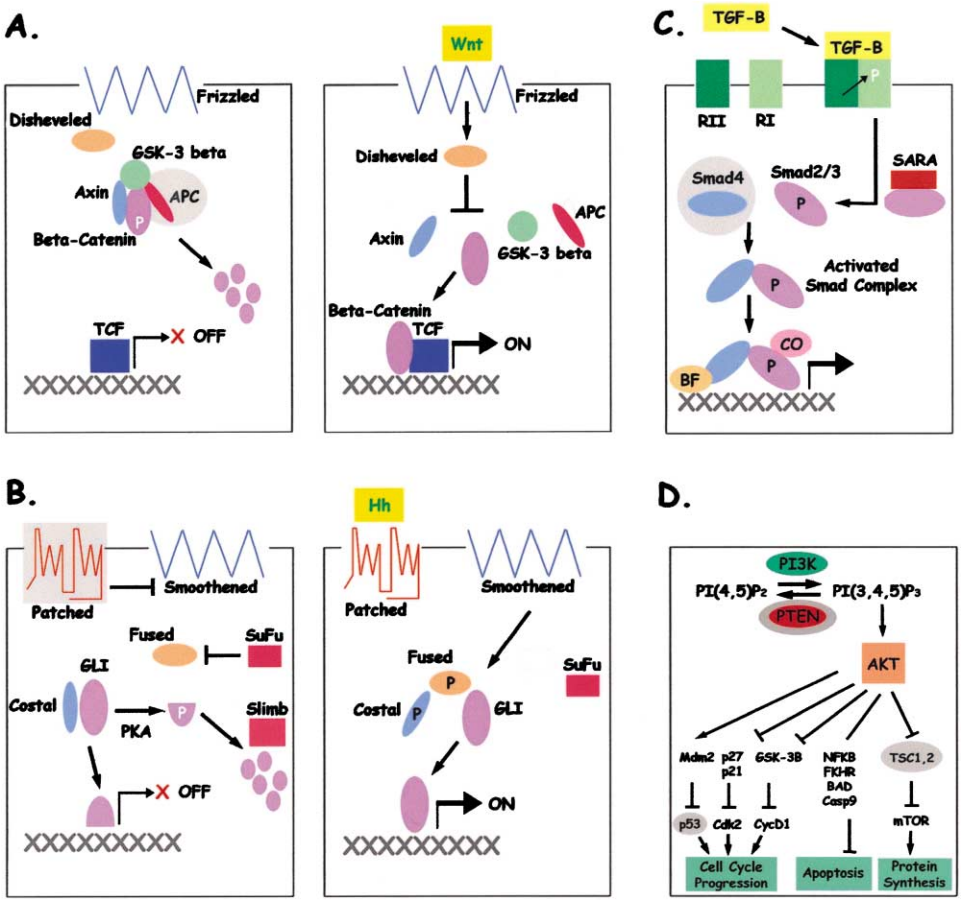

Figure 2. Tumor Suppressor Proteins Regulating Ligand-Mediated Signaling Pathways

The tumor suppressor proteins APC, PTCH, Smad4/DPC4, PTEN, TSC1,2, and p53 depicted in different images are highlighted by gray shading.

(A) Wnt signaling. In the absence of Wnt ligand (left image), β-catenin binds to a destruction complex containing APC, Axin, and GSK-3β. Phosphorylation of β-catenin facilitates its recognition by a unbiquitin-conjugating E3 ligase (SCF^{TRCP}) that targets it for proteasomal degradation. When Wnt binds to its receptor (right image), signaling via Frizzled and Disheveled prevents β-catenin phosphorylation and destruction. Import of β-catenin and its binding to TCF/LEF transcription factors induces expression of Wnt target genes (adapted from Polakis, 1997; Fearnhead et al., 2001). Inactivation of APC mimics the effects of the Wnt signal.

