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Critical Review

INK4 Proteins, a Family of Mammalian CDK Inhibitors with Novel Biological Functions

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Summary

The cyclin D-Cdk4-6/INK4/Rb/E2F pathway plays a key role in controlling cell growth by integrating multiple mitogenic and antimitogenic stimuli. The members of INK4 family, comprising $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, block the progression of the cell cycle by binding to either Cdk4 or Cdk6 and inhibiting the action of cyclin D. These INK4 proteins share a similar structure dominated by several ankyrin repeats. Although they appear to be structurally redundant and equally potent as inhibitors, the INK4 family members are differentially expressed during mouse development. The striking diversity in the pattern of expression of INK4 genes suggested that this family of cell cycle inhibitors might have cell lineage-specific or tissue-specific functions. The INK4 proteins are commonly lost or inactivated by mutations in diverse types of cancer, and they represent established or candidate tumor suppressors. Apart from their capacity to arrest cells in the G1-phase of the cell cycle they have been shown to participate in an increasing number of cellular processes. Given their emerging roles in fundamental physiological as well as pathological processes, it is interesting to explore the diverse roles for the individual INK4 family members in different functions other than cell cycle regulation. Extensive studies, over the past few years, uncover the involvement of INK4 proteins in senescence, apoptosis, DNA repair, and multistep oncogenesis. We will focus the discussion here on these unexpected issues.

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Keywords cell cycle; cancer; INK4; DNA repair; apoptosis; senescence.

INTRODUCTION

The most critical decision that every proliferating mammalian cell must make is whether to continue another round of cell division or to exit the cell cycle and reach a quiescent state. Likewise, quiescent cells must decide whether to continue in their non proliferative state or to re-enter the cell cycle. All cells have the capacity to enter quiescence and all quiescent cells, except those that have reached a state of terminal differentiation, have the capacity to re-enter the cycle. So, which are the mechanisms that control these important decisions and how can small imbalances in these regulatory pathways lead to cancer?

The basic cell cycle is divided into four phases. During two of these phases, cells execute the two basic events in cell division: generation of a single and faithful copy of its genetic material (S phase) and partitioning of all the cellular components between two identical daughter cells (M phase). The two other phases of the cycle, G1 and G2, represent 'gap' periods, during which cells prepare themselves for the successful completion of the S and M phases, respectively. When cells cease proliferation, either due to specific antimitogenic signals or to the absence of proper mitogenic signalling, they exit the cycle and enter a non-dividing quiescent state known as G0. To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one (*1, 2*).

Progression through each phase of the cell cycle is governed by cyclin-dependent kinases (Cdks). The Cdks are a family of Ser/Thr protein kinases, which are activated by phosphorylation/dephosphorylation events and binding to regulatory subunits or cyclins to form heterodimers. Mitogen stimulation induces entry and progression through G1 in part by ligand binding to receptors, activating multiple signalling pathways that converge on the transcription of immediate early genes, D-type cyclins, and cyclin assembly to Cdk4/6 kinases (*3*).

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Activated cyclin D-Cdk4/6 complexes preferentially phosphorylate retinoblastoma protein (pRb) and pRb-related proteins p107 and p130 (*4*). This initial pRb phosphorylation is followed by additional phosphorylation by the cyclin E-CDK2 holoenzyme. Once phosphorylated, pRb and related proteins release tethered E2F transcriptions factors that, in complex with DP subunits DP1 and DP2, either activate (E2F-1, 2, and 3) or repress (E2F- 4 and 5) gene transcription essential for the G1 to S phase transition and commitment to mitosis (*5*).

Cyclin-Cdk complexes are negatively regulated by small polypeptides, the Cdk-inhibitors (CKIs). In mammals, CKIs are divided into two families. The INK4 family, that specifically bind and inhibit Cdk4/6, includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. The Cip/Kip family comprises $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. Whereas Cip/Kip proteins act as negative regulators of cyclin E- and cyclin A-Cdk2 and cyclin B-Cdk1 holoenzymes in a 1:1 stoichiometry, they also act as positive regulators of cyclin D-Cdk4/6 complexes by mediating their assembly early in G1 (*3*). This results in the titration of $p27^{Kip1}$ from cyclin E-CDK2, and the activation of this complex. In turn, $p27^{Kip1}$ is phosphorylated by cyclin E-Cdk2, targeting it for degradation. Instead, INK4 proteins compete with D-type cyclins for binding Cdk4/6. Enforced expression of INK4 proteins can lead to G1 arrest by promoting the redistribution of Cip/Kip proteins and blocking cyclin E-Cdk2 activity. Thus, in cycling cells, there is a reassortment of Cip/Kip proteins between Cdk4/6 and Cdk2 as cells progress through G1 alternately acting as positive or negative regulators of Cdk activity (*3, 6*).

The four proteins of the INK4 family share a similar structure dominated by several ankyrin repeats. Although they appear to be structurally redundant and equally potent as inhibitors, the INK4 family members are differentially expressed during mouse development. The striking diversity in the pattern of expression of INK4 genes suggested that this family of cell cycle inhibitors might have cell lineage-specific or tissue-specific functions. Apart from their physiological roles, the INK4 proteins are commonly lost or inactivated by mutations in diverse types of cancer, and they represent established or candidate tumor suppressors (*7, 8*). Extensive studies over the recent past years uncover the involvement of INK4 proteins in different functions other than cell cycle regulation. We will focus the discussion here on these unexpected issues.

