The Molecular Balancing Act of p16\(^{\text{INK4a}}\) in Cancer and Aging

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Running Title: p16\(^{\text{INK4a}}\) in Cancer and Aging
Key Words: INK4/ARF, senescence, CDKN2A, polycomb
Financial Support: This work was supported by NIH R00AG036817 (C.E.B) and American Cancer Society IRG-67-003-50 (C.E.B.).
Conflict of Interest Disclosure: The authors declare no conflicts of interest.

Word Count: 8,619
Figure Count: 5 Figures
Abstract

Located on chromosome 9p21.3, p16<sup>INK4a</sup> seems lost amongst a cluster of neighboring tumor suppressor genes. While best known for inhibiting cyclin dependent kinase (CDK) activity, p16<sup>INK4a</sup> is not a one trick pony. Long term p16<sup>INK4a</sup> expression pushes cells to enter senescence, an irreversible cell cycle arrest that prevents the growth of would-be cancer cells, but also contributes to aging. Loss of p16<sup>INK4a</sup> is one of the most frequent events in human tumors and allows pre-cancerous lesions to bypass senescence. Therefore, precise regulation of p16<sup>INK4a</sup> is essential to tissue homeostasis, maintaining a tight balance between tumor suppression and aging. Here, we outline the pathways required for proper p16<sup>INK4a</sup> regulation and highlight the critical functions of p16<sup>INK4a</sup> in cancer, aging and human physiology that make this gene special.
Introduction

Every day we depend on our cells to make the right decision – to divide or not to divide. Proliferation is essential for tissue homeostasis, but when deregulated can both promote cancer and lead to aging. For this reason, the decision to replicate is tightly controlled by a complex network of cell cycle regulatory proteins. In the early 1990s, it was clear that the catalytic activity of cyclin dependent kinases (CDKs) was required to drive cellular division. Less obvious were the signals that regulate CDK activity and how these became altered in neoplastic disease. In an attempt to address this very question, Beach and colleagues made the observation that CDK4 bound a distinct, 16 kilodalton protein in cells transduced with a viral oncogene (1). Biochemical characterization of this protein, later named p16\textsuperscript{INK4a}, placed it amongst the INK4-class of cell cycle inhibitors, which bind directly to CDK4 and CDK6, blocking phosphorylation of the retinoblastoma tumor suppressor (RB) and subsequent traversal of the G1/S cell cycle checkpoint ((2, 3); Fig. 1A). In the presence of various stressors (e.g. oncogenic signaling, DNA damage), p16\textsuperscript{INK4a} expression blocks inappropriate cellular division, and prolonged induction of p16\textsuperscript{INK4a} leads to an irreversible cell cycle arrest termed ‘cellular senescence’.

The gene encoding p16\textsuperscript{INK4a}, \textit{CDKN2A}, lies within the \textit{INK4/ARF} tumor suppressor locus on human chromosome 9p21.3 (Fig. 1B). \textit{CDKN2A} encodes two transcripts with alternative transcriptional start sites (4). Both transcripts share exons 2 and 3, but are translated in different open reading frames to yield two distinct proteins: p16\textsuperscript{INK4a} and ARF (p14\textsuperscript{ARF} in humans, p19\textsuperscript{ARF} in mice). In addition to \textit{CDKN2A}, the \textit{INK4/ARF} locus encodes a third tumor suppressor protein, p15\textsuperscript{INK4b}, just upstream of the \textit{ARF} promoter (3). Discovered through homology-based cDNA library screens, p15\textsuperscript{INK4b} functions analogously to p16\textsuperscript{INK4a}, directly blocking the interaction of CDK4/6 with D-type cyclins (2, 3). In contrast to p15\textsuperscript{INK4b} and p16\textsuperscript{INK4a} which function to inhibit RB phosphorylation, ARF expression stabilizes and thereby activates another tumor suppressor, p53 (5, 6). Like the INK family of inhibitors, p53 functions to block inappropriate proliferation and cellular transformation. Through a poorly understood mechanism, likely...
dependent upon cell type and transcriptional output, p53 activation can trigger either apoptosis or cell cycle arrest (7). A fourth INK4/ARF transcript, ANRIL (Anti-sense Non-coding RNA in the INK4/ARF Locus), was recently discovered in a familial melanoma kindred with neural system tumors (8). The ANRIL transcript runs anti-sense to p15INK4b and encodes a long, non-coding RNA elevated in prostate cancer and leukemia (9, 10). ANRIL is proposed to function as an epigenetic regulator of INK4/ARF gene transcription, targeting histone modifying enzymes to the locus (See discussion below). In summary, the INK4/ARF locus is a relatively small (110kb), but complex locus essential to the proper maintenance of cell cycle control and tumor suppression. In this review, we focus on the founding member of the INK4/ARF locus, p16INK4a, and discuss what is known and unknown about p16INK4a regulation in cancer and aging.

CDK4/6-Independent Roles of p16INK4a

Several lines of evidence suggest that p16INK4a may function both through CDK4/6-dependent and -independent mechanisms to regulate the cell cycle. CYCLIN D-CDK4/6 complexes are stabilized by interactions with the CDK2 inhibitors, p21\(^\text{CIP1}\), p27\(^\text{KIP1}\) and p57\(^\text{KIP2}\), and serve to titrate these proteins away from CDK2 (11-14). Subsequent expression of p16INK4a or p15INK4b causes these complexes to disassociate, releasing sequestered CDK2 inhibitors (15). This process, known as ‘CDK inhibitor re-shuffling’, has been documented in a growing list of cell lines, and several lines of evidence support the biological relevance of this model. Mice harboring kinase-dead Cdk4 or Cyclin D1 alleles that retain p27\(^\text{KIP1}\) binding capacity (Cyclin D1\(^{112E}\), Cdk4\(^{D158N}\)) display heightened CDK2 activity (16-18) and fewer developmental defects than Cyclin D1 knockouts. The same observation holds true for a Cyclin D1 knock-in mutation incapable of binding RB (Cyclin D1\(^{Δ\text{LxCxE}}\))(19). As such, it is not surprising that p27\(^\text{KIP1}\) deletion can rescue the retinal hypoplasia and early mortality phenotypes of Cyclin D1-null mice (20, 21).
More recently the biological relevance of CDK inhibitor re-shuffling has come under scrutiny. Knock-in mice harboring p16\textsuperscript{INK4a}-insensitive Cdk4 and Cdk6 alleles still capable of binding p27\textsuperscript{KIP1} (Cdk6\textsuperscript{R31C} and Cdk4\textsuperscript{R21C}, respectively) do not display the phenotypes predicted by this model (18, 22). The decreased p16\textsuperscript{INK4a} binding capacity of these mutants should promote p27\textsuperscript{KIP1} sequestration and enhanced CDK2 activity, but cells from the liver and testes of Cdk4\textsuperscript{R21C} mice show no change in the composition of CDK2-Cyclin complexes, nor do thymocytes harboring the Cdk6\textsuperscript{R31C} allele (18, 22). These data suggest that, in at least a subset of cell types, the kinase activity of CDK4/6 is predominantly responsible for proliferative control. Knockout mice lacking a single CDK4/6 inhibitor (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b} or p19\textsuperscript{INK4d}) develop normally and are born at expected Medelian ratios ((23-25). In contrast, p18\textsuperscript{INK4c} knockouts are characterized by organomegaly, yet the association of p27\textsuperscript{KIP1} with CDK2 complexes is unchanged in these animals (26). Work examining combined loss of p15\textsuperscript{INK4b} and p18\textsuperscript{INK4c} (24) or p27\textsuperscript{KIP1} and p18\textsuperscript{INK4c} (26) in mice suggests that distinct mechanisms are used by each inhibitor to control cellular proliferation. This result is in contrast to the CDK inhibitor re-shuffling model wherein co-deletion would be predicted to concordantly promote CDK2 activity. However, it is important to note that none of these publications contest the fact that CDK2 inhibitors bind CYCLIN D-CDK4/6 complexes and are released upon p16\textsuperscript{INK4a} expression. Moreover, recent findings suggest that p16\textsuperscript{INK4a} may contribute to cell cycle regulation through additional CDK-independent mechanisms. Specifically, expression of p16\textsuperscript{INK4a} has been reported to stabilize p21\textsuperscript{CIP1}, and may inhibit the AUF1-dependent decay of p21\textsuperscript{CIP1}, cyclin\textit{D1} and \textit{e2f1} mRNA (27, 28). As a whole, these data provide evidence that the cell cycle-related functions of p16\textsuperscript{INK4a} may extend beyond CDK4/6 inhibition to include the regulation of other CDK-CYCLIN targets.