(B) Hedgehog signaling. Most of the molecules involved in transmitting the hedgehog (Hh) signal have been genetically identified in *Drosophila*, and some as yet have no counterparts in mammalian cells. In the absence of Hh (left image), the Patched receptor (PTCH) negatively regulates Smoothened (Smo). The Ci transcription factor in flies (homologous to three GLI genes in mammals) is tethered by the kinesin-like molecule, Costal-2, to a microtubule-anchored cytoplasmic complex. Following its phosphorylation by protein kinase A (PKA), Ci is recognized by a Slimb-containing ubiquitin E3 ligase and degraded. It is inferred that Gli is subject to similar control. A fragment of Ci is imported into the nucleus where it binds to DNA to repress target genes. Binding of Hh to Patched relieves suppression of Smo, disrupts the cytoplasmic complex, and stabilizes Ci/GLI. Hh signaling also antagonizes a suppressor of the Fused kinase (SuFu), leading to phosphorylation (P) of different components and facilitating Ci/GLI activation (adapted from Murone et al., 1999). Loss of *PTCH* constitutively activates the pathway.

(C) TGF-β signaling. Binding of TGF-β to type I (RI) and type II (RII) receptors triggers phosphorylation (P) of RII and its serine-threonine protein kinase activity. Receptor (R)-Smads 2 and 3 are phosphorylated and relieved from negative regulation by SARA. The R-Smads bind Smad4 to form a complex that is imported into the nucleus and mediates expression of TGF-β-responsive genes. Specificity is dictated by other DNA binding factors (BF) and by either coactivators or corepressors (CO) that determine the nature of the transcriptional response (adapted from Siegel and Massagué, 2003). Disruption of *SMAD4/DPC4* renders cells resistant to inhibition by TGF-β.

(D) Phosphoinositide 3-kinase (PI3K) signaling. PI3K, which is activated via many growth factor receptors, catalyzes the conversion of phosphatidylinositol (4,5) bis-phosphate [$PI(4,5)P_2$] to PI $(3,4,5)P_3$. The activity of PI3K is opposed by the PTEN lipid phosphatase. $PI(3,4,5)P_3$ recruits the AKT (PKB) kinase to the plasma membrane where it undergoes phosphorylation by PDK1 (not shown) and activation. AKT phosphorylates substrates that foster cell cycle progression, cancel apoptosis, and facilitate translation of capped mRNAs. The tuberous sclerosis complex [TSC1 (hamartin) and TSC2 (tuberin)] antagonizes the function of a G protein (Rheb, not shown) whose activity is required for activity of the mTOR kinase and its ability to promote translation (adapted from Sulis and Parsons, 2003; Tee et al., 2003). Loss of PTEN upregulates signaling.

of hundreds of target genes that can coordinately restrain epithelial cell proliferation (Siegel and Massagué, 2003). This endows TGF-β with the ability to govern complex biological effects, including tissue morphogenesis, angiogenesis, cell migration and adhesion, and apoptosis. The direct phosphorylation of Smad proteins by ligand-activated TGF-β receptors facilitates the assembly of heterooligomeric Smad transcription factor complexes that bind, together with other specificity factors, to the promoters of TGF-β-responsive genes. This

can result in downregulation of certain genes, such as c-*Myc*, that are required for cell proliferation, as well as the induction of others, such as those encoding Cdk inhibitors, that slow cell cycle progression (Shi and Massagué, 2003). Smad-4, a component of the active transcription complex, was first identified in the guise of a tumor suppressor gene deleted in pancreatic cancer (*DPC4*) (Hahn et al., 1996b). Mutations in *SMAD4/DPC4* were subsequently identified in colon cancers (Schutte et al., 1996), and germline mutations are associated with familial juvenile polyposis (Howe et al., 1998). In turn, mutations affecting the TGF-β Type II receptor have also been detected in colon cancers (Grady et al., 1999). Hence, desensitizing epithelial cells in the pancreas and colon to the growth inhibitory properties of TGF-β strongly contributes to carcinogenesis.