TUMORIGENESIS

The *INK4a/ARF/INK4b* locus is deleted in a wide variety of tumors including melanoma, pancreatic adenocarcinoma, glioblastoma, leukemias and bladder carcinoma. In a relative small 35 kb region of the human genome, the locus encodes three related genes (*ARF*, *p15INK4b* and *p16INK4a*) that instruct for distinct tumor suppressor proteins (*9*). While *p15INK4b* has its own open reading frame that is physically distinct, *p16INK4a* 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 these polypeptides are not isoforms and do not share any amino acid homology. Both $p16^{INK4a}$ and $p15^{INK4b}$ have appropriate biochemical properties for tumor suppression by acting as inhibitors of the Cdk4/6 that regulate progression through the G1 phase of the cell cycle. The tumor suppressor activity of ARF is largely ascribed to its ability to regulate p53 in response to aberrant growth or oncogenic stresses. ARF binds to and inactivates the MDM2 protein, and MDM2 in turn negatively regulates p53 (*9*). As human cancers frequently harbor homozygous deletions of the *INK4a/ARF/INK4b* locus that abrogate expression of all three proteins, considerable dispute has focused on which member of the locus represents the principal tumor suppressor activity located at human chromosome 9p21. Knockout studies of mice specifically deficient for Arf, $p15^{INK4b}$ and $p16^{INK4a}$ have revealed that all three strains are more prone to spontaneous cancers than wild-type littermates, but that each of these single knockouts appears less tumor prone than animals lacking both $p16^{INK4a}$ and Arf (*10*). 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 (*11*). Overexpression of the *INK4a/Arf/INK4b* locus in mice also supports its role in tumor suppression. Recently, mice carrying a transgenic copy of the entire *INK4a/Arf/INK4b* locus were characterized (*12*). Cells derived from these mice have increased resistance to *in vitro* immortalization and oncogenic transformation. Importantly, 'super *INK4a/Arf* mice' manifest higher resistance to cancer compared to normal, nontransgenic, mice and display normal aging and lifespan. Therefore, the authors suggest that an increase in tumor suppression can be achieved by increased *INK4a/Arf/INK4b* activity without increased aging.

Specific somatic loss of $p16^{INK4a}$, through point mutation or small deletion, has been reported in thousands of human cancers (*13, 14*). Similarly, at least 56 distinct germline mutations targeting only *p16INK4a*, and sparing *ARF* and *p15INK4b*, have been described in unrelated kindreds that are cancer prone. Finally, *p16INK4a* 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. Definitely, $p16^{INK4a}$ is an important suppressor of human cancer. On the other hand, specific genetic lesions of *p15INK4b*, which do not also inactivate *p16INK4a* or *ARF*, are not well described. Specific epigenetic silencing by hypermethylation of $p15^{INK4b}$ gene has been demonstrated in rare glial tumors and certain hematologic neoplasma including leukemia and myelodysplasia (*15*). In myelodysplasia, *p15INK4b* hypermethylation has been reported in the absence of *p16INK4a* hypermethylation and in some of these

cases the expression of $p15^{INK4b}$ can be reactivated in response to treatment with inhibitors of DNA methyltransferase (*16*). Furthermore, because of their overlapping biochemical function, co-deletion of *p15INK4b* with *p16INK4a* 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) appear to show very high frequencies of homozygous deletion of 9p21 (targeting all three proteins) rather than specific inactivation of p16INK4a or ARF. Therefore, $p15^{INK4b}$ is probably an important suppressor of human cancers too, particularly in the hematopoietic lineages.

Genetic alterations of *p18INK4c* gene are rare events in human tumors. However, several studies reported that $p18^{INK4c}$ is a haploinsufficient tumor suppressor in mice, suggesting the possibility that a quantitative decrease of this INK4 may be involved in human tumorigenesis (*17*). In this regard, a recent work demonstrates that activation of PKC promotes human cancer cell growth through downregulation of $p18^{INK4c}$ in an AP-1 activation-independent manner (*18*). Furthermore, a large variety of structurally and mechanistically distinct PKC inhibitor was reported to have potent antitumor activity *in vitro* and *in vivo*. Some of these agents are now undergoing clinical evaluation (*19*). Therefore, it will be of great interest to investigate the expression levels of $p18^{INK4c}$ in human tumors with stimulated PKC activity, and to examine the role of $p18^{INK4c}$ in anti-PKC-based therapy for human malignancies. Additionally, p18INK4c gene silencing by promoter methylation has been observed in Hodgkin lymphoma (*20*) and medulloblastoma (*21*).

p19INK4d null mice do not develop tumors or other types of proliferative disorders. Furthermore, they are not more susceptible to tumor formation than wild-type mice in response to carcinogenic treatment. These results suggest that $p19^{INK4d}$ is not a tumor supresor (*8*). The only significant phenotype observed in *p19INK4d* knock out mice is testicular atrophy, albeit the animals remain fertile (*22, 23*). This phenotype is associated with increased apoptosis of germ cells, a result that correlates well with the high expression levels of $p19^{INK4d}$ in this tissue (*24*).

