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## Contrasting behavior of the p18<sup>INK4c</sup> and p16<sup>INK4a</sup> tumor suppressors in both replicative and oncogene-induced senescence

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### Abstract

The CDK inhibitors, p18<sup>INK4c</sup> and p16<sup>INK4a</sup>, both have the credentials of tumor suppressors in human cancers and mouse models. For p16<sup>INK4a</sup>, the underlying rationale is its role in senescence but the selective force for inactivation of p18<sup>INK4c</sup> in incipient cancer cells is less clear. Here we show that in human fibroblasts undergoing replicative or oncogene-induced senescence, there is a marked decline in the levels of p18<sup>INK4c</sup> protein and RNA, which mirrors the accumulation of p16<sup>INK4a</sup>. Down-regulation of *INK4c* is not dependent on p16<sup>INK4a</sup> and RAS can promote the loss of *INK4c* without cell cycle arrest. Down-regulation of p18<sup>INK4c</sup> correlates with reduced expression of menin and E2F1 but is unaffected by acute cell cycle arrest or inactivation of the retinoblastoma protein (pRb). Collectively, our data question the idea that p18<sup>INK4c</sup> acts as a backup for loss of p16<sup>INK4a</sup> and suggest that the apparent activation of p18<sup>INK4c</sup> in some settings represents delayed senescence rather than increased expression. We propose that the contrasting behavior of the two very similar INK4 proteins could reflect their respective roles in senescence versus differentiation.

### Keywords

INK4; CDK inhibitors; senescence; E2F1; menin

### Introduction

The INK4 family of cyclin-dependent kinase (CDK) inhibitors, p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup> and p19<sup>INK4d</sup>, are structurally and functionally similar (1). They comprise between 3.5 and 5 ankyrin-type repeats and bind directly to CDK4 and CDK6, thereby preventing or destabilizing the interaction of these kinases with regulatory D-type cyclins. Although the INK4 proteins demonstrate equivalent affinity for CDK4 and CDK6 in vitro and when over-expressed (2), the prevailing impression is that they associate preferentially with CDK6 in physiological settings (3-6).

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The prototypic member of the family, p16<sup>INK4a</sup>, is an accredited tumor suppressor that sustains inactivating germline mutations in familial melanoma and is incapacitated by diverse mechanisms in a variety of sporadic cancers (1). Its tumor suppressor functions are best explained by its role in senescence, either in response to an activated oncogene or in cells that reach the end of their proliferative lifespan (7). The *INK4b* gene, which is tandemly linked to *INK4a* on human chromosome 9p21, is also implicated in senescence (7) and has credentials as a tumor suppressor in some contexts, but inactivating mutations of p15<sup>INK4b</sup> are very rare (1).

The same applies to p18<sup>INK4c</sup> and p19<sup>INK4d</sup> but there has recently been renewed interest in the potential role of *INK4c* in tumor suppression. The most compelling evidence comes from mice carrying null alleles of *Ink4c*. These animals develop spontaneous pituitary adenomas late in life (8) but when *Ink4c* deficiency is combined with other gene knockouts, the range and frequency of tumors becomes more extensive. For example, mice lacking both p18<sup>INK4c</sup> and p27<sup>Kip1</sup> develop a tumor spectrum reminiscent of human multiple endocrine neoplasia type 1 (MEN1) (9). Critically, menin, the product of the *MEN1* gene, has been shown to activate p27<sup>KIP1</sup> and p18<sup>INK4c</sup> by enhancing the binding of trithorax group (TrxG) complexes to the respective promoters (10, 11). Crossing of the knockout mice implicates *Ink4c* as the critical target of menin in this system (12). Another important example is the combination of *Ink4c* and p53 deficiency that results in early onset hemangiosarcoma and medullablastoma and nicely models the genetic background of the human disease (13, 14).