Many receptor systems activate the serine/threonine-specific Akt protein kinase (protein kinase B) whose activity enhances protein synthesis, cell growth (mass), cell cycle progression, and survival (Figure 2D). Akt activation depends upon phosphoinositides produced by PI 3-kinase (Cantley, 2002), a process negatively regulated by the lipid phosphatase, PTEN (Maehama and Dixon, 1999; Sulis and Parsons, 2003). PTEN is ubiquitously expressed in eukaryotes, and its inactivation in somatic cells results in constitutively elevated levels of $PI(3,4,5)P_3$. Complete loss of *Pten* in flies and mice leads to early embryonic lethality, and the ability of hypomorphic *Akt* alleles to rescue the lethality of *Pten* null *Drosophila* embryos emphasizes the importance of Akt as an effector of this pathway (Stocker et al., 2002). Germline mutations of *PTEN* cause four rare human diseases with similar clinical features (Sulis and Parsons, 2003), one of which (Cowden syndrome) is associated with malignant tumor development. Homozygotic inactivation of *PTEN* occurs frequently in glioblastoma multiforme, endometrial, and advanced prostate cancers. Given the pleiotropic effects of this signaling pathway in influencing cell growth (size) and proliferation, as well as in countering the effects of other proapoptotic tumor suppressors, such as p53 (Figure 2D), it is not surprising that *PTEN* inactivation is a frequent event in many forms of human cancer.

Cumulatively, these four examples reveal the diversity of biochemical mechanisms that can be used to derail cell growth control signaling pathways. They also illustrate how the developmental history of particular cell types determines the identity of the pathways that are disrupted in different tumor types.

The DNA Damage Response and Genome Instability

Persons carrying germline mutations affecting gene products that sense or repair DNA damage are particularly prone to cancer. Hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) represents a family of disorders stemming from mutations in genes required for DNA mismatch repair (Ionov et al., 1993; Fishel et al., 1993). HNPCC accounts for 1%–3% of all cases of colorectal cancer, in which patients inheriting a germline mutation develop colon cancers associated with loss of the remaining wild-type allele (Chung and Rustgi, 2003). Although not all individuals inheriting a mismatch repair gene defect will develop cancer, disease penetrance is high with a lifetime risk of about 80%. Errors in DNA replication involve either single base mispairing or unfaithful copying of microsatellite DNA sequences composed of mononucleotide or dinucleotide repeats. If the latter errors are uncorrected, microsatellite instability ensues. The mismatch repair system includes MutS (*MSH2*, *MSH3*, *MSH6*) and MutL (*MLH1*, *MLH3*, *PMS1*, *PMS2*) genes involved in mismatch recognition and repair, respectively. The MSH2 protein assembles with either MSH6 or MSH3 to recognize single or larger loops of mismatched DNA, respectively, which are then excised by the MLH1/PMS1 complex (Figure 3A). Most cases (95%) of HNPCC arise from mutations in *MLH1* and *MSH2* (common to both pathways) (Chung and Rustgi, 2003). Approximately 15% of sporadic colorectal tumors also exhibit microsatellite instability most commonly due to epigenetic inactivation of *MLH1*. Importantly, disruption of the mismatch repair system leads to a "mutator" phenotype in which resulting genetic instability ultimately targets other oncogenes and tumor suppressor genes to induce tumor formation (Lengauer et al., 1998; Loeb et al., 2003).

Like many epithelial tumors, most colon cancers, whether of familial or sporadic origin, exhibit a high degree of aneuploidy resulting from inappropriate segregation of chromosomes during mitosis. Normal cells do not progress through mitosis until chromosomes are appropriately aligned on the mitotic spindle, but the "spindle checkpoint" that monitors the fidelity of this process is frequently disrupted in those colon cancers that exhibit chromosomal, as opposed to microsatellite, instability (Cahill et al., 1998). Other mitotic miscues or defective cytokinesis can also lead to ploidy changes. In the presence of functional p53, such cells arrest in G1 phase, but in its absence, they enter S phase and soon become aneuploid after subsequent divisions (Lanni and Jacks, 1998; Nigg, 2002). Aneuploidy in advanced cancers can also result from telomere dysfunction, which leads to end-to-end chromosome fusions and fusion-bridge-breakage cycles that induce p53-dependent apoptosis (Artandi et al., 2000). Again, p53 loss enables such cells to survive, resulting in the outgrowth of tumors that exhibit many unbalanced translocations. Although genes that regulate mitotic progression, cytokinesis, or telomerase activity in somatic cells can protect against tumor progression, they have not as yet been implicated in familial cancer syndromes and are not listed in Table 1.