One common question is why *p16INK4a* is frequently mutated in human cancers whereas the other three *INK4* genes are rarely altered. Several pieces of evidence indicate that the basis for the physiological differences between individual INK4 most likely lies in their upstream regulation. Detailed description of the elements and factors involved in INK4 transcription has been recently reviewed (*6, 25*).

Despite that *INK4b*/*ARF*/*INK4a* locus encodes three tumor suppressors which together constitute one of the main anti-oncogenic defenses of mammalian organisms, little is known about the mechanisms that govern the expression of this locus. A new mechanism of inactivation of the *INK4b*/*ARF*/*INK4a* locus has been reported very recently (*26*). The authors have identified a conserved DNA element located near the transcription initiation site with the capacity to regulate the locus in a global manner. Inactivation of this element, which the authors named $RD^{INK4/ARF}$, results in the silencing of the entire locus. Interestingly, $RD^{INK4/ARF}$ is both a transcriptional regulatory element and a replication origin. The replication protein Cdc6 binds to $RD^{INK4/ARF}$ and is able to recruit histone deacetylases that, in turn, result in the heterochromatinization and repression of the *INK4b*/*ARF*/*INK4a* locus. This model, although unprecedented in vertebrates, has striking parallelisms with the silencing of the yeast mating-type *HM* locus of the yeast *S. cerevisiae* (*27*). The Cdc6 protein is aberrantly overexpressed in some human cancers and its oncogenicity could be related to the functional inactivation of the locus (*28, 29*). Thus, this proposal represents a novel oncogenic mechanism that connects the replication machinery with the inactivation of tumor suppressors.

SENESCENCE

According to a current hypothesis on aging, senescence cells accumulate in the organism and this result in failure of organ homeostasis and function. Cellular senescence – characterized by the permanent arrest of cell proliferation – is detrimental to the regenerative capacity of organs during aging. However, the senescence checkpoint is also considered to be a major mechanism for suppressing tumors, protecting the organism from cancer during early life (*30*). A number of stimuli have been identified as inducing senescence and these include telomere shortening, DNA damage, oxidative stress, sustained mitogen stimulation, and other cellular stresses. Senescence induced by telomere shortening has been called 'replicative senescence' and is a result of DNA damage-like signals generated by dysfunctional telomeres (*31*). In addition to telomere attrition, several other mechanisms can abruptly induce senescence independently of telomere length, termed 'premature senescence', including, overactivation of mitogenic pathways such as Ras, Raf, or MEK, or overexpression of E2F (*32*).

Recent evidence from several groups, have confirmed seminal observation of Sherr and colleagues (*33*), indicating 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. 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.

Senescent cells remain permanently insensitive to mitogenic signals, owing to the combined action of the p53 and $p16^{INK4a}$-Rb tumor suppressor networks. Although the stability of senescence presumably contributes to its anti-neoplasic activities, the molecular machinery that drives the cell into a permanent state of proliferative arrest is poorly understood (*34*). In some cell types, senescence is associated with global changes in chromatin structure, leading to the accumulation of heterochromatin protein 1 (HP1) and histone H3 trimethylated on lysine 9 in senescence-associated heterochromatin foci (SAHF) and on the promoters of certain cell cycle genes (*35*). Such changes could promote senescence by producing a repressive chromatin environment that prevents transcription of growth-promoting genes, thereby producing an extreme insensitivity to mitogenic stimulation. In this regard, authors of a recent reports claim that High-Mobility Group A (HMGA) proteins, which are typically associated with gene activation and have been linked to proliferation and tumorigenesis, accumulate on the chromatin of senescent fibroblasts and are essential structural components of SAHF (*36*). Thus HMGA proteins act in a mutually reinforcing manner with $p16^{INK4a}$ to promote SAHF formation and contribute to the stable repression of E2F target genes (*37*). Accordingly, suppression of both HMGA and $p16^{INK4a}$ in growing cells acts synergistically to enable cells to bypass senescence and leads to the reactivation of some E2F target genes and S phase re-entry in senescent cells.

Recently, Krishnamurthy et al. attempted to identify molecular markers of aging in mice and rats by exploiting the expression levels of several known cell cycle inhibitors (*38*). They show that the expression levels of both $p16^{INK4a}$ and ARF mRNA markedly increase with aging in most murine tissues, whereas there is no significant change in the expression levels of other related cell cycle inhibitors such as $p15^{INK4b}$, $p21^{Cip1}$ and $p27^{Kip1}$ with aging. At the level of transcription, the expression of $p16^{INK4a}$ is modulated by three principal regulators, ETS1, inhibitor of DNA binding 1, and beta lymphoma Mo-MLV insertion region (BMI1) (*39*). The evidence presented by the authors indicates that the age-related increase in $p16^{INK4a}$ and ARF expression can be attributed largely to the expression of ETS1. These data suggest that, in addition to its role as senescence effector, $p16^{INK4a}$ is a robust biomarker of mammalian aging (*40*).

In cancer the levels of BMI1 or the histone methyltransferase EZH2 are frequently found to be up-regulated, and have been associated with cancer development (*41*). A recent communication presents evidence demonstrating that the repression of the *INK4b-ARF-INK4a* locus by BMI1 requires its direct association and is dependent on the continued presence of the EZH2-containing Polycomb-Repressive Complex 2 (PCR2) complex (*42*). Significantly, EZH2 is down-regulated in stressed and senescing populations of cells, coinciding with decreased levels of associated trimethylation of lysine 27 of histone 3, displacement of BMI1, and activation of transcription. These results provide a model for how Polycomb proteins contribute to cancer and how they are related to senescence condition.

