Cell

The Regulation of *INK4/ARF* in Cancer and Aging

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DOI 10.1016/j.cell.2006.10.003

Loss of the *INK4a/ARF/INK4b* locus on chromosome 9p21 is among the most frequent cytogenetic events in human cancer. The products of the locus—p15^{INK4b}, p16^{INK4a}, and ARF—play widespread and independent roles in tumor suppression. Recent data also suggest that expression of p16^{INK4a} induces an age-dependent decrease in the proliferative capacity of certain tissue-specific stem cells and unipotent progenitors. Here, we discuss the regulation and role of p16^{INK4a}, ARF, and p15^{INK4b} in cancer and aging.

Regulated cellular proliferation is required for mammalian homeostasis, but uncontrolled proliferation is the hallmark of cancer. Therefore, an important question in cancer biology is how a tumor suppressor protein distinguishes malignant from physiological growth? This is no mean feat. Physiologic growth can have many of the properties associated with the worst malignancies: it can be rapid, disordered, unexpected, and invasive (for example, an inflammatory response to a wound). Cells harboring oncogenic mutations in vivo often respond by activating expression of the *INK4a/ARF/INK4b* locus, which encodes critical tumor suppressor proteins. This indicates that cells understand at a very early stage of transformation that something has gone awry. Understanding in molecular terms what regulates the *INK4a/ARF/INK4b* locus should help to elucidate the properties a would-be cancer cell recognizes as malignant. This question has taken on additional importance given recent evidence that at least one product of the locus, p16^{INK4a}, also contributes to the decline in replicative potential of self-renewing cells during aging. These observations suggest the provocative but unproven notion that mammalian aging results in part from the beneficial efforts of tumor suppressor proteins to interdict cancer. In this review, we discuss the regulation and function of *INK4a/ARF/INK4b* with regard to cancer and aging.

The INK4 Family and ARF

The *INK4a/ARF/INK4b* locus (also known as *CDKN2a* and *CDKN2b*) is deleted in a wide spectrum of tumors including melanoma, pancreatic adenocarcinoma, glioblastoma, certain leukemias, non-small cell lung cancer, and bladder carcinoma. In a small 35 kb stretch of the human genome, the locus encodes three related genes (*ARF* [also known as *p19^{ARF}* and *p14^{ARF}*], *p15^{INK4b}*, and *p16^{INK4a}*) that encode distinct tumor suppressor proteins (reviewed in Sherr, 2000). Whereas *p15^{INK4b}* has its own open reading frame that is physically distinct, *p16^{INK4a}* and *ARF* have different first exons that are spliced to a common second and third exon. Although exons 2 and 3 are shared by *p16^{INK4a}* and *ARF*, the proteins are encoded in alternative reading frames. As a consequence p16^{INK4a} and ARF are not isoforms and do not share any amino acid homology (Figure 1).

The INK4 class of cell-cycle inhibitors p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INKd} (the latter not to be confused with p19^{ARF}) are homologous inhibitors of the cyclin-dependent kinases, CDK4 and CDK6, which promote proliferation. In particular, p15^{INK4b} and p16^{INK4a} are 85% similar at the amino acid level and little biochemical distinction has been made between these proteins (reviewed in Sharpless, 2005). The binding of the INK4 proteins to CDK4 and CDK6 induces an allosteric change that abrogates the binding of these kinases to D-type cyclins, inhibiting CDK4/6-mediated phosphorylation of retinoblastoma (Rb) family members. Thus, expression of p15^{INK4b} or p16^{INK4a} maintains Rb-family proteins in a hypophosphorylated state, which promotes binding E2F to effect a G1 cell-cycle arrest.

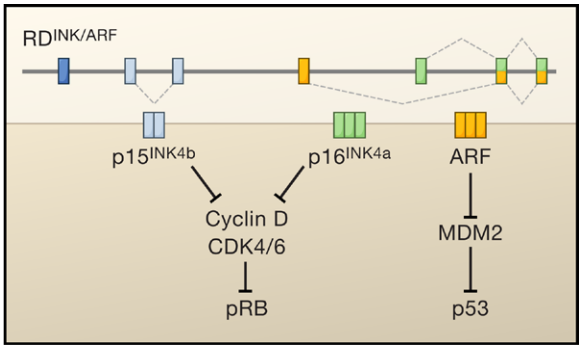

Figure 1. The *INK4a/ARF/INK4b* Locus
The *INK4a/ARF/INK4b* locus encodes three genes within 35 kilobases: *ARF*, *p15^{INK4b}*, and *p16^{INK4a}*. Members of the INK4 family of cyclin-dependent kinase inhibitors bind to and inactivate CDK4/6. ARF inhibits MDM2, resulting in p53 stabilization. RD$^{INK/ARF}$ indicates a newly discovered origin of replication 5′ to *p15^{INK4b}* that participates in the transcriptional silencing of the *INK4a/ARF/INK4b* locus.

The tumor suppressor activity of ARF is largely ascribed to its ability to regulate p53 in response to aberrant growth or oncogenic stresses such as c-MYC activation (discussed below). ARF binds to and inactivates the MDM2 protein, and MDM2 in turn negatively regulates p53. One mechanism that has been proposed to explain how MDM2 regulates p53 is that it acts as an E3 ubiquitin ligase to target p53 for proteasomal degradation. Although strong biochemical and genetic evidence link ARF and p53 in tumor suppression, several p53-independent functions of ARF have also been reported. For example, Arf expression is required to induce vascular regression in the developing eye, which does not appear to require p53 (McKeller et al., 2002), and overexpression of Arf induces a cell-cycle arrest in murine embryo fibroblasts (MEFs) lacking *p53* (Weber et al., 2000). Moreover, ARF has been reported to interact with multiple proteins other than MDM2, including E2F-1, MDMX, HIF-1α, topoisomerase I, MYC, and nucleophosmin (NPM).