Transcriptional, Translational and Epigenetic Regulation of p16\textsuperscript{INK4a}
To maintain tissue homeostasis and prevent cancer, the ability of p16<sup>INK4a</sup> to inhibit cellular proliferation must be tightly controlled. In this section, we discuss the role of chromatin, transcriptional co-factors and RNAs in maintaining proper p16<sup>INK4a</sup> expression. In addition, we highlight the complexity and redundancy of p16<sup>INK4a</sup> regulatory pathways required for proper proliferative control.

**p16<sup>INK4a</sup> Repression by Polycomb Group Complexes**

Chromatin modifications by the polycomb group complexes (PcGs), PRC1 and PRC2, are critical to the homeostatic regulation of INK4/ARF gene expression (Fig. 2A). The PRC2 complex is made up of four core components: EZH1/2, EED, SUZ12 and RBAp46/48. EZH1 or 2 serves as the catalytic subunit of PRC2 and functions only in the presence of EED and SUZ12 to compact chromatin through the di- and tri-methylation of histone H3 lysine 27 (H3K27me2/3) (29, 30). H3K27me3 is recognized by a chromodomain-containing CBX protein family member associated with PRC1. In this manner, PRC1 is recruited to the INK4/ARF locus, where it catalyzes the ubiquitylation of histone H2A lysine 119 (H2AK119ub), resulting in further chromatin compaction and gene silencing (31). Multiple variants of the PRC1 complex have been identified in vivo, each containing homologs of the *Drosophila* Posterior Sex Comb (Psc; NSPC1/PCGF1, MEL-18/PCGF2, RNF3/PCGF3, BMI1/PCGF4, RNF159/PCGF5, RNF134/PCGF6), Polycomb (Pc; CBX2, CBX4, CBX6, CBX7, CBX8), Sex Combs Extra (RING1, RING2) and Polyhomeotic proteins (Ph; HPH1, HPH2, HPH3) (32). PRC1 complexes contain a single Psc and Pc homolog, yet Maertens et al. observed binding of MEL-18, BMI-1, CBX7 and CBX8 to the repressed INK4/ARF locus (32). Further investigation revealed that multiple PRC1 variants bind to the p16<sup>INK4a</sup> promoter, working in a concerted manner to control gene expression (32). It remains to be determined whether recently reported non-canonical PRC1 complexes that lack a Pc homolog, contain RYBP/YAF2, and are recruited to chromatin independent of H3K27me3 (33) can also function to regulate INK4/ARF gene expression. However, regardless of their composition, PRC1 and 2 complexes clearly bind
throughout the INK4/ARF locus, and repress $p16^{INK4a}$ expression in young, unstressed cells (31). Maintenance of this repression may be partially dependent on the ubiquitin-specific protease, USP11, which was also shown by Maertens et al. to bind and stabilize the PRC1 complex (34). In their work, depletion of USP11 caused polycomb complexes to dissociate from the INK4/ARF locus leading to subsequent de-repression of $p16^{INK4a}$.

The importance of PRC1 and PRC2 for proper $p16^{INK4a}$ regulation may be best exemplified by the phenotypes of polycomb knockout mice. Deletion of the PRC1 component, $Bmi1$, results in homeotic skeletal transformations, lymphoid and neurological defects (35). Many of these phenotypes are attributable to the deregulation of homeobox gene expression, however, the lymphoid and neurological defects observed in $Bmi1$ knockout mice can be almost completely rescued by INK4/ARF deletion (36).

Here, INK4/ARF loss reverses the self-renewal defects of $Bmi1$-null hematopoietic and neuronal progenitors (37, 38). Together, these data show that PRC1 regulation of $p16^{INK4a}$ expression is required for proper development, stem cell maintenance and homeostasis. In contrast to PRC1-null animals which survive gestation, deletion of the PRC2 members, $Ezh2$ or $Suz12$, is embryonic lethal (39, 40). For this reason, conditional knockout alleles are required to assess the biological functions of PRC2. Using such alleles to delete $Ezh2$ in the brain, pancreas and embryonic skin, phenotypic outcomes have been observed. Specifically, loss of $Ezh2$ in murine pancreatic islets causes a diabetic phenotype associated with increased β-cell expression of both $p16^{INK4a}$ and ARF (41). In contrast, $Ezh2$ deletion in the brain and skin resulted in only mild differentiation defects (42, 43). The recent observation that EZH1 is expressed in many adult tissues and also catalyzes histone H3 methylation led to the hypothesis that EZH1 may compensate for EZH2 loss in some settings. Indeed, deletion of both $Ezh1$ and $2$ caused severe defects in murine skin morphogenesis associated with a >70-fold increase in $p16^{INK4a}/ARF$ expression (43).
Supporting a role for EZH2 in maintaining proliferative homeostasis, human germline mutations in EZH2 give rise to Weaver syndrome, a congenital disorder characterized by uncontrolled and rapid growth (44). While this phenotype could be attributed to p16<sup>INK4a</sup> silencing, few reports have attempted to functionally characterize the EZH2 missense mutations commonly associated with Weaver syndrome (44). Instead, work has focused on recurring point mutations reported in B-cell lymphoma. These mutations localize to tyrosine 641 of the EZH2 SET domain, resulting in the production of a neomorphic protein with enhanced H3K27 di- and tri-methylation activity (45). Of importance, not all cancer-associated EZH2 mutants are gain-of-function alleles. In myeloid neoplasms, missense, nonsense and frameshift mutations in EZH2 have been described which lack a catalytic SET domain (46). In addition, the expression of wildtype EZH2 has been reported to cause p16<sup>INK4a</sup> silencing in SNF5-deficient malignant rhabdoid tumors (MRTs) (47). Deletion or pharmaceutical inhibition of EZH2 activity in cells from these tumors increases p16<sup>INK4a</sup> expression, resulting in cell cycle inhibition (47, 48). Together, these observations suggest that EZH2 activity must be tightly controlled in order to maintain proliferative homeostasis and proper p16<sup>INK4a</sup> regulation.

Recruiting Polycomb to CDKN2A

In Drosophila melanogaster, polycomb group proteins are recruited to defined DNA binding sites termed, Polycomb repressive elements (PREs) (Reviewed in: (49)). In contrast, few mammalian PREs have been identified to date, leading many to speculate that other DNA binding proteins or RNAs must guide polycomb complexes to target genes like the INK4/ARF locus. Recent work suggests that long non-coding RNAs (lncRNAs) can serve as scaffolds for polycomb recruitment and epigenetic gene silencing. The discovery of disease-linked polymorphisms within ANRIL prompted investigation into whether this lncRNA could function in a similar manner to regulate INK4/ARF gene transcription. Through RNA binding assays, a study by Yap et al. showed that CBX7 interacts with both ANRIL and
H3K27me3 to promote INK4/ARF gene silencing (Fig. 2A and (10)). Subsequently, ANRIL binding to SUZ12, a component of the PRC2 complex, was reported to promote silencing of p15\textsuperscript{INK4b}, but not p16\textsuperscript{INK4a} (50). Together with these data, a report showing that MOV10, a putative RNA helicase and PRC1 binding partner, is required for p16\textsuperscript{INK4a} repression, supports a role for ANRIL in polycomb recruitment to the INK4/ARF locus (51). Whether MOV10 binds ANRIL to facilitate PRC1 interaction with the INK4/ARF locus has yet to be determined. However, together these data make a strong case for the role of ANRIL in epigenetic silencing of p16\textsuperscript{INK4a}.