Even though numerous aging-related stimuli have been reported to increase *p16INK4a* gene expression (*32*), less is known about the posttranslational regulation of its products (*43*). In a recent report, authors demonstrate the presence of an instability determinant within the 3′-untranslated region (UTR) of the $p16^{INK4a}$ mRNA human diploid fibroblasts (*44*). The $p16^{INK4a}$ 3′-UTR was found to be specific target of AUF1, an RNA binding protein implicated in promoting mRNA decay. Both AUF1 levels and AUF1-$p16^{INK4a}$ mRNA associations were strikingly more abundant in early-passage than late-passage fibroblast cultures. Moreover, short interfering RNA-based reductions in AUF1 levels increased the stability of $p16^{INK4a}$ 3′-UTR containing transcripts, elevated the expression of $p16^{INK4a}$ and accentuate the senescent phenotype.

The role of $p16^{INK4a}$ in senescence was challenged in a recent report. Reactive oxygen species (ROS) serve many cellular functions; for example, second messenger, antibacterial agent, mutagen, aging-accelerant and growth stimulant. With regard to neoplasia, the view has generally been that ROS cause cancer through a number of mechanisms, including the induction of DNA damage and alteration of intracellular signalling (*45*). A provocative new report, however, suggests that ROS have an unexpected role in inducing and maintaining senescence-induced tumor suppression (*46*). The authors make a particularly tantalizing speculation as to how this process may work: Rb, through E2F transcription factors, regulates enzymes involved in ROS production and metabolism. Therefore, mitogenic stimulation induces a growth-related production of ROS, while concomitant Rb activation induces an enzymatic production of ROS and impedes ROS degradation, suggesting that these cellular signals converge to produce sharply elevated ROS levels. In agreement with this hypothesis, the authors showed that p16INK4a expression in normal fibroblasts, which presumably does little more than activate Rb, induced ROS levels to almost the same levels as the strong proliferative signals associated with the ectopic expression of oncogenic Ras. Because of convincing data linking ROS and aging, many experts in the field have wondered whether ROS promotes senescence by inducing $p16^{INK4a}$ expression and Rb activation. Although this may be, the authors show that, in fact, $p16^{INK4a}$-Rb activation can induce ROS.

Tissue repair and regeneration are essential for longevity in complex animals, and often depend on the proliferation of unspecialized cells known as stem or progenitor cells. In many tissues, the regenerative capacity of such cells declines with age, and it is thought that this decline drives many age-related symptoms. But stem/progenitor-cell proliferation is a double-edged sword. Although it ensures tissue repair and regeneration, it also puts tissues at risk of hyperproliferative diseases,

the most deadly of which is cancer (*47*). This risk is mitigated by tumour-suppressor mechanisms, which either eliminate potential cancer cells by programmed cell death (apoptosis) or prevent their proliferation, often by permanently halting the cell division cycle (senescence). Therefore, the benefits to longevity afforded by stem/progenitor-cell proliferation might be compromised by mechanisms that prevent life-threatening cancer (*48*).

Present 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 self-renewing compartments *in vivo*. Three papers, recently published, uncover a new role for the $p16^{INK4a}$ in promoting aging, a role shared by the p53 tumor suppressor. These papers, using $p16^{INK4a}$-deficient and overexpressing mice to study self-renewal in three distinct tissues: hemoatopoietic stem cells (HSC), neural stem cells (NSC), and pancreatic islets, have suggested that $p16^{INK4a}$ expression is one cause of aging in these tissues (*49–51*). 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 activity 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 displayed 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.

APOPTOSIS AND DNA REPAIR

To ensure survival and propagation of accurate copies of the genome on to subsequent generations, eukaryotic cells respond to damaged DNA by a multifaceted response that coordinates cell cycle progression with DNA repair, chromatin remodelling, transcriptional programs and other metabolic adjustments or cell death (*52, 53*).

In spite of these mechanisms, the enzymes responsible for DNA repair do not always properly fix damaged DNA, and some lesions may remain unrepaired or be misrepaired. Residual lesions can lead to apoptosis. However, if apoptosis is inhibited for some reason, this increases the risk of chromosomal instability at several levels, and cells that are sufficiently robust to survive can be at a growth advantage, which ultimately can lead to cancer. Genetic instabilities, including changes to chromosome numbers and chromosomal aberrations, are frequent in human cancers and are thought to be required for the generation of the multiple genetic hits necessary for malignant transformation (*54*). The maintenance of a switching mechanism that shifts the cell from DNA repair to apoptosis, as appropriate in the presence of excessive DNA damage, appears to be of central importance for avoiding progression to cancer (*55*). There is much evidence supporting the fact that apoptosis might relate to DNA repair. It may be accomplished through proteins that participate in the detection of DNA damage and in DNA repair and can directly relay to the apoptotic machinery (*56*).