In addition to these mouse models, loss or silencing of human p18<sup>INK4c</sup> has been recorded in a number of human malignancies (14-23). Glioblastoma multiforme (GBM) is an intriguing example because homozygous deletions and inactivating mutations of *INK4c* are observed in a tumor that is frequently associated with defects in the p16<sup>INK4a</sup>-CDK4-pRb pathway (20-23). As a number of the samples showed concordant loss of both *INK4a* and *INK4c*, implying that the selection against these components is not mutually exclusive, some have proposed that p18<sup>INK4c</sup> acts as a backup for p16<sup>INK4a</sup> (21, 23).

In considering what drives the inactivation of p18<sup>INK4c</sup> in human cancers, we investigated whether it contributes to replicative or oncogene-induced senescence. Remarkably, the accumulation of p16<sup>INK4a</sup> in senescent human fibroblasts is accompanied by the virtual elimination of p18<sup>INK4c</sup>. Loss of p18<sup>INK4c</sup> correlates with a reduction in the total levels of menin and E2F1, which positively regulate *INK4c*, but we saw no inverse correlation between *INK4c* and *pRb* status in human cancer cells, or between p16<sup>INK4a</sup> and p18<sup>INK4c</sup> levels as previously suggested. Rather, depletion of p16<sup>INK4a</sup> delays the onset of senescence and concomitant down-regulation of p18<sup>INK4c</sup>.

## Materials and Methods

### Cell culture and retroviral infection

Conditions for the serial propagation and retroviral infection of human diploid fibroblasts (Hs68, TIG3, BF, IMR90, Leiden and Q34) and mouse embryo fibroblasts (MEF) were as previously described (24-27). As these are primary cells with a finite lifespan, they are considered to be genetically stable and have not been authenticated. The panel of human

tumor cells lines, described previously (28) were authenticated by DNA fingerprinting by the in-house Cell Services facility. Retroviral vectors encoding HA-tagged p16<sup>INK4a</sup>, p18<sup>INK4c</sup>, p21<sup>CIP1</sup>, and SV40 large T-antigen were as described (24, 29) and the E2F1:ER, ER:RAS, RAF:ER and Bmi1 vectors were kindly provided by Kristian Helin, Paul Khavari, Martin McMahon and Maarten van Lohuizen, respectively. The various ER fusions proteins were induced by addition of either 100 or 500nM 4-hydroxytamoxifen (OHT). For the cell cycle arrest experiments, cells were treated with hydroxyurea (1mM), nocodazole (0.3μM), or RO 3306 (9 μM) for 24 h or with the CDK4/6 inhibitor PD-033299 (500ng/ml) for up to 20 days. In some experiments, cells were treated with MG132 (20μM) for 24h or with cycloheximide (20μM). Staining for SA-β-galactosidase activity and DAPI foci followed standard protocols (24).

### Immunoprecipitation and immunoblotting

Cell lysates were prepared in TNN buffer (50mM Tris.HCl, pH 7.5, 120 mM NaCl, 5mM EDTA, 0.5% NP40) with complete EDTA free protease inhibitor (Roche) plus 1mM PMSF, and analyzed essentially as described previously (24). Primary antibodies were as follows: CDK4 (sc-601), CDK6 (sc-177), E2F1 (sc-22820), p18<sup>INK4c</sup> (sc-1208), mouse p16<sup>Ink4a</sup> (sc-1207), β-tubulin (sc-9104), p53 (DO-1, sc-126), cyclin E (sc-247), cyclin A (sc-601) and p21<sup>CIP1</sup> (sc-397) were from Santa Cruz; MEK1/2 (#9122) was from Cell Signalling; menin (AB2605), HRP conjugated anti-GAPDH (AB9482) and a monoclonal antibody (mAb) against CDK6 (AB77674) were from Abcam; CDK4 (DCS31) and p18<sup>INK4c</sup> (DCS118) mAbs were from Novus; the cyclin D1 mAb (DCS6) was from BD Pharmingen; a pan-Ras mAb (OP41) was from Oncogene Research; the mAb against p16<sup>INK4a</sup> (JC8) was provided by LRI Cell Services using cells originally obtained from J. Koh and E. Harlow. We also generated two rabbit antisera raised against bacterially expressed p18<sup>INK4c</sup> (FST1 and FST4) and have previously described in-house reagents against cyclin D1 (287.3), CDK6 (LBO-1) and p16<sup>INK4a</sup> (DPAR12) (28).