ANRIL-independent mechanisms are also suggested to recruit PcG complexes to the p16\textsuperscript{INK4a} locus. Recently, H2.0-like homeobox 1 (HLX1), a homeobox (HOX) protein, was shown by Martin \textit{et al.} to facilitate PRC2 recruitment to the p16\textsuperscript{INK4a} promoter (Fig. 2A and (52)). While the mechanism has yet to be defined, six other homeobox-containing proteins (HOXA9, DLX3, HOXB13, HOXC13, HOXD3 and HOXD8) were similarly reported to participate in p16\textsuperscript{INK4a} silencing (52). Given the role of HOX genes in developmental patterning, it is interesting to speculate that proteins like HLX1 and HOXA9 initiate tissue-specific silencing of p16\textsuperscript{INK4a}. Like HOX proteins, both TWIST1, a basic helix-loop-helix (bHLH) transcription factor, and KDM2B, a histone demethylase, may also facilitate polycomb-mediated silencing of the INK4/ARF locus. Ectopic expression of TWIST1 and KDM2B can cause cellular levels of EZH2 to rise, resulting in an increase in PRC2 activity (53). KDM2B was further reported to function in demethylating H3K36me2/3, a common marker for DNA polymerase II transcription (53). Furthermore, TWIST1 appears to increase BMI1 expression (55), and subsequent recruitment of BMI1 to the p16\textsuperscript{INK4a} promoter has been linked to interactions with phosphorylated RB (pRB) and zinc finger domain-containing protein 277 (ZFP277) (54, 55). In particular, the link between pRB and p16\textsuperscript{INK4a} silencing is intriguing as it suggests the presence of a feedback loop wherein cells entering S-phase repress p16\textsuperscript{INK4a} expression (54). ZFP277-mediated recruitment of BMI1 to the p16\textsuperscript{INK4a} promoter may also be linked to the cell cycle. A study by Negishi \textit{et al.} showed that reductions in ZFP277 expression caused by oxidative...
stress could lead to PRC1 dissociation from the $p16^{INK4a}$ promoter and subsequent cell cycle arrest (55). How these mechanisms of PRC recruitment interplay with ANRIL remains to be established, but certainly the complexity of $p16^{INK4a}$ silencing is indicative of the importance of this gene in maintaining tissue homeostasis.

Reversing Polycomb Silencing of the INK4/ARF locus

In order for normal cells to traverse the G1/S checkpoint, $p16^{INK4a}$ must be maintained in a repressed state. At the same time, induction of $p16^{INK4a}$ expression in the presence of stress signals is required to prevent inappropriate cell cycle progression. Linking proliferative control to epigenetic regulation of the $p16^{INK4a}$ promoter, Bracken et al. first demonstrated that RB phosphorylation during the G1 to S-phase transition releases E2F1 to transactivate $EZH2$ and $EED$ (56). Later, these data were confirmed by an independent group who showed that p53 activation represses $EZH2$ expression through RB-mediated inhibition of E2F1 activity (57). While these results explain how the activity of PRC2 might be curbed in the presence of stress, they do not explain how epigenetic silencing of the $p16^{INK4a}$ promoter is reversed. One mechanism of removing repressive histone marks is via the activity of histone demethylases. In response to oncogenic stressors, levels of the H3K27me3 demethylase, $Jmjd3$, increase, removing repressive histone marks from the $p16^{INK4a}$ promoter (Fig. 2B; (58, 59)). Following histone demethylation, the Yokoyama lab showed that Jun dimerization protein 2 (JDP2) may help maintain $p16^{INK4a}$ in an active state by binding and sequestering H3K27 away from the actions of PRC2 (60). These findings suggest that the activity of JDP2 and JMJD3 establishes a permissive state for $p16^{INK4a}$ expression. Furthermore, unmethylated H3K27 is no longer recognized by PRC1, and chromatin surrounding the $p16^{INK4a}$ promoter is decondensed. Based upon this activity, it is not surprising that JMJD3, like $p16^{INK4a}$, serves as a barrier to induced pluripotency (61, 62) (See: “$p16^{INK4a}$ as a Barrier to Pluripotency”). Clearly, interplay between PRC1, PRC2 and histone demethylases is required for
homeostatic regulation of the $p16^{INK4a}$ promoter, yet how this crucial balance is maintained is still in question. Moreover, it is possible that other histone demethylases, such as lysine-specific demethylase 6A (KDM6A/UTX), may also play a role in $p16^{INK4a}$ regulation.

In *Drosophila*, the SWI/SNF chromatin remodeling complex serves as a trithorax group activator, opposing the actions of polycomb-mediated silencing. Similarly, SWI/SNF functions to counteract PcG silencing of $p16^{INK4a}$ in mammals. Cancer cell lines deficient in the SWI/SNF component, SNF5, induce high levels of $p16^{INK4a}$ upon the restoration of SNF5 expression (63). In untransformed cell lines, SNF5 binds and directly inhibits the transcription of $EZH2$, resulting in decreased polycomb occupancy at the $p16^{INK4a}$ promoter (47). Tumor cells lacking SNF5 overexpress EZH2 and are dependent upon the activity of PRC2 to silence $p16^{INK4a}$ expression and drive proliferation (47, 64).

Chromatin Regulation of $p16^{INK4a}$ by Non-Polycomb Proteins

Epigenetic modification of the INK4/ARF locus extends beyond polycomb-mediated silencing. The well-conserved genomic insulator, CCCTC-motif binding factor (CTCF), binds throughout the INK4/ARF locus (65, 66) and functions to regulate both chromatin compaction and gene expression (Fig. 2B). Witcher et al. first reported interaction of CTCF with a chromosomal boundary ~2kb upstream of the $p16^{INK4a}$ promoter (65). In their studies of cancer cell lines, knockdown of CTCF resulted in the spread of heterochromatin DNA into the INK4/ARF locus leading to epigenetic silencing of $p16^{INK4a}$ (65). In contrast to this model, Hirosue et al. recently reported that decreases in CTCF expression associated with oncogene induced senescence promote decondensation of the INK4/ARF locus and lead to heightened levels of $p16^{INK4a}$ mRNA (66). Reconciliation of these two results is possible as rapid $p16^{INK4a}$ induction following CTCF knockdown would place enormous pressure on would-be cancer cells to epigenetically silence the INK4/ARF locus. In fact, the observation that epigenetic silencing of $p16^{INK4a}$ in
breast cancers is accompanied by CTCF disassociation from the locus (65) is consistent with a role for
CTCF in proper epigenetic regulation of INK4/ARF.

Transcriptional Activators and Repressors of $p16^{INK4a}$

**Opposing Transcriptional Regulators**

The presence of a permissive chromatin state alone is insufficient for $p16^{INK4a}$ expression. Binding of activation factors and the subsequent recruitment of RNA polymerase is required to initiate $p16^{INK4a}$ transcription (Fig. 3). Similar to the interplay between PRC2 and JMJD3, transcriptional regulation of the $p16^{INK4a}$ promoter is tightly controlled by antagonistic pathways. Serving as a classic example, induction of $p16^{INK4a}$ by the E-box binding transcription factors E-26 transformation-specific 1 (ETS1), E-26 transformation-specific 2 (ETS2) and E47, is directly opposed by Inhibitor of DNA Binding 1 (ID1) (67). In response to oncogenic and senescent signaling, ETS1, ETS2 and/or E47 bind to E-box motifs (CANNTG) within the $p16^{INK4a}$ promoter to stimulate gene expression (67, 68). This action is directly antagonized by ID1 which prevents interaction of ETS1, ETS2 and E47 with the $p16^{INK4a}$ promoter (67, 68). As such, it is not surprising that $Id1$ null MEFs undergo premature senescence in culture (69), and that age-related increases in $p16^{INK4a}$ expression correlate directly with ets-1 levels in mice and rats (70).