Several recent works have been reported that proteins from the INK4 family could be involved in the cellular response to genotoxic agents. In one of these papers, the authors show that $p16^{INK4a}$-compromised cells, osteosarcoma U2OS cell line as well as *p16INK4a* $-/-$ MEFs are sensitive to apoptosis, following treatment with ultraviolet light (*57*). On the other hand, only a small proportion of p16-proficient EH1/EH2 cells and MEF *p16INK4a* $+/+$ cells underwent UV-induced apoptosis, showing that this tumor suppressor protects cells against UV damage-mediating apoptosis in both human and mouse cells. Importantly, the UV-mediated apoptosis observed in U2OS coincided with a decrease in the level of the antiapoptotic Bcl-2 protein, with no effect on the level of the apoptosis agonist Bax protein. However, in EH1 cells the reduction in UV-induced apoptosis was associated with downregulation of Bax expression, whereas the level of Bcl-2 protein remained constant. This suggests that $p16^{INK4a}$ negatively controls UV-induced apoptosis by downregulating the apoptosis agonist Bax protein. However, in the absence of $p16^{INK4a}$, UV damage triggers apoptosis by downregulating the apoptosis antagonist Bcl-2. Therefore, $p16^{INK4a}$ would control UV-dependent apoptosis through the intrinsic mitochondrial cell death pathway, which is under the control of the p53-tumor-suppressor protein. Further evidences were reported by Le et al. (*58*) demonstrating that, after DNA damage, $p16^{INK4a}$ overexpression, like $p21^{Cip1}$ and $p27^{Kip1}$, in tumoral cells caused cell cycle arrest and inhibited genotoxic-induced apoptotic events such as cytochrome c release, mitochondrial membrane depolarization, and activation of the caspase cascade. These observations suggest that by blocking dysregulated cell cycle progression, CDK inhibitors can influence the sensitivity of the mitochondria to proapoptotic signals in DNA damage-induced cancer cells.

Recent observation contributes to this hypothesis involving $p16^{INK4a}$ in triggering apoptosis. *INK4a* $-/-$ mice possess increased thymus size and cellularity, thus suggesting the involvement of $p16^{INK4a}$ in the control of thymocyte proliferation (*59*). In these animals, authors found increased numbers of CD8 and CD4 T lymphocytes in thymus and spleen. Unexpectedly, this was not related to an increase in T-cell division rates, which were similar in lymphoid organs of

wild type mice. In contrast, T-cell apoptosis rates were significantly decreased in thymus and spleen from *INK4a* $-/-$. Moreover, whereas $p16^{INK4a}$-deficient and wild type T cells were equally sensitive to Fas or TCR-mediated apoptosis, the former were clearly more resistant to apoptosis induced by oxidative stress or gamma irradiation. These results indicate that $p16^{INK4a}$ function is associated with T-cell apoptosis, and subsequently contributes to the control of T-cell population size in lymphoid organs.

On the other hand, in our laboratory, we obtained strong evidence that another INK4 member, $p19^{INK4d}$, plays a crucial role in regulating genomic stability and overall cell viability under conditions of genotoxic stress (*60, 61*). By using different cell lines, like human neuroblastoma derived SH-SY5Y cells, mouse Leydig tumor MA-10 cells, and hamster fibroblast BHK21 cells, proficient or deficient in $p19^{INK4d}$ expression, we have demonstrated that $p19^{INK4d}$-overexpressing cells exhibited enhanced DNA repair activity after UV irradiation, whereas p19-deficient cells showed diminished ability to repair DNA lesions. Notably, improvement of DNA repair in these cells inversely correlated with the apoptosis triggered by DNA damage. In this regard, our results showing that $p19^{INK4d}$ overexpression confers resistance to cells exposed to UV irradiation suggest that, in addition to its role in improving DNA repair, $p19^{INK4d}$ would negatively modulate DNA damage-induced apoptosis in mammalian cells. On this matter, recent studies performed in *p19INK4d* null mice, demonstrated that knockdown of $p19^{INK4d}$ rendered cells sensitive to apoptotic and autophagic cell death and suggest a possible role of $p19^{INK4d}$ induction in chemoprevention (*62, 63*).

We do not know yet whether $p19^{INK4d}$ exerts a direct inhibitory effect over some step of apoptotic pathways or if it plays an indirect role that reflects the increased efficiency of the mechanisms involved in DNA damage removal. However, the action of a protein allowing a more robust response against DNA injury and, besides, diminishing apoptotic cell death would be physiologically relevant. Given that both DNA repair and apoptosis are energy-demanding processes, the proper utilization of the available ATP in the cell is of vital importance (*64*). If repair of DNA damage is prolonged in any given cell an 'energy catastrophe' will occur if, concomitantly, apoptosis is triggered in the meantime. Thus, induction of $p19^{INK4d}$ in response to DNA damage would be directed to the maintenance of genomic stability by increasing DNA repair effectiveness and to the prevention of a considerable investment of energy by delaying the commitment to programmed cell death. Finally, how might $p19^{INK4d}$ exert its effect in response to DNA damage? Our results strongly support a CDK4-binding independent mechanism, suggesting that the role of $p19^{INK4d}$ in DNA repair is distinct from its cell cycle regulatory role and described a new function of this protein (*60, 61*). Considering its function as cell cycle inhibitor, as a whole, these results suggest that $p19^{INK4d}$ may itself play a role in DNA repair, in cell cycle checkpoint response, or in integrating apoptosis and DNA repair response.