### Quantitative reverse transcription and PCR

Total RNA was extracted using the Ultra Pure RNA extraction Kit (Roche) (Roche). Reverse transcription was carried out with 0.5–1 μg of RNA using MultiScribe reverse transcriptase and random hexamer primers (Applied Biosystems). One fiftieth of the cDNA was used as a template for quantitative real-time PCR (qRT-PCR) on ABI 7900HT (Applied Biosystems) using Power SYBR Green Master Mix (Applied Biosystems) or TaqMan Universal PCR Master Mix (Applied Biosystems). Expression was normalized to GAPDH. For detection of the *INK4a* transcript, annealing and extension was done for 1 min at 66°C, while for other transcripts this step was performed at 60°C. Sequences of the primers can be found in Supplementary Table 1

### RNA interference

The validated shRNA against p16<sup>INK4a</sup> in the pRetroSuper vector was as described (30). Three different shRNAs targeting human p18<sup>INK4c</sup> were generated in pRetroSuper using the following 19-nucleotide sequences: A- 5'-CTGGTTTCGCTGTCATTCA-3', B- 5'-

GGGAACCTGCCCTTGCCT-3 and C- 5'-GGGGGACACCGCCTGTGAT-3'. The C sequence was the most effective.

### Chromatin immunoprecipitation

Cells were harvested and processed for chromatin immunoprecipitation as described (31). After sonication, the chromatin was centrifuged to pellet the debris and pre-cleared at 4°C for 2h with protein A-agarose and protein G-sepharose. The samples were immunoprecipitated with 4µg of relevant antibody or a control IgG at 4°C overnight, followed by a 3h incubation with blocked protein A- or protein G-sepharose. The antibody-chromatin complexes were extensively washed with ice-cold IP buffer (150mM NaCl, 50mM Tris-HCl (pH 7.5), 5mM EDTA, 0.5% NP40 and 1% Triton X-100) and eluted with 1% SDS, 100mM NaHCO<sub>3</sub>, 10mM DTT at 65°C for 5min and RT for 15min. Crosslinking was reversed by incubation at 65°C overnight following the addition of NaCl to 150mM. The samples were treated with 100 µg/ml RNase (Roche) for 30 min at 37 °C and DNA was purified using a Qiagen PCR cleanup kit. Antibodies against H3K4me3 (clone 07-473 or MC315) and H3K27me3 (clone 07-449) were from Millipore, and Menin (AB2605) from Abcam.

### Gel filtration

The procedures for gel filtration and associated data were as described in ref (32).

## Results

### Down-regulation of p18<sup>INK4c</sup> in replicative and oncogene-induced senescence

When primary human diploid fibroblasts (HDFs) reach the end of their proliferative lifespan in culture, there is a substantial accumulation of p16<sup>INK4a</sup>, preceded by a peak in p21<sup>CIP1</sup> expression which subsequently declines (25). However, to our knowledge, there have been no reports on the expression of p18<sup>INK4c</sup> in this classical model of senescence. We found that whereas p18<sup>INK4c</sup> levels remained relatively constant with cumulative population doublings, they declined dramatically when the cells became senescent, essentially the mirror image of p16<sup>INK4a</sup> expression levels (Fig. 1A). This pattern of expression was recapitulated in different HDFs irrespective of their maximum lifespan and basal levels of p16<sup>INK4a</sup> and p18<sup>INK4c</sup> (data not shown).