Individuals carrying mutations in a number of other DNA damage response genes are also highly tumor prone. Genes regulating such processes that have been identified in inherited disorders predisposing to various forms of cancer include *ATM*, *NBS1*, *BRCA1*, *BRCA2*, *CHK2*, and the Fanconi anemia (FA) complex (Figure 3B).

The ATM kinase, whose loss of function results in ataxia telangiectasia, acts as a sensor of DNA damage, being activated specifically in response to double-stranded DNA breaks (Figure 3B). Patients with defective ATM function are exquisitely sensitive to ionizing radiation, although they show no such sensitivity to certain other DNA damage signals such as UV irradiation, which is sensed by the ATM-related kinase ATR. ATM belongs to an evolutionarily conserved family of proteins

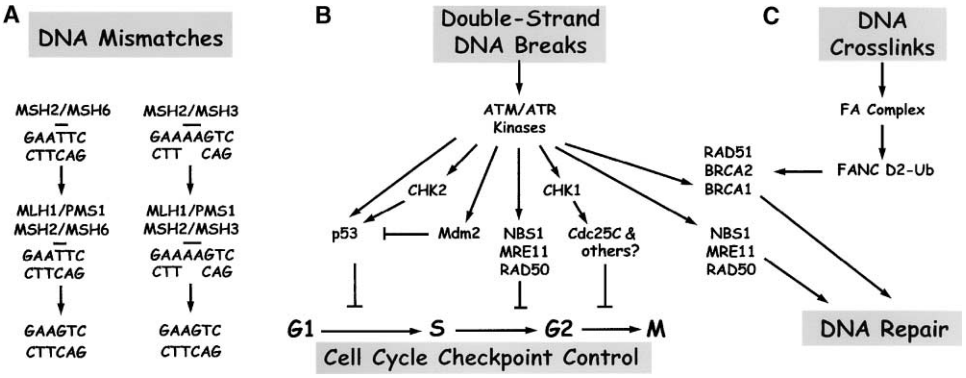

Figure 3. Tumor Suppressors Involved in the DNA Damage Response

(A) MSH complexes recognize replication errors resulting in single nucleotide (left) or di/trinucleotide (right) mismatched pairing and recruit MLH complexes that excise mispaired nucleotides, leading to repair (adapted from Chung and Rustgi, 2003).

(B) Double-strand DNA breaks activate the ATM and ATR kinases, which phosphorylate numerous substrates involved in both checkpoint control and DNA repair. Phosphorylation of p53 and Mdm2 induce G1 phase arrest. NBS1 phosphorylation inhibits late origin firing and prevents progression through S phase. Phosphorylation of Cdc25C inhibits the activity of cyclin B/Cdk1 to prevent entry into mitosis. Phosphorylation of NBS1 and BRCA1 trigger their recruitment to DNA damage foci to facilitate homologous and nonhomologous modes of DNA repair. Activation of BRCA1 also induces p53 (not shown) (adapted from Kastan and Lim, 2000).

(C) DNA crosslinks activate the Fanconi anemia complex (consisting of the Fanc A, C, E, F, and G proteins) which ubiquitylate Fanc D2, enable its interaction with BRCA2 (equivalent to Fanc D1), and facilitate repair via homologous recombination (adapted from D'Andrea and Grompe, 2003). As indicated, NBS1 enters into complexes with MRE11 and RAD50, whereas BRCA1 binds to BRCA2, which interacts with the RecA homolog RAD 51.

that also includes the DNA-dependent protein kinase DNA-PK_{CS}; each of these plays distinct roles in responses to DNA damage. Following DNA double-strand breakage, ATM phosphorylates a number of proteins (including p53, CHK2 kinase, NBS1, BRCA1, and FANCD2) to initiate both cell cycle checkpoint responses and DNA repair processes (Figure 3B). Activation of virtually all of the ATM kinase in a cell can be induced by very few double-strand DNA breaks, implying either that ATM is upregulated in response to global damage-induced changes in chromosome structure or through some other amplifying mechanism (Bakkenist and Kastan, 2003). In turn, activation of the ATM substrates p53, CHK2, and NBS1 inhibits cell proliferation, presumably allowing cells an opportunity to repair damaged DNA. Indeed, *CHK2*, like *p53*, is inactivated in some Li-Fraumeni families (Bell et al., 1999), whereas *NBS1* is mutated in the Nijmegen breakage syndrome, a disease that closely mimics ataxia telangiectasia (Shiloh and Kastan, 2001). Although ATR controls a broader spectrum of DNA damage responses than does ATM, both *ATR* and *CHK1* are essential genes and, hence, not tumor suppressors.