CONCLUSIONS

It has been just over a decade since the discovery of the INK4 family of CDK inhibitors. The extensive studies during these last few years have contributed significantly to our current understanding of mammalian G1 control at the cellular and biochemical levels. However unresolved and controversial issues still remain. Given their emerging roles in fundamental physiological as well as pathological processes, the biochemical similarities and evolutionary homology of the INK4 family members raise and important question about redundant versus non-overlapping features among them. Recent evidence, discussed here, support that, in addition to their role as cell cycle regulators, the individual INK4 family members would perform diverse and distinct cellular tasks. This statement is not surprising and there is a good reason to believe that we will be promptly uncovering new functions for the INK4 proteins in diverse cellular mechanisms. Inevitably, various cellular pathways, such as cell growth, differentiation, senescence, DNA repair, apoptosis and various checkpoint controls, must interact with the pathways that regulate the progression through the G1 phase of the cell cycle.

An open question still remains: which are the mechanisms involved in these INK4 novel functions? Some of them, like its roles in tumorigenesis or senescence are closely related to their ability to inhibit CDK4/CDK6. In this case the common structural motif implicated in CDK4/CDK6 binding could suffice to confer such properties. Thus, INK4 specific functions could rely on its upstream regulation. However, other recently described functions of INK4 proteins, like those related to apoptosis and DNA repair seem to be dissociated from their role in CDK4/CDK6 modulation. Why certain INK4 exert these functions and other does not? Perhaps, the study of minor, although potentially relevant variations in INK4 structure or differential postranslational modifications would reveal key determinants that allow delineation of their emerging individual roles.

REFERENCES

1. Roussel, M. F. (1999) The INK4 family of cell cycle inhibitors in cancer. *Oncogene* **18**, 5311–5317.
2. Malumbres, M. and Barbacid, M. (2001) To cycle or not to cycle: a critical decision in cancer. *Nat. Rev. Cancer* **1**, 222–231.
3. Massague, J. (2004) G1 cell-cycle control and cancer. *Nature* **432**, 298–306.
4. Sherr, C. J., and Roberts, J. M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501–1512.
5. Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* **98**, 859–869.