Arguably, the best characterized of these interactions is between ARF and NPM (reviewed in Lindstrom and Zhang, 2006). NPM, also known as B23, is a protein that shuttles between the nucleolus and cytoplasm and is involved in several cellular processes including ribosome processing and centrosome duplication. The ARF-NPM interaction modulates ARF protein stability as suggested by the finding of reduced levels in cells lacking NPM. Additionally, it appears that this interaction sequesters ARF in the nucleolus thus preventing it from binding MDM2. Specific point mutations of the C terminus of NPM are noted in ~30% of cases of primary adult acute myelogenous leukemia (AML) (Colombo et al., 2006; Falini et al., 2005). These mutant forms of NPM appear constitutively localized to the cytoplasm but retain the ability to bind ARF. Therefore, these mutations are believed to compromise the ARF-p53 pathway both by decreasing ARF's protein stability and by mislocalizing ARF to the cytoplasm where it is unable to inactivate MDM2 (Colombo et al., 2006; Falini et al., 2005). Although a compelling hypothesis, it is worth noting that NPM functions in many critical cellular processes related to growth and proliferation and therefore the "true" cancer-promoting activities of mutant NPM have not been fully determined.

The unusual genomic arrangement of the *INK4a/ARF/INK4b* locus is assuredly a weakness in our anti-cancer defenses, as it renders three crucial regulators of the RB and p53 tumor suppressor pathways vulnerable to a single, relatively small deletion. This observation can be used to draw one of two opposite conclusions: either tumor formation provides no evolutionary selection pressure (and the overlapping *INK4a/ARF/INK4b* locus is not selected against) or tumorigenesis provides such a strong pressure that an entire suite of tumor suppressor genes has been selected for at the *INK4a/ARF/INK4b* locus to prevent cancer. The finding that polymorphisms of the $p15^{INK4b}/p16^{INK4a}$ homolog of *Xiphophorus* (sword-tail fish) segregate with melanoma susceptibility (Nairn et al., 1996) in this species suggests that INK4 proteins have played such a role in tumor suppression for more than 350 million years. In mammals and birds, therefore, the evolutionarily older, INK4-based system appears to have been further improved by the more recent addition of an ARF-based anti-cancer response. Implicit in the latter interpretation, however, is the notion that the physical proximity and shared sequences of the locus allow $p16^{INK4a}$ and *ARF*, and possibly $p15^{INK4b}$ as well, to sense and coordinately respond to common stimuli in nascent tumor cells. Evidence described in this review suggests that this is in fact the case—the response of the *INK4a/ARF/INK4b* locus efficiently prevents cancers that would result from the daily onslaught of oncogenic mutations suffered by long-lived mammals. Before turning to the crucial question of the in vivo regulation of the locus, we will review some of the evidence supporting this role for *INK4a/ARF/INK4b* products in the suppression of cancer and promotion of aging.

Role in Cancer and Aging

Cancer

As human cancers frequently harbor homozygous deletions of the *INK4a/ARF/INK4b* locus that abrogate expression of all three proteins, significant debate has focused on which member or members of the locus represents the principal tumor suppressor activity located at human chromosome 9p21. Knockout studies of mice specifically deficient for Arf, $p15^{INK4b}$, or $p16^{INK4a}$ have revealed that all three strains are more prone to spontaneous cancers than wild-type littermates, but that each of these single knockouts appears significantly less tumor prone than animals lacking both $p16^{INK4a}$ and Arf (Latres et al., 2000; Sharpless et al., 2004). The finding of synergy between $p16^{INK4a}$ and Arf loss in murine cancers has been established in several tumor-prone models including response to certain carcinogens, melanoma, glioblastoma, and pancreatic cancer (reviewed in Sharpless, 2005; see also Bardeesy et al., 2006). Compound mutant animals lacking $Arf/p15^{INK4b}$, $p15^{INK4b}/p16^{INK4a}$, or the entire *Ink4a/Arf/Ink4b* locus have not yet been reported.

Overexpression of the *Ink4a/Arf/Ink4b* locus in mice also supports its role in tumor suppression. Mice harboring an allele of the *Ink4a/Arf/Ink4b* locus on a bacterial artificial chromosome display modest overexpression of $p16^{INK4a}$, $p15^{INK4b}$, and Arf (the "super-*Ink4/Arf*" mice) (Matheu et al., 2004). These mice demonstrate a 3-fold reduction in the incidence of spontaneous cancers, without an acceleration in the appearance of a few age-induced phenotypes such as kyphosis of the spine. Therefore, the authors suggest that an increase in tumor suppression can be achieved by increased *Ink4a/Arf/Ink4b* activity without increased aging. As cancer is the principal cause of death of mice on this genetic background, however, one might argue that the marked tumor resistance of the super-*Ink4/Arf* mice would also

be expected to increase longevity, yet the super-*Ink4*/*Arf* mice demonstrate a normal life span. Therefore, the fact that the super-*Ink4a*/*Arf* mice do not live longer suggests that increased *Ink4a*/*Arf*/*Ink4b* function and diminished tumor incidence may come at the cost of excess mortality from nonmalignant causes related to aging. Thus, although these data confirm the potent tumor suppressor activity of the *Ink4a*/*Arf*/*Ink4b* locus, we believe they also elegantly demonstrate a tradeoff between overly zealous tumor suppression and accelerated aging.