Homozygous mutations in *ATM* and *NBS1* predispose to lymphomas relatively early in life (Shiloh and Kastan, 2001). Their involvement most likely reflects the requirement for gene rearrangements during early lymphoid development, in which repair errors increase the frequency of chromosomal translocations that are the hallmarks of T and B cell malignancies. *ATM* is somatically inactivated in nonfamilial lymphoreticular malignancies, including T cell prolymphocytic leukemia, B-cell chronic lymphocytic leukemia, and mantle cell lymphoma, further highlighting its role in protecting developing lymphocytes from aberrant genomic rearrangements.

The BRCA genes (in a complex with RAD51) and NBS1 (in a complex with MRE11 and RAD50) play key functions in homologous recombination and nonhomologous end joining, respectively, two distinct processes used to repair DNA breaks (Figure 3B). Inherited *BRCA1* and *BRCA2* mutations are associated with familial breast and ovarian cancers, requiring somatic loss of function of the second allele for tumors to arise. Familial syndromes account for 5%–10% of all breast cancer cases and are typified by early adult onset and a predisposition to multicentric and bilateral disease. Although most familial breast cancers are due to mutations of one of the two *BRCA* genes, such tumors can also arise in Li-Fraumeni patients and in those with inherited *PTEN* deficiency (see above). The exact biochemical functions of the BRCA proteins remain unclear, although a physical association of BRCA1 with BRCA2 and the binding of the latter to RAD51 (a bacterial RecA homolog) at chromosomal foci of DNA damage strongly implicate the complex in repairing double-strand breaks by homologous recombination (Scully and Livingston, 2000; Jasin, 2002). Whether BRCA genes are also required for nonhomologous end joining remains controversial.

Strikingly, mutations of *BRCA* genes have not been observed in sporadic breast or ovarian cancers. If BRCA loss confers only a weak selective advantage to certain cell types so that hereditary tumors arise in middle age or later, there may be little opportunity for sporadic homozygous inactivation. Because biallelic *BRCA1* loss activates cell cycle checkpoints that trigger proliferative arrest or apoptosis (Scully and Livingston, 2000), any tumors that emerge are likely to have acquired mutations in genes that regulate these processes. Possibly, there may be a restricted temporal and developmental window, during adolescence for example, when epithelial cells in the breast or ovary that sustain such mutations might not be eliminated (Elledge and Amon, 2002). Whereas overexpression of cyclin D1 and Her2/Neu, which occurs frequently in sporadic breast cancers, is

rarely seen in *BRCA1*-inactivated tumors, *p53* mutations are much more common (Rosen et al., 2003). Such findings suggest a different molecular etiology for the generation of *BRCA1* null and sporadic breast tumors, likely reflecting the efficacy by which cell cycle checkpoint controls counteract preexisting genomic instability.

Fanconi anemia complementation groups define eight different genes, one of which (*FANCD1*) appears to be identical to *BRCA2* (D'Andrea and Grompe, 2003). This implicates FA proteins in the process of homologous recombination, consistent with observations that FA patients exhibit defective repair of interstrand DNA crosslinks (Figure 3C). FA patients are predisposed to many types of cancer with acute myelogenous leukemia being the most common malignancy. Interestingly, ATM-mediated phosphorylation of one of the Fanconi anemia proteins (FANCD2) also plays a role in attenuating DNA replication by preventing late origin firing (the S phase checkpoint). Such findings further emphasize the intimate interplay between cell cycle checkpoint control and DNA repair during the DNA damage response.