As replicative senescence reflects both telomere-dependent and independent events, we asked whether a similar phenomenon occurs during oncogene-induced senescence. Expression of H-RAS<sup>G12V</sup> in HDFs caused a marked down-regulation of p18<sup>INK4c</sup>, contrasting with the anticipated up-regulation of p16<sup>INK4a</sup> (Fig. 1B). Analogous effects were observed in mouse embryo fibroblasts (MEFs) (Fig. 1C) and with other oncogenes (data not shown).

### Down-regulation of p18<sup>INK4c</sup> at senescence is independent of p16<sup>INK4a</sup>

One simple explanation for these observations would be that the accumulation of p16<sup>INK4a</sup> at senescence might displace p18<sup>INK4c</sup> from CDK complexes, resulting in its turnover. Indeed, ectopic expression of HA-tagged p16<sup>INK4a</sup> in early passage HDFs caused a marked

reduction of p18<sup>INK4c</sup> (Fig. 1D). However, there are several reasons why this explanation is untenable. First, both p18<sup>INK4c</sup> and p16<sup>INK4a</sup> are stable proteins with half-lives in excess of 15 hours as judged by blocking protein synthesis with cycloheximide or by pulse-chase analyses (Suppl. Fig. S1) and (5, 28, 33, 34). Second, the down-regulation of p18<sup>INK4c</sup> was not reversed by treatment with the proteasome inhibitor MG132 (Fig. 1E). Third, extinction of p18<sup>INK4a</sup> at senescence was observed in the Leiden strain of human fibroblasts (24), which lack functional p16<sup>INK4a</sup> (Fig. 1F).

### Down-regulation of p18<sup>INK4c</sup> RNA at senescence

As these data imply that the loss of p18<sup>INK4c</sup> at senescence is not caused by protein turnover, we asked whether the down-regulation of *INK4c* occurs at the RNA level. When different strains of HDF, including p16<sup>INK4a</sup>-deficient strains, were passaged to senescence, there was a marked reduction in the levels of *INK4c* RNA relative to early passage cells, as assessed by qRT-PCR (Fig. 2A and 2B). *INK4c* RNA levels also declined in cells infected with an H-RAS<sup>G12V</sup> retrovirus (Fig. 2C) or following addition of OHT to HDFs transduced with a RAS:ER fusion protein (Fig. 2D). As expected, *INK4a* RNA levels were substantially increased under the same conditions. Unlike other members of the *INK4* family, the *INK4c* gene specifies two distinct transcripts that originate from alternative promoters but encode the same protein (35). Using primers that discriminate between the two transcripts, we confirmed that both are down-regulated by RAS (Suppl. Fig. S2).

HDFs arrested by over-expression of p16<sup>INK4a</sup> or other members of INK4 and CIP/KIP families acquire many features of senescence although they do not display the senescence-associated secretory phenotype associated with chronic DNA damage (36, 37). We therefore investigated the fate of p18<sup>INK4c</sup> in these circumstances. Interestingly, ectopic expression of p21<sup>CIP1</sup>, p16<sup>INK4a</sup> or p18<sup>INK4c</sup> itself resulted in down-regulation of the endogenous *INK4c* gene (Fig. 2E). These findings suggest that transcriptional silencing of *INK4c* is a consistent feature of senescent cells but make it unlikely that the loss of p18<sup>INK4c</sup> has a causal role in the phenotype.

### Regulation of p18<sup>INK4c</sup> by menin

The *INK4a* locus is subject to regulation by the Polycomb group (PcG) of transcriptional repressors (7) via trimethylation of histone H3 on lysine 27 (H3K27me3). Although published datasets indicate that *INK4c* is also occupied by PcG complexes in embryonic stem cells (38), we found no evidence for the H3K27me3 mark at *INK4c* in human fibroblasts under conditions in which it is readily detectable at *INK4a* (Suppl. Fig. S3). As previously shown (26), H-RAS<sup>G12V</sup> caused a loss of H3K27me3 at *INK4a*.