In human cancers, the goal of defining which protein(s) at the locus represent the relevant tumor suppressor has led to genetic searches of specific tumor types in an attempt to identify specific lesions that target one member of the locus in the absence of mutation of the others. Such analyses make their strongest case for $p16^{INK4a}$. For example, in a few malignancies where recurrent 9p21 deletions have been most carefully mapped, the minimal region of deletion appears to center on exons 1α and 2, but these analyses have not been comprehensive in most tumor types and will certainly soon be improved by ongoing high-resolution, genome-wide efforts at tumor genotyping. Specific somatic loss of $p16^{INK4a}$, through point mutation or small deletion, has been reported in thousands of human cancers (Forbes et al., 2006). Similarly, at least 56 distinct germline mutations targeting only $p16^{INK4a}$, and sparing *ARF* and $p15^{INK4b}$, have been described in unrelated kindreds that are cancer prone (Greenblatt et al., 2003). Finally, $p16^{INK4a}$ was one of the first genes noted to be silenced epigenetically in human cancers, and silencing through promoter methylation is well described at high frequency in numerous types of human cancers (Esteller et al., 2001). Indisputably, $p16^{INK4a}$ is an important suppressor of human cancer.

On the other hand, specific genetic lesions of $p15^{INK4b}$, which do not also inactivate $p16^{INK4a}$ or *ARF*, are not well described. Specific epigenetic silencing by hypermethylation of the $p15^{INK4b}$ gene has been demonstrated in rare glial tumors and certain hematologic neoplasms including leukemia and myelodysplasia (Esteller et al., 2001; Herman et al., 1996; Uchida et al., 1997). In myelodysplasia, $p15^{INK4b}$ hypermethylation has been reported in the absence of $p16^{INK4a}$ hypermethylation and in some of these cases the expression of $p15^{INK4b}$ can be reactivated in response to treatment with inhibitors of DNA methytransferase. Furthermore, because of their overlapping biochemical function, co-deletion of $p15^{INK4b}$ with $p16^{INK4a}$ may be more oncogenic in certain tissues than loss of either alone. Such redundancy might explain why a few malignancies (e.g., T cell acute lymphoblastic leukemia [ALL]) appear to show very high frequencies of homozygous deletion of 9p21 (targeting all three proteins) rather than specific inactivation of $p16^{INK4a}$ or ARF. Therefore, $p15^{INK4b}$ is probably an important suppressor of human cancers too, particularly in the hematopoietic lineages.

In murine cancer, the data show that the loss of Arf is potently oncogenic (Kamijo et al., 1997; Sharpless et al., 2004), but selective inactivation of ARF, in the absence of a concomitant loss of $p15^{INK4b}$ and $p16^{INK4a}$, has only been reported in a small number of cases of human cancer. For example, three distinct germline mutations that do not affect $p16^{INK4a}$ have been described in kindreds with familial melanoma and astrocytoma (Hewitt et al., 2002; Randerson-Moor et al., 2001; Rizos et al., 2001). Additionally, somatic ARF-specific mutations and promoter methylation have been reported in studies of colon cancer (Burri et al., 2001; Esteller et al., 2001), but such specific targeting events appear less common in human cancer than those affecting only $p16^{INK4a}$. Some have argued that the relatively infrequent finding of lesions selectively targeting ARF indicates species differences in the relative importance of $p16^{INK4a}$ and ARF in tumor suppression; that is, Arf is more important in mice, and $p16^{INK4a}$ more important in humans. We, however, suggest that these data can also be explained by a consideration of the biochemical nature of the ARF-MDM2 interaction, which only requires a relatively small portion of the highly basic N terminus of ARF (Korgaonkar et al., 2002). Therefore, missense mutations that disable ARF's principal anticancer activity may be very improbable. Instead, we believe that the human and murine genetic data considered as a whole establish that the *INK4a*/*ARF*/*INK4b* locus encodes at least two ($p16^{INK4a}$ and ARF), and probably three, major human tumor suppressor proteins, although their relative and combinatorial importance in any given tumor type has not been fully resolved.

Aging

Recent evidence from several groups has suggested a role for the senescence machinery in mammalian aging. These experiments were motivated by the seminal observation of Sherr and colleagues (Zindy et al., 1997), later confirmed by several groups (Krishnamurthy et al., 2004; Melk et al., 2004; Nielsen et al., 1999), that the expression of $p16^{INK4a}$ increases markedly with aging in many tissues of rodents and humans. This finding has now been extended to a large number of aging human tissues in health and disease and has led to the proposal that $p16^{INK4a}$ expression could be used as a biomarker of physiologic, as opposed to chronologic, age (Krishnamurthy et al., 2004). Moreover, as aging is characterized in part by a reduced ability of reservoirs of self-renewing tissue stem cells to regenerate lost or damaged cells, this observation has suggested the possibility that an age-induced increase in $p16^{INK4a}$ expression contributes to the decline of replicative potential of certain self-renewing compartments with aging.