Protein Ubiquitination, Turnover, and Suppression of Tumor Angiogenesis

Tumor growth and metastasis depend upon angiogenesis, a process by which quiescent vasculature is induced to sprout new capillaries. Avascular tumors cannot expand in size because of a lack of blood supply and oxygen, but their ability to switch on the production of angiogenic factors explains how they can trigger neovascularization, maintain oxygen-dependent ATP production, expand, and metastasize (Hanahan and Folkman, 1996).

Activation of hypoxia-inducible transcription factor complexes (HIFs) in response to oxygen deprivation drives the expression of many genes important for angiogenesis, red cell production, and glycolysis. These include key vascular and hematopoietic growth factors, such as vascular endothelial growth factor (VEGF), erythropoietin, platelet-derived growth factor (PDGF), and TGFα (Semenza, 1999). HIF subunits are transcribed constitutively. However, HIF-α proteins undergo prolyl hydroxylation under normoxic conditions, targeting them for ubiquitination by the von Hippel-Lindau (VHL) protein-containing E3 ligase and for subsequent destruction by the proteasome (Kaelin, 2002). Within this multiprotein complex, the VHL protein provides the recognition motif for prolyl hydroxylated HIF-α (Figure 4). Under hypoxic conditions, unhydroxylated HIF-α subunits are not recognized by VHL and are therefore stabilized, enabling them to form transcriptionally active complexes with HIF-β subunits to drive transcription of hypoxia-induced promoters. Loss of *VHL* function results in constitutive HIF stabilization and predisposes to particular tumors, such as renal clear cell carcinomas, cerebellar hemangioblastomas, retinal angiomata, and pheochromocytomas, all of which have a major vascular component. Tumor formation in the hereditary setting (VHL syndrome) stems from a loss of heterozygosity at the *VHL* locus with retention of the mutant *VHL* allele. Somatic *VHL* mutations, deletions, and gene silencing can also be observed in sporadic renal cell carcinomas and cerebellar hemangiosarcomas.

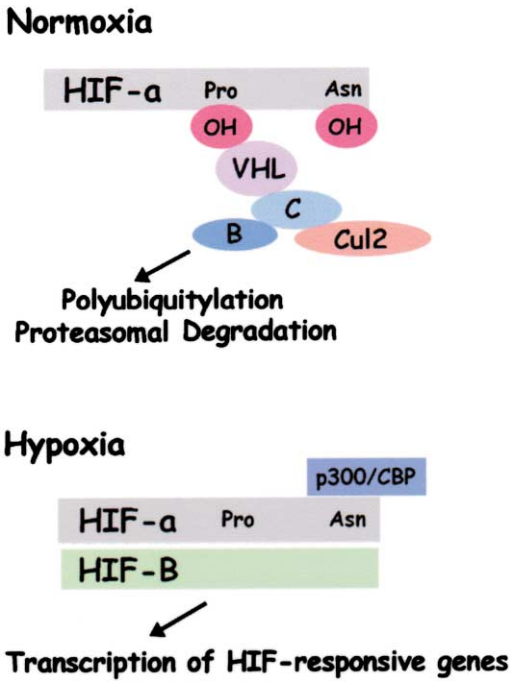

Figure 4. VHL Regulates HIF Activity to Govern Responses to Hypoxia

Under normoxic conditions (top) HIF-α subunits are hydroxylated on both prolyl and asparaginyl residues. Prolyl hydroxylation facilitates HIF-α recognition by the VHL-containing E3 ligase containing Cul2, and elongins B and C, whereas asparaginyl hydroxylation prevents binding of p300/CBP coactivators. During hypoxia, unhydroxylated HIF-α subunits are stabilized, bind to HIF-β, assemble with coactivators, and activate transcription (adapted from Kaelin, 2002).

Key features exemplified by studies of VHL are that most, if not all, of the systems that prevent tumor development are exquisitely sensitive to levels of protein expression, and that much of the relevant circuitry is controlled by ubiquitin-mediated proteolysis. Many pathways governing protein degradation are subverted in cancer cells. For example, the rates of turnover of β-catenin and Ci/Gli transcription factors (Figure 2) are crucial in establishing the thresholds of Shh and Wnt required for productive signaling. The p53 negative regulator Mdm2 is an E3 ubiquitin ligase that is negatively regulated by Arf binding and ATM phosphorylation. Disruption of these signaling pathways can be affected by mutation of motifs ("degrons") necesssary for degradation (e.g., VHL, β-catenin) or by altered expression of E3 ubiquitin ligases (e.g., Mdm2 amplification). The fact that changes in protein turnover can either promote or interfere with a signaling pathway suggests that other recently discovered components that regulate ubiquitination will eventually be revealed to act as oncogenes or tumor suppressors.