Similar to ID1, TWIST1 is reported to oppose transcriptional activation of $p16^{INK4a}$. The relationship between ID1 and TWIST1 was initially suggested in studies examining the progression of benign human nevi to melanoma. In general, nevi are non-proliferative and express elevated levels of $p16^{INK4a}$; yet, upon progression to melanoma these lesions frequently silence $p16^{INK4a}$ (71). Analysis of TWIST1 and $p16^{INK4a}$ expression in nevi and melanomas revealed an inverse correlation between these two proteins, putting forth the hypothesis that they function in antagonistic pathways (72). Preliminary work suggested that this antagonism might be mediated through direct interaction of TWIST1 with ETS2.
however, in a recent publication by Cakouros et al., TWIST1 was reported to inhibit $p16^{\text{INK4a}}$ transcription by decreasing E47 expression (73). Clearly, further work is required to fully understand the physiological relationship between $p16^{\text{INK4a}}$ and TWIST1. In addition, it will be of interest to assess the potential role of $p16^{\text{INK4a}}$ in classical TWIST1 pathways including epithelial to mesenchymal transition, stem cell maintenance and tumor metastasis.

In line with the relationship between TWIST, ID1 and E-box binding transcription factors, antagonistic interplay has also been described between members of the Activator Protein-1 (AP-1) family of transcription factors, c-JUN and JUNB. While JUNB serves to activate $p16^{\text{INK4a}}$ transcription by binding to three identified AP1-like sites within the $p16^{\text{INK4a}}$ promoter (74), c-JUN limits $p16^{\text{INK4a}}$ expression (75). Certainly, a delicate balance between inhibitory (ID1, TWIST, c-JUN) and stimulatory (JUNB, ETS, E47) pathways is required for proper regulation of $p16^{\text{INK4a}}$. Upsetting this balance through the overexpression of inhibitors or repression of $p16^{\text{INK4a}}$ activators promotes the bypass of senescence and can lead to cancer (72, 73, 76-78).

The HOX family of proteins could also be viewed as antagonistic $p16^{\text{INK4a}}$ regulators. Above, we discussed the potential role of HLX1, HOXA9, DLX3, HOXB13, HOXC13, HOXD3 and HOXD8 in polycomb-mediated repression of the $\text{INK4/ARF}$ locus. In contrast, the HOX proteins, VENTX, MEOX1 and MEOX2 have each been reported to bind the $p16^{\text{INK4a}}$ promoter and activate gene transcription (79-81). This proposed interplay between HOX genes and $p16^{\text{INK4a}}$ regulation is suggestive of a role for CDK4/6 inhibition in embryonic development. In spite of this, developmental defects are not observed in $p16^{\text{INK4a}}$ knockout mice or melanoma-prone kindreds harboring germline $p16^{\text{INK4a}}$ deficiencies (25, 82). Whether compensatory mechanisms are required to combat the functional loss of $p16^{\text{INK4a}}$ during development is still to be determined.

Role of Acetyltransferases and Deacetylases in $p16^{\text{INK4a}}$ Regulation
Histone acetylation facilitates chromatin decondensation and subsequent gene transactivation. As such, transcriptional coactivators often harbor or recruit histone acetyltransferase (HAT) activity to target gene promoters. In a pair of recent publications, Wang et al. describe how the transcription factors, SP1 and HMG box-containing protein 1 (HBP1), recruit p300, a well-known HAT, to the p16\textsuperscript{INK4a} promoter (83, 84). In their studies, acetylation of local chromatin as well as HBP1, promoted decondensation of the p16\textsuperscript{INK4a} promoter and subsequent gene transactivation. However, numerous targets of p300 acetylation have been identified to date, including B-MYB, a putative repressor of p16\textsuperscript{INK4a} transcription (83, 85, 86). Therefore, the p300-p16\textsuperscript{INK4a} relationship is likely complex and may be dependent upon the available pool of transcriptional co-factors within a given cell type.

HAT activity is opposed by histone deacetylases (HDACs) which promote transcriptional silencing. In human cell lines, HDACs 1-4 have all been reported to bind and repress transcription from the p16\textsuperscript{INK4a} promoter (52, 87-89). Most of these interactions have been linked to bridging transcription factors such as Lymphoid Specific Helicase (LSH), HLX1 and ZBP-89 (87, 89); albeit one report suggested that HDAC2 may directly bind the p16\textsuperscript{INK4a} promoter (88). While loss of Hdac1, 2, 3 or 4 causes developmental abnormalities and lethality in mice, none of these phenotypes have been attributed to defects in p16\textsuperscript{INK4a} regulation (90).

Age-related Signaling Pathways Influence p16\textsuperscript{INK4a} Expression

The observation that p16\textsuperscript{INK4a} levels are high in senescent cells has prompted several investigations into the connection between pro-senescent signaling and p16\textsuperscript{INK4a} regulation. For example, age-related metabolic pathologies have long been associated with activation of the peroxisome proliferator-activated receptors (PPARs). It is now known that these nuclear receptors directly bind and activate the p16\textsuperscript{INK4a} promoter leading to subsequent cell cycle arrest (91, 92). Similarly, alterations in TGF-β signaling have been linked to a variety of age-related diseases including
cancer, osteoarthritis, cardiovascular disease and Alzheimer’s (93). Work by the Conboy lab has demonstrated that elevated TGF-β signaling reduces the capacity of muscle stem cells to regenerate (94). Here, phospho-SMAD3 has been shown to directly bind the p16INK4a promoter, stimulating gene transcription and cell cycle arrest (95). Due to crosstalk between the TGF-β and β-CATENIN/WNT signaling pathways, it is not surprising that aberrant WNT signaling is also associated with age-related disease (95). β-CATENIN can directly bind and activate the p16INK4a promoter in both human and murine cells (96-98), and recent evidence links the induction of p16INK4a by reactive oxygen species (ROS) to β-CATENIN/WNT signaling (99). Together these findings suggest a strong association between age-promoting, ‘gerontogenic’ signals and p16INK4a expression.

Cellular Structure and p16INK4a Expression

An emerging theme in p16INK4a regulation is the potential for cytoskeletal rearrangements to influence INK4/ARF gene transcription. In an siRNA screen of over 20,000 genes, Bishop and colleagues recently identified GLI2, a member of the Hedgehog signaling pathway, as an activator of p16INK4a expression (100). Interestingly, GLI2 partially localizes to a non-motile cytoskeletal protrusion called the primary cilium, and cultured human mammary epithelial cells with a primary cilium expressed lower levels of p16INK4a than those without (100). Upon ablation of p16INK4a, the number of cells with a primary cilium increased, suggesting a link between cellular structure and p16INK4a expression (100). Supporting this observation, the ACTIN nucleating enzymes ARP2 and 3 have been implicated in a second connection between cytoskeletal structure and Ink4/Arf gene transcription. Here, the generation of stable ARP2/3 knockdown cells required concomitant Ink4/Arf deletion (101). These data suggested that lamellipodial dysfunction triggers growth inhibitory Ink4/Arf gene transcription; however the specific role of p16INK4a in this process has yet to be examined.
The Role of microRNAs and RNA Binding Proteins in p16INK4a Regulation

While literature defining transcriptional and epigenetic regulators of p16INK4a is extensive, far fewer studies have examined post-transcriptional mechanisms of p16INK4a regulation. Discovered in an unbiased search for miRNAs silenced during senescence (102), two independent groups have reported that miR-24 binds and inhibits p16INK4a translation (102, 103). Consistent with this observation, antagonists of miR-24 cause a moderate decrease in the proliferation of normal human keratinocytes (103). In a similar manner, knockdown of miR-31 has been proposed to regulate cell cycle progression in murine embryo fibroblasts with altered nuclear structure (104). Here, loss of Lamin B1 was associated with increased miR-31 expression and p16INK4a instability (104). This work suggests another possible connection between cellular structure and p16INK4a regulation, linking changes in nuclear integrity to p16INK4a expression. Together, the associations between miR-31, miR-24 and p16INK4a suggest that miRNAs function to control proliferation via interactions with p16INK4a mRNA; however, it is important to note that other cell cycle regulators have been identified as miR-24 targets (e.g. cdk4, cyclin A2, cyclin B1, myc, e2f2, p14ARF and p27kip1; (103, 105, 106)) and miR-31 (e.g. ets1, cdk1; (107)). Therefore, cell cycle defects caused by perturbations in miRNA expression likely reflect the outcome of both p16INK4a-dependent and -independent pathways.