Several lines of evidence suggest that $p16^{INK4a}$ expression participates in cell-autonomous aging in vivo. Much of this work to date has studied hematopoietic stem cells (HSCs), the best characterized adult stem cell. Surprisingly, murine HSC number does not necessarily decline with aging, and in some genetic strains, old mice actually harbor an increased number of HSCs based on immunophenotype (de Haan and Van Zant, 1999; Morrison et al., 1996). Transplantation studies from old

donors into young recipients, however, have established that a decline in HSC function does occur with aging, that this decline is cell autonomous, and that it is not rescued by ectopic telomerase expression (Allsopp et al., 2003; Morrison et al., 1996). Additionally, genotoxic stimuli such as ionizing radiation or busulfan exposure, which induce a durable compromise of HSC function, are known to potently induce $p16^{INK4a}$ expression in HSCs (Meng et al., 2003; Wang et al., 2006). Oxidative stress has also been suggested to induce an *Ink4a/Arf*-dependent decline in HSC function (Ito et al., 2004, 2006). The *Ink4a/Arf* locus has been identified as a possible effector of a cell-autonomous decline in HSC function, as loss of both $p16^{INK4a}$ and Arf, but not Arf alone, increases the ability of HSCs to serially transplant and repopulate irradiated recipient mice (Stepanova and Sorrentino, 2005). In aggregate, these results demonstrate that HSCs carry the memory of prior aging, serial transplantation, and other age-promoting genotoxic exposures and suggest that $p16^{INK4a}$ expression is a mechanism whereby HSCs recognize that they are old.

These studies, however, do not directly address the question of whether $p16^{INK4a}$ plays a causal role in the age-induced decline of replicative function in HSCs or other self-renewing compartments in vivo. Recent work from our lab and collaborators using $p16^{INK4a}$-deficient and overexpressing mice to study self-renewal in three distinct tissues (HSCs, neural stem cells [NSCs], and pancreatic islets) has suggested that $p16^{INK4a}$ expression is one cause of aging in these tissues (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). These particular self-renewing tissues were chosen for analysis because expression of $p16^{INK4a}$ markedly increases in each with aging, and these tissues appear to require cdk4 or cdk6 for proliferation. In all three cell types, $p16^{INK4a}$ deficiency partially abrogated the age-induced decline in proliferation. This decline in proliferation was accompanied by functional effects. For example, HSCs from old, but not young, $p16^{INK4a}$-deficient animals demonstrated an enhanced ability to serially transplant or competitively repopulate irradiated recipient mice compared to wild-type cells from littermate mice. Moreover, $p16^{INK4a}$ deficiency increased neural progenitor function and neurogenesis in old but not young mice. Lastly, older $p16^{INK4a}$-deficient mice demonstrated an age-dependent enhancement of islet regeneration after chemical ablation of β cells when compared to littermate wild-type mice. Enhanced islet regeneration correlated with resolution of diabetes and improved survival. Therefore, these data from disparate systems suggest that $p16^{INK4a}$, in part, promotes aging by limiting proliferation and self-renewal. A caveat to these results, however, is that they derive from the study of mice with a germline deletion of $p16^{INK4a}$. Therefore, some of the age-promoting effects of $p16^{INK4a}$ expression observed in these systems may result from mechanisms that are not cell autonomous. Future studies in mice using tissue-specific inactivation of $p16^{INK4a}$ will address whether $p16^{INK4a}$ induces aging in a given tissue in a cell-autonomous manner.

The effects of $p16^{INK4a}$ loss were remarkably consistent across three self-renewing tissues of vastly different biologic properties. Expression of $p16^{INK4a}$ exerted age-dependent antiproliferative effects in true stem cells (HSCs and NSCs) as well as unipotent progenitors (pancreatic β cells). Therefore, $p16^{INK4a}$ appears capable of promoting aging in disparate tissues that are developmentally distinct. Secondly, in no organ studied (bone marrow, brain, or endocrine pancreas) did $p16^{INK4a}$ loss completely abrogate the effects of aging. For example, brains from mice lacking $p16^{INK4a}$ still demonstrated reduced neurogenesis with aging, and β cells from old $p16^{INK4a}$ knockout mice demonstrated less regenerative capacity than those of young wild-type mice. Therefore, $p16^{INK4a}$-independent aging occurs in these compartments. The molecular basis of this $p16^{INK4a}$-independent aging is unknown, although Arf is one obvious candidate as its expression is potently antiproliferative and increases in many murine tissues with aging to a degree comparable to $p16^{INK4a}$. Further experiments will be required to determine which age-induced phenotypes involve $p16^{INK4a}$ and *Arf* and which are *Ink4a/Arf* independent.

The Regulation of *INK4a/ARF/INK4b* Expression

Given the importance of products of the *INK4a/ARF/INK4b* locus in tumor suppression and aging, regulation of the locus has been an area of intense study. Evidence suggests that *INK4a/ARF* expression increases at an early stage of tumorigenesis (reviewed in Sherr, 2000), but the precise stimuli relevant to cancer that induce the expression of the locus are unknown. Additionally, the molecular characteristics of aging that lead to increased $p16^{INK4a}$ expression are similarly unclear. Therefore, it has not been possible to determine if the beneficial signals that serve to prevent malignancy by inducing *INK4a/ARF/INK4b* early in the life of a would-be cancer cell are the same undesirable signals that promote aging by inducing $p16^{INK4a}$. Work in these fields, however, has proceeded at a rapid pace, and several recent developments have enhanced our understanding of the control of *INK4a/ARF/INK4b* expression.