6. Trimarchi, J. M., and Lees, J. A. (2002) Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell. Biol.* **3**, 11–20.
7. Pei, X. H., and Xiong, Y. (2005) Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues. *Oncogene* **24**, 2787–2795.
8. Ortega, S., Malumbres, M., and Barbacid, M. (2002) Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim. Biophys. Acta* **1602**, 73–87.
9. Kim, W. Y., and Sharpless, N. E. (2006) The regulation of INK4/ARF in cancer and aging. *Cell* **127**, 265–275.
10. Sharpless, N. E., Ramsey, M. R., Balasubramanian, P., Castrillon, D. H., and DePinho, R. A. (2004) The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene* **23**, 379–3785.
11. Bardeesy, N., Aguirre, A. J., Chu, G. C., Cheng, K. H., Lopez, L. V., Hezel, A. F., Feng, B., Brennan, C., Weissleder, R., Mahmood, U., Hanahan, D., Redston, M. S., Chin, L., and Depinho, R. A. (2006) Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. *Proc. Natl. Acad. Sci. USA* **103**, 5947–5952.
12. Matheu, A., Pantoja, C., Efeyan, A., Criado, L. M., Martin-Caballero, J., Flores, J. M., Klatt, P., and Serrano, M. (2004) Increased gene dosage of Ink4a/Arf results in cancer resistance and normal aging. *Genes Dev.* **18**, 2736–2746.
13. Ruas, M., and Peters, G. (1998) The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim. Biophys. Acta* **1378**, F115–177.
14. Forbes, S., Clements, J., Dawson, E., Bamford, S., Webb, T., Dogan, A., Flanagan, A., Teague, J., Wooster, R., Futreal, P. A., and Stratton, M. R. (2006) Cosmic 2005. *Br. J. Cancer* **94**, 318–322.
15. Esteller, M., Corn, P. G., Baylin, S. B., and Herman, J. G. (2001) A gene hypermethylation profile of human cancer. *Cancer Res.* **61**, 3225–3229.
16. Aggerholm, A., Holm, M. S., Guldberg, P., Olesen, L. H., and Hokland, P. (2006) Promoter hypermethylation of p15INK4B, HIC1, CDH1, and ER is frequent in myelodysplastic syndrome and predicts poor prognosis in early-stage patients. *Eur. J. Haematol.* **76**, 23–32.
17. Bai, F., Pei, X. H., Godfrey, V. L., and Xiong, Y. (2003) Haploinsufficiency of p18(INK4c) sensitizes mice to carcinogen-induced tumorigenesis. *Mol. Cell. Biol.* **23**, 1269–1277.
18. Matsuzaki, Y., Takaoka, Y., Hitomi, T., Nishino, H., and Sakai, T. (2004) Activation of protein kinase C promotes human cancer cell growth through downregulation of p18(INK4c). *Oncogene* **23**, 5409–5414.
19. Virchis, A., Ganeshaguru, K., Hart, S., Jones, D., Fletcher, L., Wright, F., Wickremasinghe, R., Man, A., Csermak, K., Meyer, T., Fabbro, D., Champain, K., Yap, A., Prentice, H. G., and Mehta, A. (2002) A novel treatment approach for low grade lymphoproliferative disorders using PKC412 (CGP41251), an inhibitor of protein kinase C. *Hematol. J.* **3**, 131–126.
20. Sanchez-Aguilera, A., Delgado, J., Camacho, F. I., Sanchez-Beato, M., Sanchez, L., Montalban, C., Fresno, M. F., Martin, C., Piris, M. A., and Garcia, J. F. (2004) Silencing of the p18INK4c gene by promoter hypermethylation in Reed-Sternberg cells in Hodgkin lymphomas. *Blood* **103**, 2351–2357.
21. Uziel, T., Zindy, F., Xie, S., Lee, Y., Forget, A., Magdaleno, S., Rehg, J. E., Calabrese, C., Solecki, D., Eberhart, C. G., Sherr, S. E., Plimmer, S., Clifford, S. C., Hatten, M. E., McKinnon, P. J., Gilbertson, R. J., Curran, T., Sherr, C. J., and Roussel, M. F. (2005) The tumor suppressors Ink4c and p53 collaborate independently with Patched to suppress medulloblastoma formation. *Genes Dev.* **19**, 2656–2667.
22. Zindy, F., den Besten, W., Chen, B., Rehg, J. E., Latres, E., Barbacid, M., Pollard, J. W., Sherr, C. J., Cohen, P. E., and Roussel, M. F. (2001) Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors p18(Ink4c) and p19(Ink4d). *Mol. Cell. Biol.* **21**, 3244–3255.
23. Zindy, F., van Deursen, J., Grosveld, G., Sherr, C. J., and Roussel, M. F. (2000) INK4d-deficient mice are fertile despite testicular atrophy. *Mol. Cell. Biol.* **20**, 372–378.
24. Bartkova, J., Thullberg, M., Rajpert-De Meyts, E., Skakkebaek, N. E., and Bartek, J. (2000) Lack of p19INK4d in human testicular germ-cell tumours contrasts with high expression during normal spermatogenesis. *Oncogene* **19**, 4146–4150.
25. Gil, J., and Peters, G. (2006) Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat. Rev. Mol. Cell. Biol.* **7**, 667–677.
26. Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., Mendez, J., Antequera, F., and Serrano, M. (2006) Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. *Nature* **440**, 702–706.
27. Kirchmaier, A. L., and Rine, J. (2006) Cell cycle requirements in assembling silent chromatin in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **26**, 852–862.
28. Karakaidos, P., Taraviras, S., Vassiliou, L. V., Zacharatos, P., Kastrinakis, N. G., Kougiou, D., Kouloukoussa, M., Nishitani, H., Papavassiliou, A. G., Lygerou, Z., and Gorgoulis, V. G. (2004) Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability–evidence of E2F-1 transcriptional control over hCdt1. *Am. J. Pathol.* **165**, 1351–1365.
29. Murphy, N., Ring, M., Heffron, C. C., King, B., Killalea, A. G., Hughes, C., Martin, C. M., McGuinness, E., Sheils, O., and O'Leary, J. J. (2005) p16INK4A, CDC6, and MCM5: predictive biomarkers in cervical preinvasive neoplasia and cervical cancer. *J. Clin. Pathol.* **58**, 525–534.
30. Campisi, J. (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* **120**, 513–522.
31. Kim, S. H., Kaminker, P., and Campisi, J. (2002) Telomeres, aging and cancer: in search of a happy ending. *Oncogene* **21**, 503–511.
32. Ben-Porath, I., and Weinberg, R. A. (2005) The signals and pathways activating cellular senescence. *Int. J. Biochem. Cell. Biol.* **37**, 961–976.
33. Zindy, F., Quelle, D. E., Roussel, M. F., and Sherr, C. J. (1997) Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* **15**, 203–211.
34. Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M., Beach, D., and Serrano, M. (2005) Tumour biology: senescence in premalignant tumours. *Nature* **436**, 642.
35. Zhang, R., Poustovoitov, M. V., Ye, X., Santos, H. A., Chen, W., Daganzo, S. M., Erzberger, J. P., Serebriiskii, I. G., Canutescu, A. A., Dunbrack, R. L., Pehrson, J. R., Berger, J. M., Kaufman, P. D., and Adams, P. D. (2005) Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Dev Cell* **8**, 19–30.
36. Zhang, R., Chen, W., and Adams, P. D. (2007) Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol. Cell. Biol.* **27**, 2343–2358.
37. Narita, M., Narita, M., Krizhanovsky, V., Nunez, S., Chicas, A., Hearn, S. A., Myers, M. P., and Lowe, S. W. (2006) A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. *Cell* **126**, 503–514.
38. Krishnamurthy, J., Torrice, C., Ramsey, M. R., Kovalev, G. I., Al-Regaiey, K., Su, L., and Sharpless, N. E. (2004) Ink4a/Arf expression is a biomarker of aging. *J. Clin. Invest.* **114**, 1299–307.
39. Ohtani, N., Zebedee, Z., Huot, T. J., Stinson, J. A., Sugimoto, M., Ohashi, Y., Sharrocks, A. D., Peters, G., and Hara, E. (2001) Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. *Nature* **409**, 1067–1070.