The let-7 family of microRNAs has also been implicated in p16INK4a regulation, proliferative control and stem cell aging. The Morrison group first reported that murine let-7b expression increased with age in neuronal stem cells (108). Although let-7b did not bind p16INK4a mRNA directly, overexpression of let-7b reduced the expression of High-Mobility Group AT-Hook 2 (HMGA2), causing p16INK4a levels to increase (108). In addition to hmg2a, a growing number of RNAs involved in growth and proliferative control have been identified as let-7b targets, and therefore, it is not surprising that the levels of most let-7 family members decrease during tumor progression (109).
In addition to miRNAs, RNA binding proteins can also regulate $p16^{\text{INK4a}}$ translation. Interaction of the Hu RNA binding protein, HuR, with the $p16^{\text{INK4a}}$ 3’UTR have been reported to destabilize the transcript in an miRNA-independent fashion (110). Work by Zhang et al. suggests that this action is opposed by the tRNA methyltransferase, NSUN2, which methylates the 3’UTR of $p16^{\text{INK4a}}$ to prevent HuR binding and subsequent mRNA degradation (111). In this way, interplay between HuR and NSUN2 may tightly control $p16^{\text{INK4a}}$ translation. For example, in the presence of oxidative stress, NSUN2 levels appear to increase, tipping the balance towards mRNA stability, $p16^{\text{INK4a}}$ expression and subsequent cell cycle arrest (111).

The Function and Significance of $p16^{\text{INK4a}}$ Expression in Cancer

In the absence of $p16^{\text{INK4a}}$, both mice and humans are predisposed to cancer (25, 82). Loss of $p16^{\text{INK4a}}$ function can occur through gene deletion, methylation or mutation, and therefore, comprehensive genetic analyses are required to determine the frequency of $p16^{\text{INK4a}}$ silencing in cancer. Adding to the challenge of assessing $p16^{\text{INK4a}}$ status in human tumors, few studies distinguish between the two $CDKN2A$ transcripts: $p16^{\text{INK4a}}$ and ARF. Here, we compiled data from the Cancer Genome Atlas (TCGA) to reveal that functional inactivation of $CDKN2A$ is a frequent event in most tumor types ((112-118); Fig. 4). We probed 16 tumor types to determine the percent of cases wherein mutational and/or copy number changes disrupted genes critical to the $p16^{\text{INK4a}}$ tumor suppressor pathway (i.e. $RB1$, $CDKN2A$, $CCND1$ (CYCLIN D1), $CCND2$ (CYCLIN D2), $CCND3$ (CYCLIN D3), $CCNE1$ (CYCLIN E1), and $CCNE2$ (CYCLIN E2)). Tumor types displaying a high percentage of $p16^{\text{INK4a}}$ tumor suppressor pathway alterations included urothelial carcinoma (BLUCA; 77%), glioblastoma multiforme (GBM; 77%), head and
neck squamous cell carcinoma (HNSC; 63%), lung squamous cell carcinoma (LUSC; 58%) and cutaneous melanoma (SKCM; 58%) (Fig. 4A). Examination of individual tumor profiles revealed infrequent overlap between CDKN2A and RB1 deletion, implying that these are often mutually exclusive tumorigenic events (Fig. 4B). In support of these data, an inverse correlation between $p16^{\text{INK4a}}$ and RB inactivation was previously described in lung cancer cell lines (119) and human tumors (120). In contrast to RB, we frequently noted alterations within other components of the $p16^{\text{INK4a}}$ tumor suppressor pathway (i.e. Cyclin amplification) in CDKN2A mutant tumors from the TCGA dataset (Fig. 4B). However, in cases where the $p16^{\text{INK4a}}$ tumor suppressor pathway appeared genetically intact, high levels of RB1 or CDKN2A methylation were observed (e.g. kidney cancers (KIRP, KIRC), colorectal adenocarcinomas (COAD/READ), low grade glioma (LGG), and acute myeloid leukemia (AML); Fig. 4C). Combining these genetic and epigenetic data suggests that functional inactivation of the $p16^{\text{INK4a}}$-RB axis approaches 100% in many cancer types. A prior study by Schutte et al. supports this claim, demonstrating inactivation of the $p16^{\text{INK4a}}$/RB-axis in 49 of 50 pancreatic carcinomas (121). However, further comprehensive assessment of $p16^{\text{INK4a}}$ functionality in a wide variety of tumors will require thorough experimental and bioinformatic analyses.

$p16^{\text{INK4a}}$ Expression in Tumors

In most tumor types, $p16^{\text{INK4a}}$ inactivation occurs early in tumorigenesis. For example, pancreatic intraepithelial neoplasias frequently inactivate $p16^{\text{INK4a}}$ upon progression to invasive disease (122). Pressure to silence $p16^{\text{INK4a}}$ presumably stems from oncogenic engagement of the RB tumor suppressor pathway. Therefore, tumors with early defects in RB signaling continue to express $p16^{\text{INK4a}}$ independent of progression (Reviewed in: (123)). Intense activation of the $p16^{\text{INK4a}}$ promoter is believed to represent a futile attempt by the cell to curb oncogenic proliferation in the absence of a functional G1/S checkpoint. In the clinic, immunohisotchemical $p16^{\text{INK4a}}$ staining is used to identify cervical and...
head and neck tumors driven by oncogenic human papilloma virus (HPV) infection. Here, the HPV viral
oncoprotein, E7, functionally inactivates RB leading to increases in p16\(^{INK4a}\) expression (124). Although it
is believed that RB inactivation is requisite for the elevation of p16\(^{INK4a}\) expression in cancer, aberrations
in the RB pathway are not obvious in every tumor. The RB checkpoint is deregulated by multiple
mechanisms independent of \(RB1\) mutation, deletion or methylation. Viral oncogene expression and
cyclin gene amplification represent just two possible forms of alternative RB inactivation.
Unfortunately, there is a paucity of studies that comprehensively examine the status of the RB pathway
in multiple human tumor types. Therefore, the critical role of this p16\(^{INK4a}\)-induced checkpoint may be
underestimated.

Limited analyses of the RB pathway in human tumors have revealed that p16\(^{INK4a}\) expression can
be predictive of both tumor subtype and therapeutic response. Elevated p16\(^{INK4a}\) levels discern small
cell lung cancer from lung adenocarcinoma (119, 125) and characterize the basal-like breast cancer
subtype (126). Given that tumor subtypes often show distinct therapeutic response profiles, it is not
surprising that p16\(^{INK4a}\) expression can predict therapeutic efficacy. For example, elevated p16\(^{INK4a}\) levels
predict a higher initial response to radiation therapy in prostatic adenocarcinoma (127). However, these
same p16\(^{INK4a}\) positive tumors are the most likely to fail androgen-deprivation therapy (128). In
oropharyngeal cancer, p16\(^{INK4a}\) serves as a marker of oncogenic HPV infection and is predictive of
improved therapeutic response and patient survival (129). While p16\(^{INK4a}\) expression clearly serves as a
relevant clinical marker, combined analysis of multiple RB pathway members may be of additional value.
Likewise, recent data suggests that the localization of p16\(^{INK4a}\) staining within a tumor may provide
further clinical insight (see below).