The Basis of Tissue Specificity?

Despite our increasing understanding of the cellular functions of many tumor suppressor proteins, we do not know why the loss of a particular gene contributes to tumors in some tissues but not others. Understanding the involvement of these proteins in specific biochemi-

cal pathways provides some insights. For example, Wnt and Shh signaling are central to developmental programs that affect the formation of the intestine and cerebellum (Korinek et al., 1998; Taipale and Beachy, 2001; Wechsler-Reya and Scott, 2003), and disruption of these pathways predisposes to colorectal cancers and medulloblastoma, respectively. But things are not so tidy. Although inactivation of $p16^{INK4a}$ or RB, which function in the same biochemical pathway, is observed in many different tumor types, the fact that small cell lung cancers preferentially acquire *RB* mutations while lung adenocarcinomas sustain *INK4a* loss points to a far greater degree of cell type specificity that defies explanation. In the case of the DNA damage response, genes such as *ATM* and *NBS1* specifically detect double-strand breaks, and their contribution to lymphoid malignancies logically reflects a need for fidelity of repair during the processes of gene rearrangement that generate the immune repertoire. But, why then does loss of BRCA1 or BRCA2, which seem to play central roles in checkpoint and repair responses to DNA damage, specifically predispose to breast and ovarian cancers? It may prove that a functional redundancy of key signaling pathways can protect many tissues from dire consequences of tumor suppressor gene inactivation, whereas particular cell types that lack such compensatory mechanisms are at greater risk to undergo transformation. An improved understanding of tumor suppressor functions, particularly as they relate to processes of tissue-specific expression, cell differentiation, and tissue development will require much more investigation.

Haploinsufficiency—When Only One Hit Is Enough

Because of their recessive nature, the traditional approach to identifying tumor suppressor genes has been to pinpoint small chromosomal regions of loss-of-heterozygosity (LOH) that occur in particular tumor types (and frequently in familial cancers), to narrow the critical region by deletion mapping, and finally to search the intact homologous chromosomal segment for mutated genes whose functions can be demonstrated to protect against cancer development. However, this strategy will fail under circumstances in which the second allele is epigenetically silenced or when the targeted gene is haploinsufficient for tumor suppression, a situation in which functional loss of only one allele confers a selective advantage for tumor growth (Cook and McCaw, 2000; Quon and Berns, 2001). Only a few haploinsufficient tumor suppressors have been identified so far, but this may not reflect their actual number—just the difficulties in finding them. Indeed, the ease of pinpointing prototypic tumor suppressors within regions of LOH seems to have diminished over time, pointing to the possibility that a larger haploinsufficient class exists but still eludes detection (Quon and Berns, 2001; Balmain et al., 2003).

One well-defined instance of haploinsufficiency in the mouse involves the Cdk inhibitor, $p27^{Kip1}$ (Fero et al., 1998). Animals lacking one copy of *Kip1* develop tumors spontaneously late in life and are highly sensitive to tumor induction by chemical carcinogens; however, the tumors that arise retain the normal *Kip1* allele, which encodes a fully functional protein. Although tumors arise faster in *Kip1* null animals, these data argue that a reduced dosage of $p27^{Kip1}$, rather than its absolute absence, can contribute to cancer susceptibility. The latter concept has been reinforced in investigations of human cancers, in which hemizygous loss of *Kip1* and/or reduced levels of protein expression confer a poor prognosis (Blain et al., 2003). There is some evidence that other Cdk inhibitors, including $p21^{Cip1}$, $p57^{Kip2}$, and $p18^{Ink4c}$ might function in this manner, so this might prove to be a general property of this class of proteins.