Although some stimuli are known that selectively regulate $p16^{INK4a}$ but not ARF, or vice versa, the two genes are largely coregulated in rodents (Krishnamurthy et al., 2004; Zindy et al., 1997). That is, increases in $p16^{INK4a}$ in response to neoplastic signals or with aging are often mirrored by a comparable increase in Arf expression. In contrast, coregulation of $p16^{INK4a}$ and ARF in human cells is not well established. For example, in cultured human cells, senescence generally occurs in the setting of increased expression of $p16^{INK4a}$, but not ARF, and enforced RAS-RAF activation also appears to induce only $p16^{INK4a}$ in human cells (Huot et al., 2002; Michaloglou et al., 2005; Munro et al., 1999). Likewise, with human aging, only an increase in $p16^{INK4a}$ expression has been reported (Melk et al., 2004; and our own unpublished observations), although studies of ARF expres-

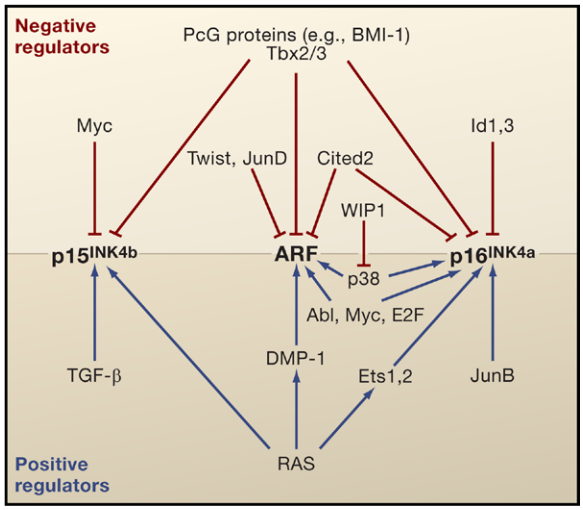

Figure 2. Regulation of the INK4a/ARF/INK4b Locus
Positive and negative regulators of *p15*^{INK4b}, *ARF*, and *p16*^{INK4a} expression. Some proteins specifically influence the expression of a single locus member whereas others influence multiple genes. Repressors (red) are shown in the upper part of the figure and activators (blue) are shown in the lower half.

sion in human aging have not been comprehensive. Expression of p15^{INK4b} does not correlate with that of p16^{INK4a} and/or Arf across a large panel of normal rodent tissues (Krishnamurthy et al., 2004; Zindy et al., 1997), but induction and repression of p15^{INK4b} has been noted in response to a few signaling events, such as RAS activation, that also induce *INK4a/ARF* expression (Figure 2 and described below).

Numerous noxious stimuli have been reported to induce p16^{INK4a} and/or ARF expression in vitro and in vivo. In particular, expression of p16^{INK4a} increases after several DNA-damaging stimuli including UV light (Pavey et al., 1999; Piepkorn, 2000), oxygen radicals (Chen et al., 2004; Ito et al., 2004, 2006; Stockl et al., 2006), ionizing radiation (Meng et al., 2003; Wang et al., 2006), chemotherapeutic agents (Meng et al., 2003; Robles and Adami, 1998), and telomere dysfunction (Jacobs and de Lange, 2004). It is important to note, however, that in most of these systems, detectable induction of p16^{INK4a} occurs 2 to 4 weeks after the DNA-damaging insult (see for example Jacobs and de Lange, 2004; Robles and Adami, 1998; Wang et al., 2006). Moreover, the induction of p16^{INK4a} in response to these stressors is often associated with MAPK activation (Bulavin et al., 2004; Ito et al., 2006; Iwasa et al., 2003). This delayed response of p16^{INK4a} compared to the very rapid response of p53 to DNA damage may explain the relationship between p16^{INK4a} and p21^{CIP}, a transcriptional target of p53, in serially passaged cultures of human fibroblasts undergoing senescence in response to telomere dysfunction. Under these circumstances, peak expression of p21^{CIP} precedes that of p16^{INK4a} by a few weeks (Stein et al., 1999), and the expression of the two cell-cycle inhibitors do not colocalize in individual cells of such cultures (Herbig et al., 2004). Stimuli that induce Arf have been best characterized in murine embryo fibroblasts and include oncogene activation as well as the act of culture itself. Regulation of p15^{INK4b} in response to stressors has not been as thoroughly studied. Increased expression of p15^{INK4b} is not seen with aging (Krishnamurthy et al., 2004; Zindy et al., 1997) but has been noted with TGF-β signaling (Reynisdottir et al., 1995) and in some models of RAS-induced senescence (Collado et al., 2005) (Figure 2).

As for molecular regulators, several tumor-relevant and/or stress signaling pathways are known to influence expression of the *INK4a/ARF/INK4b* locus (Figure 2). Arguably the best studied of these molecular signals are those that induce the ERK MAPK pathway through activating mutations of RAS and its downstream effector, B-RAF. A few models have been suggested as to how RAS activation might lead to increased *Ink4a/Arf* expression including ERK-mediated activation of Ets1/2 to induce p16^{INK4a} (Ohtani et al., 2001) and Jun-mediated activation of the transcription factor DMP1 to induce ARF expression (Sreeramaneni et al., 2005). The functional importance of RAS-RAF signaling to p16^{INK4a} and ARF is supported by the frequent finding of homozygous *INK4a/ARF* deletion in melanoma, the majority of which harbor mutations of N-RAS or B-RAF.

Additionally, a few repressors of *INK4a/ARF/INK4b* expression have been identified. For example, the T box proteins (e.g., Tbx2 [Jacobs et al., 2000]) and the polycomb group (PcG) genes (BMI-1, Cbx7, Mel18) have been reported to repress all three genes (*p16* ^{Ink4a}, *p15*^{Ink4b}, and *ARF*) (Gil et al., 2004; Jacobs et al., 1999). *Bmi1* deficiency in mice is associated with failure to maintain diverse self-renewing stem cells (e.g., HSCs and NSCs), which can in large part be rescued by *Ink4a/Arf* deficiency (Bruggeman et al., 2005; Jacobs et al., 1999; Molofsky et al., 2003, 2005; Park et al., 2003). In contrast, homeotic transformations of the axial skeleton noted in mice lacking *Bmi1* are not rescued by loss of *Ink4a/Arf* (Jacobs et al., 1999). The relationship of PcG complexes to *INK4a/ARF/INK4b* expression has been particularly provocative as BMI-1 is required for stem cell maintenance of HSCs and NSCs, and the ability of these complexes to create heritable epigenetic marks might suggest a nongenetic mechanism whereby cells could carry the memory of prior aging-related exposures. Nonetheless, it has not been possible to show a consistent decline in *Bmi1* mRNA levels with aging, and the precise mechanism whereby BMI-1 represses the *INK4a/ARF/INK4b* locus is unknown.