The repressive effects of PcG complexes can be countered by the TrxG proteins and a number of studies have shown that *INK4c* is positively regulated by the tumor suppressor menin as part of a TrxG complex (10, 11). In line with these reports, we found that in human fibroblasts undergoing H-RAS<sup>G12V</sup>-induced senescence, there was a measurable decrease in the levels of menin and H3K4me3 at the *INK4c* locus (Figs. 3A and 3B). In essentially all of the situations in which we documented a senescence-associated decline in *INK4c*

expression, there was a concomitant but less pronounced decline in the levels of menin RNA (Fig. 3C -G).

### Regulation of p18<sup>INK4c</sup> by E2F

As it seemed unlikely that the changes in menin levels could account for the dramatic down-regulation of *INK4c*, we considered the possibility that as an E2F-regulated gene (21, 39, 40), *INK4c* was simply reacting to cell cycle arrest. However, agents that cause acute arrest in different cell cycle phases had no effect on the levels of p18<sup>INK4c</sup> RNA or protein, and we saw no substantive changes in p18<sup>INK4c</sup> levels following serum stimulation of quiescent cells (Suppl. Fig. S4A and S4B, and data not shown). Moreover, p18<sup>INK4c</sup> levels were relatively unaffected by introduction of SV40 large T-antigen whereas p16<sup>INK4a</sup> expression was clearly elevated in this setting (Fig 4A). We also surveyed p18<sup>INK4c</sup> expression in a panel of human tumor cell lines that we had previously used to demonstrate the inverse correlation between pRb status and p16<sup>INK4a</sup> expression (28). There was no consistent relationship between p16<sup>INK4a</sup> and p18<sup>INK4c</sup> expression or between p18<sup>INK4c</sup> and pRb status (Fig 4B), in line with an earlier report (41).

Taken together, our data imply that p18<sup>INK4c</sup> expression is largely unaffected by the fluctuations of E2F activity that occur during the cell cycle or as a consequence of pRb inactivation. However, it was quite clearly induced by over-expression of E2F1, as confirmed using HDFs expressing a tamoxifen-regulated ER-E2F1 fusion protein (Suppl. Fig. S4C and S4D). We therefore asked whether the down-regulation of *INK4c* at senescence correlated with reduced expression of E2F1. As shown in Figure 4C-F, the levels of E2F1 RNA and protein are consistently and dramatically reduced in fibroblasts undergoing either replicative, oncogene- or p16-induced senescence.

### Distinction between activation and delayed repression of *INK4c*

It was previously reported that shRNA-mediated knockdown of p16<sup>INK4a</sup> in normal astrocytes caused an increase in the levels of p18<sup>INK4c</sup> (21). As this appeared to be at odds with our findings, we performed analogous experiments in human fibroblasts. In general, we found that shRNA-mediated knockdown of p16<sup>INK4a</sup> had no discernible impact on the levels of p18<sup>INK4c</sup> despite robust depletion of p16<sup>INK4a</sup> (Fig. 5A). This negative result was confirmed in multiple experiments with several different strains of logarithmically dividing cells (not shown). However, we occasionally noted higher levels of p18<sup>INK4c</sup> in p16<sup>INK4a</sup> - knockdown cells that had been in culture for some time. We reasoned that agents that suppress or disable the pRb-p16<sup>INK4a</sup> pathway can extend the replicative lifespan of cells and potentially delay the down-regulation of p18<sup>INK4c</sup> that occurs at senescence. Depending on when the cells are analyzed, this could give the impression that p18<sup>INK4c</sup> levels are elevated relative to the control cells.

To explore this possibility, we monitored the changes in p16<sup>INK4a</sup> and p18<sup>INK4c</sup> expression in human fibroblasts infected with a retrovirus encoding the PcG protein Bmi1. As expected (25), Bmi1 enabled the TIG3 strain of fibroblasts to grow for ~10 additional population doublings relative to the cells infected with the empty vector (Fig. 5B). It also reduced the basal levels of p16<sup>INK4a</sup> but did not prevent the eventual accumulation of p16<sup>INK4a</sup> at

senescence (Fig. 5C). Importantly, the p18<sup>INK4c</sup> levels declined much later in the Bmi1-transduced cells than in the controls. To illustrate this point more clearly, Figure 5D shows a direct comparison of cell lysates prepared on days 50 and 64 post-infection. At these time points, cells expressing Bmi1 appear to have substantially more p18<sup>INK4a</sup> than the control, giving the false impression that p18<sup>INK4c</sup> is being activated by Bmi1. Similar effects were observed in cells whose lifespan was extended by over-expression of CDK4 or p16-shRNA (data not shown).