Several other examples of haploinsufficiency for tumor suppression have been demonstrated (Cook and McCaw, 2000). For example, mice hemizygous for *p53* can develop tumors that retain and express wild-type p53 protein (Venkatachalam et al., 1998). In the absence of a functional *Ink4a* allele, animals lacking a single *Arf* allele are strikingly more prone to melanoma development (Krimpenfort et al., 2001). *Pten* haploinsufficiency in mice accelerates tumor progression, and loss of one *PTEN* allele with preservation of the second is also common in human tumors, although its role in these settings remains controversial (Sulis and Parsons, 2003). A recent study of mice engineered to express a mutant series of Pten alleles with incrementally decreasing activity and penetrance argues strongly that Pten is haploinsufficient for tumor suppression in prostate cancer (Trotman et al., 2003). In short, bona fide tumor suppressors such as *p53*, *Arf*, and *PTEN* may well manifest haploinsufficient effects, particularly when combined with collaborating mutations affecting additional oncogenes or tumor suppressors.

Tumor Susceptibility and Resistance

The vast majority of human cancers show no obvious familial inheritance, and it has been suggested that multiple, low penetrance genes segregating in the human population confer cancer susceptibility and resistance to environmental carcinogens (Balmain et al., 2003; Loeb et al., 2003). These low penetrance genes might only act combinatorially in a dosage-dependent manner to determine cancer predisposition, incrementally affecting processes such as carcinogen metabolism, DNA repair efficiency, inflammation, and the immune response to provide relative degrees of tumor resistance.

Finding this class of genes presents a great challenge. In principle, a set of polymorphisms used to define haplotypes in genes of potential interest can be associated with cancer development, but this approach requires that the appropriate interacting loci are suspected and tested. Mouse models of cancer susceptibility provide a way to pinpoint such genes by enriching for combinations of alleles that control a specific disease phenotype, something that is not feasible in humans. Interbreeding cancer-resistant and -sensitive mouse strains can provide information about the number and chromosomal location of genes involved in susceptibility to various forms of cancer (Balmain and Nagase, 1998). Such mapping is time consuming and labor intensive, but rapid advances in the human and mouse genome projects have helped to accelerate progress in detecting modifier loci.

In Short...

Mechanistic insights stemming from studies of tumor suppressor genes, like those gained from work on oncogenes, have helped to provide us with a conceptual framework for understanding the genetic basis of cancer.

- Prototypic tumor suppressors are recessive. Their involvement in familial cancer syndromes implies that they are highly penetrant. At the top of the list is p53, being implicated in more than 50% of human cancers. In principle, the signaling pathways controlled by genes like p53 that are so frequently disrupted in cancer are ideal targets for therapeutic intervention.
- Tumor suppressor genes govern a wide range of normal cellular activities. Although their raison d'etre is not to protect animals against cancer, their involvement in cell cycle checkpoint control, mitogenic signaling pathways, protein turnover, DNA damage, hypoxia, and other stress responses illustrates the broad spectrum of cell-autonomous processes that can be deregulated in cancer cells.
- Despite our appreciation of their biochemical functions, we have no deep understanding of why many tumor suppressors are associated with certain cancers but not others. Possibly, a lack of functional redundancy of signaling pathways in particular cell populations might aggravate the consequences of tumor suppressor gene inactivation. Moreover, there may be restricted temporal and spatial windows throughout organismal development and tissue renewal during which mutations that disable these genes can lead to tumor formation. Elucidating the basis of this tissue specificity presents a significant future challenge.
- Some nonclassical tumor suppressors are haploinsufficient. This category likely includes many more genes than those so far recognized, but they are much more difficult to identify.
- Lower penetrance genes that determine cancer incidence and response to conventional therapy are likely to be even more numerous and collectively may be very important in governing the appearance of sporadic cancers in the human population. These polygenic determinants of cancer would best be called tumor susceptibility and resistance genes, since mutations involving any one of them alone are unlikely to reveal visible phenotypes. In turn, the pathways that these genes regulate may prove poorer targets for therapeutic intervention than those governed by more highly penetrant tumor suppressor genes.

I end where I began. The biochemical role of p53 was discerned less than 15 years ago. There are 30,000 journal citations that refer to this one tumor suppressor gene in the National Library of Medicine. Enough said!

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