The Significance of Stromal p16\(^{INK4a}\) Expression
While a myriad of signals are linked to $p16^{INK4a}$ regulation, most of these are triggered by intrinsic, cell-autonomous events. Employing a recently developed luciferase reporter mouse, $p16_{LUC}$, we have observed a second, cell-non-autonomous mechanism of $p16^{INK4a}$ activation (130). The $p16_{LUC}$ allele expresses firefly luciferase from the endogenous $p16^{INK4a}$ promoter, allowing investigators to dynamically track $p16^{INK4a}$ transcription in live animals. When the $p16_{LUC}$ allele was crossed with genetically engineered mouse models (GEMMs) of human cancer, a luminescent signal appeared only in the location of future tumor formation (130). These luminescent foci were visible weeks before tumors could be visualized or palpated, providing a significant detection advantage over traditional monitoring methods. This incredible sensitivity, along with the observation that tumors maintained luciferase activity during progression, led to the hypothesis that $p16^{INK4a}$ transcription is activated in the surrounding tumor stroma. Indeed, this was the case as syngeneic transplantation of six $p16_{LUC}$-negative tumor cell lines harboring a wide variety of oncogenic drivers caused stromal induction of $p16_{LUC}$ (Fig. 5A and (130)). Although the specific stromal cell types responsible for this observation have yet to be identified, bone marrow transplantation studies suggest that the $p16_{LUC}$ signal originates in part from bone marrow-derived cells. Therefore, it appears that alterations in the milieu surrounding a growing neoplasm can promote the expression of $p16^{INK4a}$ in a cell-non-autonomous fashion (Fig. 5B).

Mounting evidence supports a role for stromal $p16^{INK4a}$ expression in tumor initiation and progression. Fibroblasts ectopically expressing $p16^{INK4a}$ have altered cellular metabolism and over-produce high-energy mitochondrial fuels such as L-lactate (131). In xenograft studies, co-injection of these fibroblasts with MDA-MB-231 breast cancer cells increased tumor size 2 fold, suggesting that altered stromal metabolism caused by $p16^{INK4a}$ expression promotes tumor growth (131). In support of this observation, elevated $p16^{INK4a}$ levels in the stroma of human mammary ductal carcinoma in situ (DCIS) lesions are predictive of disease recurrence independent of other histopathological markers such as ER positivity (132). It remains to be determined whether the promotion of tumorigenesis by $p16^{INK4a}$-
positive stromal cells is solely a reflection of altered local metabolism. Studies from the p16\textsuperscript{LUC} mouse model suggest that p16\textsuperscript{INK4a} induction in tumor infiltrating immune cells may also promote cancer progression by dampening the anti-tumor response. Regardless of the mechanism, these data make it clear that p16\textsuperscript{INK4a} expression in the tumor stroma should not be ignored.

### Therapeutic Mimicry of p16\textsuperscript{INK4a}

Pharmaceutical efforts to mimic the function of p16\textsuperscript{INK4a} arose after the anti-tumor effects of flavopiridol were linked to CDK inhibition (Reviewed in: (133)). Flavopiridol (alvocidib) was originally touted as an inhibitor of epidermal growth factor receptor (EGFR), but later shown to inhibit the growth of a wide range of cancer cells at an IC\textsubscript{50} much lower than that required to block EGFR activation. Broad inhibition of cyclin dependent kinases (CDKs), including CDK4 (IC\textsubscript{50} <120nM), was later identified as the mechanism of flavopiridol action (134). After several promising phase I trials, flavopiridol failed in phase II, showing significant activity only in cases of relapsed chronic lymphocytic leukemia (135). Based upon these disappointing results, Sanofi-Aventis halted production of flavopiridol in 2010. For other non-selective CDK inhibitors, multiple toxicities and poor solubility prevented successful transition to the clinic. However, recent efforts to generate potent and selective CDK inhibitors appear more fruitful.

Of particular interest to the pharmaceutical industry, selective CDK4/6 inhibitors have shown promise in early clinical trials (Reviewed in: (136)). Knowledge of p16\textsuperscript{INK4a} regulation and function has bolstered these efforts by providing biomarkers of therapeutic efficacy and identifying patient populations wherein response is likely. Often clinical trials of CDK4/6 inhibitors now exclude RB null tumors, which are naturally resistant to the effects of ectopic p16\textsuperscript{INK4a} expression. In addition, the observation that p16\textsuperscript{INK4a} is markedly upregulated in response to RB-inactivating viral oncoproteins (e.g. E7, T-Antigen) has prompted the exclusion of tumors expressing high levels of p16\textsuperscript{INK4a}. Finally, studies of CDK knockout mice are assisting these efforts, identifying cell and tumor types which are more reliant
upon the activity of CDK4/6 to drive proliferation than that of CDK2. Employing this knowledge, CDK4/6 inhibitors are showing efficacy, and a number of drugs have now entered phase II and III clinical trials (Phase 2- LEE011(Novartis) and Ly2835219(Lilly); Phase 3- MK-7965/dinaciclib (Merck) and PD-0332991/palbociclib (Pfizer)) (137).

$p16^{INK4a}$ selectively binds CDK4 and 6 in vitro (2), however achieving such therapeutic specificity is more challenging. In fact, Merck attributes the success of dinaciclib in chronic lymphocytic leukemia to inhibition of CDK9, not CDK4/6 (137). Moreover, the observation that $p16^{INK4a}$ may contribute to CDK4/6-independent cell cycle regulation (i.e. through CDK inhibitor reshuffling) suggests that pharmaceutical CDK4/6 inhibitors may not fully recapitulate the potency of $p16^{INK4a}$. While adverse effects associated with CDK4/6-selective inhibitors are mild (i.e. limited bone marrow suppression) (136), concerns about the long-term efficacy of such therapeutics is rising. In particular, mechanisms to bypass the requirement for CDK4/6 are already known including: RB loss and increased CDK2 activity. How quickly tumors will exploit these pathways to subvert therapeutic treatment is unknown. Initial trials of PD-0332991 in mantle cell lymphomas speak to this concern. While 89% of study participants showed reduced phospho-RB staining at 3 weeks, only 18% of tumors responded to therapy, suggesting that resistance was rapidly acquired (138). In addition to concerns about resistance, the role of $p16^{INK4a}$ expression in the tumor stroma remains undefined and inhibition of the local immune response or altered cellular metabolism may function to promote growth and metastasis. In fact, fibroblasts exposed to PD-0332991 adapt a tumor promoting, metabolic phenotype similar to that of fibroblasts overexpressing $p16^{INK4a}$ (131). Therefore, therapeutic mimetics could have similar, detrimental consequences on the tumor microenvironment. A final concern is the long-term effects of CDK4/6 inhibition on cellular senescence. Prolonged expression of $p16^{INK4a}$ promotes senescence and decreases regenerative capacity (See below). In a similar manner, CKD4/6 inhibitors may influence tissue aging. However, under normal physiological conditions, stem cell populations are by and large quiescent and
thus unlikely altered by CDK4/6 inhibitors. It may be that stress and/or mitogenic signaling, which often accompany p16\textsuperscript{INK4a} induction \textit{in vitro}, is required to elicit senescence, and therefore the effects of CDK4/6 inhibition on aging will be minimal. As more than half of all cancers are diagnosed in people 65 and older, determining whether CDK4/6 therapeutics exacerbate age-related disease will be of significant interest.

The Role of p16\textsuperscript{INK4a} in Senescence and Aging

p16\textsuperscript{INK4a} Marks Biological Senescence

As noted first by Sherr and colleagues (139), p16\textsuperscript{INK4a} levels increase with aging. In fact, detailed quantification has demonstrated a direct increase in p16\textsuperscript{INK4a} expression with chronological age in all mammalian species tested to date (140). Such increases in p16\textsuperscript{INK4a} are exponential, rising \textasciitilde16 fold during the average human lifespan and making p16\textsuperscript{INK4a} one of the most robust aging biomarkers characterized to date (141). The induction of senescence and p16\textsuperscript{INK4a} expression is traditionally associated with a wide variety of intrinsic cellular stressors including: DNA damage, telomere erosion, reactive oxygen species, and stalled replication forks (Reviewed in: (142)). However, use of the p16\textsuperscript{LUC} reporter mouse has provided evidence that undefined, extrinsic signals can also trigger p16\textsuperscript{INK4a} transcription in a cell non-autonomous fashion (130). Using this same reporter to compare the dynamics of p16\textsuperscript{INK4a} expression in mice with that observed in humans showed a direct correlation between the rate of p16\textsuperscript{INK4a} accumulation and lifespan (130). Furthermore, data from progeroid and calorically restricted rodents suggest that p16\textsuperscript{INK4a} serves as a marker of biological rather than chronological aging (70, 143, 144). Supporting this observation in humans, smoking and chemotherapy
are associated with elevated $p16^{INK4a}$ expression in the human population (141, 145). Moreover, skin biopsies from long-lived nonagenarian cohorts have fewer $p16^{INK4a}$ positive cells than their age-matched partners (146). Clinically, use of $p16^{INK4a}$ as a marker of biological aging could provide quantitative measures of patient fitness including immune function and chemotherapeutic tolerance (145, 147). Assessment of $p16^{INK4a}$ levels prior to organ transplantation may also aid in the identification of biologically ‘younger’ donor tissues with increased potential for success (148-150).