Oncogenic stress has been associated with *Ink4a/Arf* expression, and several molecules (e.g., MYC, ABL, AKT) that induce growth and proliferation have been reported to regulate one or more products of the *INK4a/ARF/INK4b* locus. At least two mechanisms whereby such proliferative signals induce *INK4a/ARF/INK4b* expression have been suggested. First, the E2F tran-

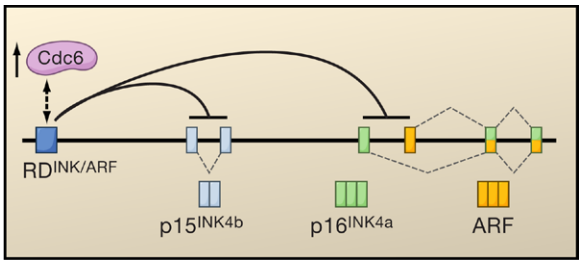

Figure 3. CDC6 Repression of the *INK4a/ARF/INK4b* Locus
Interaction of CDC6 with the origin of replication $RD^{INK/ARF}$ results in the recruitment of other members of the pre-replication complex and histone deacetylases, resulting in the heterochromatinization of the $p15^{INK4b}$, *ARF*, and $p16^{INK4a}$ promoters and silencing of gene expression.

scription factors that regulate the expression of numerous genes critical to the cell cycle have been attractive candidates in this regard. Both the Arf and $p16^{INK4a}$ promoters harbor putative E2F binding sites, and overexpression of E2F1 induces Arf activation (Bates et al., 1998; DeGregori et al., 1997). In accord with these findings, a recent report has demonstrated in MEFs that the binding of E2F3b to the Arf promoter represses Arf, whereas the binding of the "proliferative" E2Fs (E2F1 and E2F3a) enhances Arf expression (Aslanian et al., 2004). These findings suggest a mechanism by which Arf expression is coupled with the cell-cycle machinery that regulates S phase entry. A second link between cell-cycle control and expression of the *INK4a/ARF/INK4b* locus has been suggested by the recent finding of a CDC6-mediated coupling between DNA replication and *INK4a/ARF/INK4b* activation (see below). Whether these mechanisms function independently or in concert has not been resolved.

Although many genetic alterations and stimuli regulate the mRNA expression of the *INK4a/ARF/INK4b* locus (Figure 2), less is known about the posttranslational regulation of its protein products. Both $p16^{INK4a}$ and Arf have been reported to be polyubiquitinated on nonlysine residues in their N termini by yet to be defined E3 ubiquitin ligases (Ben-Saadon et al., 2004; Kuo et al., 2004). Ubiquitination of Arf occurs efficiently in cells lacking MDM2 and p53, implying that neither is involved in its ubiquitination. Further work is required to establish the importance of these posttranslational events in tumor suppression.

Silencing of the *INK4a/ARF/INK4b* Locus by Cdc6

A particularly intriguing recent finding with regard to *INK4a/ARF/INK4b* regulation suggests a coordination of transcription at the locus and DNA replication. Although DNA replication and transcription are generally considered distinct processes, coupling of the processes has been described in yeast. In *S. cerevisiae*, the transcriptional silencing of mating loci is mediated by the binding of a silencer protein complex (SPC) to specific DNA origins of replication (Fox and McConnell, 2005). However, some of the protein subunits that comprise the SPC complex differ from the complex involved in DNA replication. Furthermore, some of the dual functions of common proteins involved in both DNA replication and transcriptional silencing are separable as shown by studies of spontaneous revertants and complementation studies in yeast (Fox and McConnell, 2005). Therefore, the processes of DNA replication and mating loci silencing partially, but not completely, overlap in yeast. Such coupling of DNA replication and transcriptional silencing has not been previously described in vertebrates.

Gonzalez and colleagues have now reported a similar coordination between silencing of the *INK4a/ARF/INKb* locus and DNA replication. The authors identified a DNA replication origin ($RD^{Ink/ARF}$) in close proximity to the *INK4a/ARF/INK4b* locus that appears to transcriptionally repress $p15^{INK4b}$, $p16^{INK4a}$, and ARF expression in a manner dependent on CDC6 (Gonzalez et al., 2006) (Figure 3). In DNA replication, CDC6 interacts with the origin recognition complex and recruits factors that unwind the DNA helix. The authors first showed that heterochromatinization of $RD^{Ink/ARF}$ induced by RNA interference (RNAi) against complementary genomic DNA in cells (Kawasaki and Taira, 2004; Morris et al., 2004) led to transcriptional repression of the *INK4a/ARF/INKb* locus. In addition, they showed that CDC6, along with other members of the pre-recognition complex, specifically bound to $RD^{Ink/ARF}$. Finally, the authors found that ectopic expression of cdc6 was associated with reduced expression of $p15^{INK4b}$, Arf, and $p16^{INK4a}$ and the recruitment of histone deacetylases to the $RD^{Ink/ARF}$ as well as to the promoters of $p16^{INK4a}$ and Arf. Therefore the authors hypothesized that cdc6 expression might regulate both DNA replication and transcription at $RD^{Ink/ARF}$. The authors provided further functional data of this silencing: Expression of cdc6 in MEFs significantly increased foci formation and enhanced transformation by oncogenic RAS. Importantly, neither short-hairpin RNA (shRNA) to $RD^{Ink/ARF}$ nor cdc6 overexpression had noticeable effects on the transformation of MEFs lacking *Ink4a/Arf*, suggesting that the oncogenic effects of cdc6 are mediated specifically through repression of the *Ink4a/Arf/Inkb* locus. Lastly, in an immunohistochemical analysis of human non-small cell lung cancers, there was an inverse correlation between CDC6 and $p16^{INK4a}$ protein expression, a result consistent with the model that CDC6 functions as an oncogene in human lung cancer by repressing $p16^{INK4a}$. In aggregate, these findings suggest a novel molecular connection between DNA replication and *INK4a/ARF/INK4b* transcription. It remains to be seen how this mechanism of regulation interacts with other known repressors or activators of *INK4a/ARF/INK4b* expression.