40. Ressler, S., Bartkova, J., Niederegger, H., Bartek, J., Scharffetter-Kochanek, K., Jansen-Durr, P., and Wlaschek, M. (2006) p16INK4A is a robust in vivo biomarker of cellular aging in human skin. *Aging Cell* **5**, 379–389.
41. Raaphorst, F. M. (2005) Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumors. *Hum. Mol. Genet.* **14** Spec No. 1, R93–R100.
42. Bracken, A. P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., Theilgaard-Monch, K., Minucci, S., Porse, B. T., Marine, J. C., Hansen, K. H., and Helin, K. (2007) The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes Dev.* **21**, 525–530.
43. Zhu, D., Xu, G., Ghandhi, S., and Hubbard, K. (2002) Modulation of the expression of p16INK4a and p14ARF by hnRNP A1 and A2 RNA binding proteins: implications for cellular senescence. *J. Cell. Physiol.* **193**, 19–25.
44. Wang, W., Martindale, J. L., Yang, X., Chrest, F. J., and Gorospe, M. (2005) Increased stability of the p16 mRNA with replicative senescence. *EMBO Rep.* **6**, 158–164.
45. Finkel, T. (2003) Oxidant signals and oxidative stress. *Curr. Opin. Cell Biol.* **15**, 247–254.
46. Takahashi, A., Ohtani, N., Yamakoshi, K., Iida, S., Tahara, H., Nakayama, K., Nakayama, K. I., Ide, T., Saya, H., and Hara, E. (2006) Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. *Nat. Cell Biol.* **8**, 1291–1297.
47. Campisi, J. (2003) Cancer and ageing: rival demons? *Nat. Rev. Cancer* **3**, 339–349.
48. Krtolica, A. (2005) Stem cell: balancing aging and cancer. *Int. J. Biochem. Cell. Biol.* **37**, 935–941.
49. Janzen, V., Forkert, R., Fleming, H. E., Saito, Y., Waring, M. T., Dombkowski, D. M., Cheng, T., DePinho, R. A., Sharpless, N. E., and Scadden, D. T. (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* **443**, 421–426.
50. Krishnamurthy, J., Ramsey, M. R., Ligon, K. L., Torrice, C., Koh, A., Bonner-Weir, S., and Sharpless, N. E. (2006) p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature* **443**, 453–457.
51. Molofsky, A. V., Slutsky, S. G., Joseph, N. M., He, S., Pardal, R., Krishnamurthy, J., Sharpless, N. E., and Morrison, S. J. (2006) Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* **443**, 448–452.
52. Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J., and Jackson, S. P. (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.* **8**, 37–45.
53. Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu. Rev. Biochem.* **73**, 39–85.
54. Zhivotovsky, B., and Kroemer, G. (2004) Apoptosis and genomic instability. *Nat. Rev. Mol. Cell. Biol.* **5**, 752–762.
55. Norbury, C. J., and Zhivotovsky, B. (2004) DNA damage-induced apoptosis. *Oncogene* **23**, 2797–808.
56. Hickman, M. J., and Samson, L. D. (2004) Apoptotic signaling in response to a single type of DNA lesion, O(6)-methylguanine. *Mol. Cell* **14**, 105–116.
57. Al-Mohanna, M. A., Manogaran, P. S., Al-Mukhalafi, Z. K., Al-Hussein, A., and Aboussekhra, A. (2004) The tumor suppressor p16(INK4a) gene is a regulator of apoptosis induced by ultraviolet light and cisplatin. *Oncogene* **23**, 201–212.
58. Le, H. V., Minn, A. J., and Massague, J. (2005) Cyclin-dependent kinase inhibitors uncouple cell cycle progression from mitochondrial apoptotic functions in DNA-damaged cancer cells. *J. Biol. Chem.* **280**, 32018–32025.
59. Bianchi, T., Rufer, N., MacDonald, H. R., and Migliaccio, M. (2006) The tumor suppressor p16Ink4a regulates T lymphocyte survival. *Oncogene* **25**, 4110–4115.
60. Ceruti, J. M., Scassa, M. E., Flo, J. M., Varone, C. L., and Cánepa, E. T. (2005) Induction of p19INK4d in response to ultraviolet light improves DNA repair and confers resistance to apoptosis in neuroblastoma cells. *Oncogene* **24**, 4065–4080.
61. Scassa, M. E., Marazita, M. C., Ceruti, J. M., Carcagno, A. L., Sirkin, P. F., Gonzalez-Cid, M., Pignataro, O. P., and Cánepa, E. T. (2007) Cell cycle inhibitor, p19INK4d, promotes cell survival and decreases chromosomal aberrations after genotoxic insult due to enhanced DNA repair. *DNA Repair (Amst)* **6**, 626–638.
62. Tavera-Mendoza, L., Wang, T. T., Lallemant, B., Zhang, R., Nagai, Y., Bourdeau, V., Ramirez-Calderon, M., Desbarats, J., Mader, S., and White, J. H. (2006) Convergence of vitamin D and retinoic acid signalling at a common hormone response element. *EMBO Rep.* **7**, 180–185.
63. Tavera-Mendoza, L. E., Wang, T. T., and White, J. H. (2006) p19INK4D and cell death. *Cell Cycle* **5**, 596–598.
64. Bernstein, C., Bernstein, H., Payne, C. M., and Garewal, H. (2002) DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. *Mutat. Res.* **511**, 145–178.