A Causal Role for $p16^{INK4a}$ in Aging

Several lines of evidence suggest that $p16^{INK4a}$ is not only a biomarker of aging, but also causes aging in many cell types. Using $p16^{INK4a}$ transgenic and knockout mice, the cell-autonomous role of $p16^{INK4a}$ in aging has been investigated. In murine hematopoietic stem cells, T-cells, pancreatic $\beta$-cells and neural progenitors of the subventricular zone, age-related $p16^{INK4a}$ expression causes a decline in regenerative capacity (151-154). A caveat to these initial studies was the use of germline knockout mice; however strategies employing conditional $p16^{INK4a}$ loss or siRNAs have since confirmed these findings in T-cells and pancreatic $\beta$-cells (154, 155). Supporting the idea that tissues expressing less $p16^{INK4a}$ are more biologically fit, kidney transplant success is higher in $p16^{INK4a}$-low donor organs (148-150). These data put forth the hypothesis that $p16^{INK4a}$ expression drives cellular senescence, resulting in decreased regenerative potential. As a proof of principal, work from the Van Deursen lab showed that deletion of $p16^{INK4a}$-expressing cells from a $BubR1$ deficient, progeroid mouse model reduced many aging phenotypes (e.g. sarcopenia, cataracts, loss of adiposity) (144). Further work by this group showed that both muscle and adipocyte progenitors from these mice express high levels of $p16^{INK4a}$, suggesting that even the deletion of senescent stem cells can promote improved regenerative capacity (156). Unfortunately, these animals did not live longer, owing to the development of cardiac pathologies, and a similar experiment has yet to be reported in wild type mice (144). One explanation
for why these animals were not long-lived is that the gerontogenic effects of p16<sup>INK4a</sup> expression are tissue-specific. Indeed, p16<sup>INK4a</sup> levels do not seem to influence the regenerative capacity of murine melanocyte stem cells or neuronal progenitors of the dentate gyrus (unpublished observation and (153, 157)). Other mechanisms of curbing age-related p16<sup>INK4a</sup> induction have been reported in mice. For example, activation of platelet-derived growth factor receptor (PDGFR) signaling in elderly murine pancreatic β-cells increased the regenerative capacity of these cells via repression of p16<sup>INK4a</sup> expression (158). Likewise, administration of fibroblast growth factor 7 (FGF7) reduced p16<sup>INK4a</sup> levels and increased the number of early T-cell progenitors in 15-18 month-old mice, thereby partially rescuing the known decline in thymopoiesis with age (159). Apart from these studies, analyses of p16<sup>INK4a</sup>-mediated regenerative decline are limited to a small number of tissues; therefore the potential outcome of p16<sup>INK4a</sup>-directed therapies remains uncertain. Clearly, further characterization of the relationship between p16<sup>INK4a</sup> expression, senescence and regenerative capacity in vivo would have broad implications for the therapeutic treatment of age-related disease.

The Role of p16<sup>INK4a</sup> in Intrinsic vs. Extrinsic Aging

Senescence is typically viewed as a response to detrimental, intrinsic cellular events; however, recent evidence suggests that extrinsic signals also contribute to tissue aging. Above, we discussed the potential for neoplastic transformation to initiate cell non-autonomous p16<sup>INK4a</sup> expression. Although the extracellular mediators of stromal p16<sup>INK4a</sup> induction are undefined, several signaling pathways have been linked to the expression of p16<sup>INK4a</sup> in aging tissues. In muscle satellite cells, age-associated increases in local TGF-β production activate SMAD3, which in turn binds to the p16<sup>INK4a</sup> promoter to initiate gene transcription (94). Notch signaling antagonizes TGF-β, and in doing so can alleviate age-related declines in satellite cell function (94). Similar to TGF-β signaling, changes in thymic and bone marrow structure are reported to promote aging in local progenitor cell populations (142). Together,
these findings put forth the model of “niche aging” wherein stromal changes influence the regenerative capacity of local progenitors. However, parsing the role of the senescent cell versus the niche in aging biology becomes somewhat of a chicken and egg question. After all, senescent cells themselves secrete a large number of pro-inflammatory cytokines associated with age-related disease (Reviewed in: (160)). Future studies aimed at identifying cell non-autonomous $p16^{\text{INK4a}}$ activation signals are clearly needed to better understand the induction of senescence during physiological aging.

$p16^{\text{INK4a}}$ as a Barrier to Pluripotency

Work with induced pluripotent stem cells (iPS) also implicates $p16^{\text{INK4a}}$ as a modulator of regenerative capacity. During iPS generation, senescence induced by the four factor cocktail of OCT4, SOX2, KLF4 and c-MYC serves as a barrier to efficient reprogramming (161). In cells where reprogramming is effective, silencing of $\text{INK4/ARF}$ gene transcription is observed concomitant with the induction of molecular markers indicative of stem cell phenotypes (61). Therefore, it is not surprising that iPS production efficiency is increased by cellular immortalization (162), or shRNA knockdown of $\text{INK4/ARF}$ genes (61, 161). Currently, efficient generation of iPS from older patients represents a major hurdle for regenerative medicine. Therefore, novel reprogramming approaches aimed at curbing the senescent phenotype, may improve iPS technology for the future (163).

Genome-Wide Association Studies (GWAS) Linking $p16^{\text{INK4a}}$ to Age-Related Disease

Meta-analysis of genome-wide association studies (GWAS) suggests that we have only begun to scratch the surface in understanding the role of $p16^{\text{INK4a}}$ in age-related disease. Single nucleotide polymorphisms (SNPs) in chromosome 9p21.3 have been linked to cancer, atherosclerosis, diabetes, frailty, cataracts and late onset Alzheimer’s disease (Fig. 1B and (164)). In many cases, expression of $p16^{\text{INK4a}}$ correlates directly with SNP genotype, suggesting a causal role for $p16^{\text{INK4a}}$ in diverse, age-
associated diseases (164). Several mechanistic models have been suggested to explain how SNPs, some of which are more than 100 kb from the gene promoter, influence $p16^{INK4a}$ expression. One model proposes that the activity of distal enhancer elements is modified by SNP genotype (165). Another provides evidence that $ANRIL$ expression and splicing is directly influenced by 9p21 SNPs (166). We believe that these models are not mutually exclusive.

While mechanisms by which 9p21.3 SNPs influence $p16^{INK4a}$ transcription have been proposed, the link between $p16^{INK4a}$ expression and age-related diseases is not always apparent. For example, atherosclerosis is frequently associated with lipid metabolism, yet 9p21.3 SNPs have emerged as robust indicators of atherosclerotic risk in some of the most widely replicated GWASs conducted to date (167). Evidence from animal models suggests that 9p21.3 SNPs may reduce $INK4/ARF$ gene transcription, leading to altered cellular proliferation and apoptosis which exacerbate disease progression (168-170). However, transgenic mice carrying multiple copies of the $INK4/ARF$ locus are equally susceptible to atherosclerosis (171). Therefore, the mechanisms by which 9p21.3 SNPs influence a large number of age-related human disease remain a subject of ongoing investigation.