The Functions of the INK4a/ARF/INK4b Proteins

Although we believe that the principal biochemical activities of the INK4 proteins and ARF are well understood, how these proteins prevent cancer and

promote physiologic aging is a matter of debate. A leading candidate in this regard, however, is the induction of senescence, a specialized form of growth arrest. Senescence is distinguished from other forms of growth arrest in that it is generally permanent and is associated with characteristic morphologic alterations, enzymatic activities (SA-β-galactosidase), and novel changes in chromatin architecture (SA-heterochromatic foci) (reviewed in Campisi, 2005). Although, as stated, the relative senescence-promoting activity of $p16^{INK4a}$ and ARF may differ in humans versus mice or by cell type, the activation of the *INK4a/ARF* locus is intimately associated with the induction of senescence in most systems. The increase in $p16^{INK4a}$ and/or ARF expression in cells harboring oncogenic lesions such as ABL, MYC, or RAS activation is thought to constitute an important barrier to cancer and has been termed oncogene-induced senescence (Serrano et al., 1997). Oncogene-induced senescence has recently been described in vivo in preneoplastic tissues of both the mouse and human (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Gray-Schopfer et al., 2006; Michaloglou et al., 2005). This collection of papers convincingly demonstrates an oncogene-induced growth arrest in vivo (with the characteristics of senescence) in lymphocytes, melanocytes, and epithelia of the lung and prostate. The expression of senescence markers in human nevi (cutaneous moles) is particularly provocative as it suggests that these extremely common cutaneous lesions would become melanomas if not for the activation of the senescence machinery. This finding indicates that the *INK4a/ARF* locus exerts a constant protective effect even in young humans.

A weakness of the senescence hypothesis has been a lack of understanding as to how a cell would discern cues for normal physiologic growth from signals for oncogenic proliferation. The work of Satyanarayana and colleagues (Satyanarayana et al., 2004) provides a clue as to how this might occur. In this work, the authors demonstrated that the induction of senescence by classical stimuli required concomitant ERK stimulation to efficiently induce senescence. This observation suggests that senescence requires in essence two things: signaling induced by a strong cellular stress (such as telomere dysfunction or oxygen radicals) and some coincident period of signaling to promote proliferation (such as ERK activation). This combination appears key to the induction of *INK4a/ARF* expression and senescence. Additionally, the induction of senescence appears to be a relatively slow process compared to the onset of growth arrest. For example, using a cell line in which $p16^{INK4a}$ expression can be controlled, Dai and Enders have shown that $p16^{INK4a}$ expression causes growth arrest within 24 hr of induction but does not cause senescence unless expression of $p16^{INK4a}$ is maintained for 6 days (Dai and Enders, 2000). Therefore, senescence can be considered a response of cellular desperation after a prolonged period of mixed signals including signals for both stress and growth.

Importantly, however, $p16^{INK4a}$, Arf, and $p15^{INK4b}$ need not induce senescence to prevent cancer or contribute to aging. For example, increased $p16^{INK4a}$ expression in stem cell compartments with aging may merely attenuate self-renewal by decreasing the frequency of cell-cycle entry, in the absence of permanent growth arrest. Along the lines of this model, a novel anticancer function for $p16^{INK4a}$ has been recently suggested by McDermott and colleagues (McDermott et al., 2006). Using cultures of primary human mammary epithelial cells (HMECs), the authors show that *$p16^{INK4a}$* inactivation results in centrosome duplication leading to genomic instability. The investigators showed that knockdown of $p16^{INK4a}$ in HMECs treated with the reversible S phase cell-cycle inhibitor hydroxyurea (HU) results in an increase in supernumerary centrosomes and genomic abnormalities at metaphase. Likewise, re-expression of $p16^{INK4a}$ in HU-treated HMECs lacking $p16^{INK4a}$ abrogated the formation of cells with supernumerary centrosomes. Therefore, this work suggests an additional mechanism whereby $p16^{INK4a}$ expression could decrease transformation in proliferating, and therefore nonsenescent, cells.

Future Questions

We believe the tools of mouse genetics have more to teach us about the *Ink4a/Arf/Ink4b* locus. For example, it will be important to determine the phenotypes of *$p15^{INK4b}$/$p16^{INK4a}$*, *$p15^{INK4b}$/Arf*, and *Ink4a/Arf/Ink4b* compound knockout mice to understand if there is cooperation in tumor suppression between $p15^{INK4b}$ and the products of the *Ink4a/Arf* locus. Undoubtedly, high-resolution tumor genotyping in an unbiased and genome-wide manner will supplement murine genetic studies to refine our understanding of the tumor suppressor roles of the various products of the *INK4a/ARF/INK4b* locus in human cancers. Moreover, the majority of knockout experiments performed to date have utilized mice with germline deficiencies of the products of the *Ink4a/Arf/Ink4b* locus. A limitation of this approach, however, is the possibility of developmental compensation, particularly with regard to the many known regulators of the cell cycle. For example, compensation among the RB family members is well described (Sage et al., 2000), and in particular, we have noted pronounced alterations in the expression of $p15^{INK4b}$ and $p16^{INK4a}$ in INK4-deficient MEFs (M. Ramsey and N.E.S., unpublished data). Therefore, it will be important to ascertain if tissue-specific, somatic deletion of the *Ink4a/Arf/Ink4b* products is more oncogenic than germline deficiencies.