Conclusions

Since the discovery of $p16^{INK4a}$ more than 20 years ago, numerous advances have led to an increasingly complex view of $p16^{INK4a}$ regulation and function. The role of $p16^{INK4a}$ clearly extends beyond cancer and aging. Dynamic induction of $p16^{INK4a}$ is observed during mammary involution, wound healing, nerve regeneration and infection (unpublished data and (130, 172-174)). It has been proposed that the induction of $p16^{INK4a}$ during these highly proliferative events is critical to the maintenance of
proper tissue homeostasis. Whether the same signals that trigger p16<sup>INK4a</sup> expression under physiological conditions play a role in tumorigenesis or aging is still unclear. However, p16<sup>INK4a</sup> expression is only transient during processes like mammary involution and wound healing (130, 172, 173). Are p16<sup>INK4a</sup> positive cells cleared by the immune system or can they revert to a phenotype conducive to proliferation? Understanding the role of p16<sup>INK4a</sup> in normal physiology will be critical to the development of ‘senolytic’ therapies, which aim to lengthen our healthspan by eliminating senescent cells in the body.

p16<sup>INK4a</sup> is different than the other INK4/ARF family members. The dynamics of p16<sup>INK4a</sup> expression during senescence make it a robust biomarker of mammalian aging. Human tumors silence p16<sup>INK4a</sup> with greater frequency than ARF or p15<sup>INK4b</sup> (140), suggesting that the tumor suppressor function of p16<sup>INK4a</sup> is somehow more critical than the other INK4/ARF family members. As such, the therapeutic restoration of p16<sup>INK4a</sup> activity appears to be a promising avenue for anti-neoplastic development. Ironically, while drug development teams in the field of oncology work fervidly to move CKD4/6 inhibitors into the clinic; aging biologists aim to block the accumulation of p16<sup>INK4a</sup>-positive cells. Oddly, the key to longevity likely lies in the hands of both groups, as a careful balance of p16<sup>INK4a</sup> expression is required to stave off cancer and prevent aging.

Acknowledgements

The authors thank M. Waqas, J. Gillahan, S.T. Nguyen and Drs. D. Beach (Barts, UK), C. J. Burd (OSU) and N. Sharpless (UNC) for critical reading of the manuscript. Dr. K. Hoadley (UNC) provided advice and guidance regarding the analysis of TCGA data. This work was supported by NIH R00AG036817 (C.E.B) and American Cancer Society IRG-67-003-50 (C.E.B).
References


**Figure Legends**

**Figure 1.** Function, Structure and Polymorphisms of the INK4/ARF Locus. A, p15<sup>INK4b</sup> and p16<sup>INK4a</sup> both function in the RB tumor suppressor pathway through inhibition of CDK4/6 activity. Expression of p14<sup>ARF</sup> inhibits the E3 ubiquitin ligase activity of MDM2, leading to stabilization of p53. The p53 and RB pathways play integral roles in blocking inappropriate cellular proliferation. B, Packed into 35 kilobases of chromosome 9p21.3 are three well-characterized tumor suppressor genes: p14<sup>ARF</sup>, p15<sup>INK4b</sup> and p16<sup>INK4a</sup>. GWAS have implicated 9p21.3 SNPs in cancer, heart disease, glaucoma, type II diabetes, autism and endometriosis. The majority of the SNPs lie outside of transcript and coding regions in a recently discovered long, non-coding RNA, ANRIL. Of the identified SNPs, those that have been shown to correlate with CDKN2A expression in at least one study are filled with grey. Other SNPs that have not been correlated with CDKN2A expression in validation studies, or have yet to be examined are filled with black or white, respectively.

**Figure 2.** Mechanisms of p16<sup>INK4a</sup> Regulation by Chromatin Modification. A, p16<sup>INK4a</sup> is negatively regulated by the histone modifying complexes, PRC1 and PRC2, which combine to lay down repressive marks (e.g. H3K27me3, H2AK119ub) throughout the INK4/ARF locus. Several associated proteins (APs), including RNA binding proteins (RBPs), are reported to facilitate PRC1/2 interaction with the p16<sup>INK4a</sup> promoter. APs and RBPs discussed in the text are shown. Elevated expression of the PRC2 component, EZH2, is also reported to enhance INK4/ARF gene silencing. Proteins reported to transactivate EZH2, leading to PRC-mediated silencing of p16<sup>INK4a</sup> are depicted above. B, Activation of p16<sup>INK4a</sup> is associated with decreases in PRC1/2 levels and the removal of repressive histone marks. JMJD3 demethylates H3K27me3 and subsequent chromatin decondensation promotes access by transcription factors and
p16\textsuperscript{INK4a} transcription while JDP2 binds H3K27 and prevents further methylation. CTCF maintains chromosomal boundaries and 3-dimension structure of the chromatin surrounding p16\textsuperscript{INK4a}.

Figure 3. Transcriptional Regulation of p16\textsuperscript{INK4a}. Expression of p16\textsuperscript{INK4a} requires the action of transcription factors that recruit and/or facilitate RNA polymerase association with the promoter (shown in green). Opposing this action are transcriptional repressors (shown in red). Direct interactions with the p16\textsuperscript{INK4a} promoter are depicted by a solid line, indirect interactions with a dotted line. The numbers below each binding site indicate the position of protein interaction relative to the p16\textsuperscript{INK4a} transcriptional start site. All locations correspond to the human genome unless designated by an ‘(m)’, which signifies the mouse genome. Proteins predicted to share a common binding site are depicted over top of one another.

Figure 4. Alterations in the p16\textsuperscript{INK4a} Tumor Suppressor Pathway are Frequent in Human Cancer. A, Data from TCGA was obtained and analyzed using cBioPortal (112, 113). The tumors analyzed are as follows: Bladder Urothelial Carcinoma (BLUCA), Glioblastoma Multiforme (GBM), Head and Neck Squamous Cell Carcinoma (HNSC), Lung Squamous Cell Carcinoma (LUSC), Skin Cutaneous Melanoma (SKCM), Ovarian Serous Cystadenocarcinoma (OV), Lung Adenocarcinoma (LUAD), Stomach Adenocarcinoma (STAD), Breast Invasive Cancer (BRCA), Uterine Corpus Endometrial Carcinoma (UCEC), Brain Lower Grade Glioma (LGG), Colon and Rectum Adenocarcinoma (COAD/READ), Prostate Adenocarcinoma (PRAD), Kidney Renal Papillary Cell Carcinoma (KIRP), Kidney Renal Clear Cell Carcinoma (KIRC), Acute Myeloid Leukemia (AML). The percent of tumors with mutations or copy number changes in the p16\textsuperscript{INK4a} tumor suppressor pathway are shown. For the purposes of this analysis the p16\textsuperscript{INK4a} tumor suppressor pathway was defined to contain: CDKN2A (p16\textsuperscript{INK4a}), RB1 (RB), CCND1 (CYCLIN D1), CCND2 (CYCLIN D2), CCND3.
(CYCLIN D3), CCNE1 (CYCLIN E1), and CCNE2 (CYCLIN E2). B, OncoPrints from cBioPortal show aberrations in individual tumors across the X-axis. C, Methylation of CDKN2A (black bars) and RB1 (grey bars) was quantified using HM27 or HM450 TCGA data. Genes were considered methylated if β-values exceeded 0.2.

Figure 5. Extrinsic Versus Intrinsic Activation of p16\textsuperscript{INK4a}. A, Injection of Lkb1 null endometrial cancer cells into a syngenic mouse heterozygous for the p16\textsuperscript{LUC} reporter causes stromal luciferase expression upon tumor growth. Injection of Matrigel vehicle on the opposite flank does not alter p16\textsuperscript{INK4a} expression. See also: (130) B, Intrinsic signals induces p16\textsuperscript{INK4a} expression in damaged, senescent or transformed cells. Alterations in surrounding the cellular milieu can trigger the induction of p16\textsuperscript{INK4a} in nearby undamaged cells through an unknown pathway.
Figure 4
Figure 5

A

$p16^{\text{LUC}}$ null tumor cells

$p16^{\text{LUC/WT}}$

B

Tumor Cell (Intrinsic Signaling)

↑p16^{\text{INK4a}}

Cytokines?

Δ Local Nutrients?

Structural Δ?

Stromal Cell (Extrinsic Signaling)

↑p16^{\text{INK4a}}
Molecular Cancer Research

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\textit{Mol Cancer Res} Published OnlineFirst October 17, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-13-0350

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