Understanding how $p16^{INK4a}$ promotes aging, whether by inducing senescence or decreasing the frequency of cell-cycle entry, is an important issue. The data demonstrating an increase in $p16^{INK4a}$ expression with aging

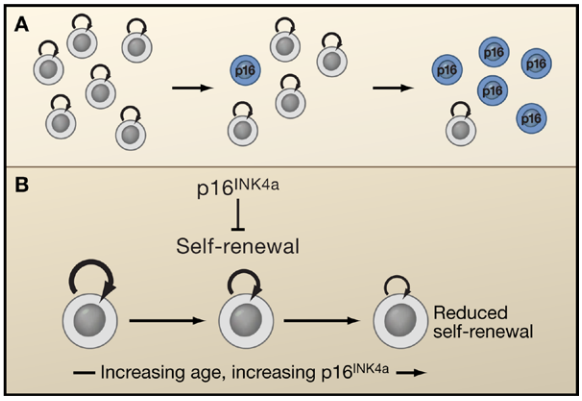

Figure 4. Models of p16^{INK4a} Function in Cellular Aging

Tissue-specific, self-renewing cells undergo repeated divisions, and p16^{INK4a} expression increases with age as a consequence of undefined stimuli.

(A) Senescence model: p16^{INK4a} expression occurs stochastically in a subpopulation of cells resulting in their senescence. In this model, the capacity for self-renewal of the nonsenescent cells is not affected.

(B) The decreased cell-cycle entry model: expression of p16^{INK4a} increases uniformly in the tissue-specific, self-renewing cells resulting in a decrease in the self-renewal capacity of the entire compartment. In either scenario, the overall regenerative capacity of the self-renewing cell compartment is compromised over time. Self-renewal is indicated by curved arrows, senescent cells are colored blue.

can be reconciled with two different models (Figure 4). With aging, stochastic activation of p16^{INK4a} expression could occur on a cell-by-cell basis in self-renewing compartments to induce senescence (Figure 4A), or expression could increase simultaneously within the majority of cells of a self-renewing compartment (Figure 4B). In the latter model, self-renewal would be impaired by p16^{INK4a} expression by decreased frequency of cell-cycle entry in the absence of senescence. The finding that with aging, there is a correlation between an increased expression of p16^{INK4a} and the expression of cellular markers of senescence in primate skin (Herbig et al., 2006), human vasculature (Matthews et al., 2006), and rodent and human kidney (Krishnamurthy et al., 2004; Melk et al., 2003, 2004) supports the senescence model (Figure 4A); but such correlative observations do not establish a causal role for senescence in aging. Addressing this question has important implications for future "anti-aging" therapies—that is, can cellular aging be reversed? The model in which cell-cycle entry is decreased (Figure 4B) suggests that the age-induced defects in proliferation could be ameliorated merely by reducing p16^{INK4a} levels or otherwise increasing CDK4/6 activity in these cells. The senescence model (Figure 4A), however, suggests that the defects in self-renewal could only be remedied through more drastic measures, for example replacement with young, self-renewing cells from an exogenous source.

Lastly, a better and integrated understanding of the in vivo regulation of the *INK4a/ARF/INK4b* locus with cancer and aging is needed. The suggestion by Gonzalez and colleagues that overexpression of CDC6 can transcriptionally silence the *INK4a/ARF/INK4b* locus through a nearby ORI is highly novel, describing a form of transcriptional repression without precedent in vertebrate biology. It is now important to reconcile this finding with the extensive list of transcription factors and other effectors (Figure 2) known to modulate *INK4a/ARF/INK4b* expression. For example, CDC6-mediated heterochromatinization might play some role in the other two incompletely understood forms of *INK4/ARF* repression: that induced by overexpression of PcG or DNA methylation.

In summary, the role of *INK4a/ARF/INK4b* products in tumor suppression is well established, and new data similarly suggest an important role for p16^{INK4a} at least in mammalian aging. Additional recent data suggest that tumor suppression by *INK4a/ARF* results from the in vivo induction of senescence, although senescence-independent anticancer functions such as a role for p16^{INK4a} in regulating tumor ploidy have also been suggested. Given these critical physiological roles, understanding the regulation of this locus has assumed new importance. Several provocative questions remain including the relationship of a recently identified coupling of DNA replication and *INK4a/ARF/INK4b* silencing with other known regulatory events that influence the locus. A holistic understanding of these many factors that communicate with the *INK4a/ARF/INK4b* locus will allow precise determination of how cells sense the earliest effects of carcinogenesis, as well as further delineate the stimuli that tell cells that they are growing old.

ACKNOWLEDGMENTS

We would like to thank Drs. Sean Morrison, Maarten van Lohuizen, Scott Hammond, and Nabeel Bardeesy for comments on the manuscript. This work was supported by Grants from the Sidney Kimmel Foundation for Cancer Research, the Ellison Medical Foundation, the Paul Beeson Physician Faculty Scholars in Aging Research Program, and the NIH.

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