### Senescence-dependent and -independent effects on p18<sup>INK4c</sup>

One of the characteristic features of senescent fibroblasts is the appearance of senescence-associated heterochromatin foci (SAHF) which cause irreversible silencing of a subset of E2F target genes (42). We wondered whether the silencing of *INK4c* at senescence was related to the formation of SAHFs. To avoid altering the levels of key components, we treated HDFs with the CDK4/6 inhibitor PD-0332991, which causes a pRb-dependent G1 arrest (43) and was recently shown to induce senescence in GBM cells that have co-deletion of *INK4a* and *INK4c* (22, 23). The stable arrest caused by PD-0332991 was accompanied by a decline in the expression of p18<sup>INK4c</sup> RNA, as well as other E2F target genes (Fig. 6A). Importantly, the cells developed characteristic features of senescence, and stained positively for SA- $\beta$ -galactosidase and SAHFs (Fig. 6B). Of note, the loss of p18<sup>INK4c</sup> expression was accompanied by down-regulation of both menin and E2F1 (Fig. 6C). However, for technical reasons, we were unable to draw a direct correlation between the appearance of SAHFs and elimination of p18<sup>INK4c</sup> at the single cell level.

Reversing the logic, we asked whether H-RAS<sup>G12V</sup> would cause down-regulation of *INK4c* in the absence of SAHFs. The Leiden strain of p16<sup>INK4a</sup>-deficient HDFs are resistant to oncogene-induced senescence under conditions that efficiently arrest other HDF strains (24). H-RAS<sup>G12V</sup> caused down-regulation of *INK4c* and up-regulation of the mutant *INK4a* in Leiden cells, although the effects were less dramatic than in the control fibroblasts (Fig. 6D and 6E). As H-RAS<sup>G12V</sup> caused down-regulation of p18<sup>INK4c</sup> in MEFs (Fig. 1D) where SAHFs are not observed (44), our data suggest that SAHFs are not required for the down-regulation of *INK4c* by H-RAS<sup>G12V</sup>. Importantly, the correlation between p18<sup>INK4c</sup>, E2F1 and menin expression still applied in Leiden cells despite the avoidance of senescence (Fig. 6F). Taken together, our data suggest that there could be two distinct pathways leading to down-regulation of *INK4c*, one instigated by CDK inhibition and the other instigated by RAS that appears to be independent of CDK inhibition.

### Consequences of p18<sup>INK4c</sup> downregulation in senescent cells

At face value, the behavior of p18<sup>INK4c</sup> at senescence is at odds with its role as a tumor suppressor. Loss or down regulation of p18<sup>INK4c</sup> would in principle make it more difficult for cells to mount a senescence-like arrest in response to oncogenic signals by depleting the total INK4 pool. To investigate this possibility, we used shRNA to knock down the expression of p18<sup>INK4c</sup> in primary HDFs. As exemplified in Figure 7, cells transduced with p18<sup>INK4c</sup> shRNA had a proliferative advantage and extended lifespan relative to control cells. Similar effects were observed in p16<sup>INK4a</sup>-deficient strains of HDF (not shown). However, the cells eventually underwent replicative senescence. Moreover, cells expressing p18<sup>INK4c</sup>

shRNA remained susceptible to H-RAS<sup>G12V</sup>-induced senescence. Interestingly, these cells had higher levels of p16<sup>INK4a</sup> at senescence, as compared to controls, suggesting an increased dependence on p16<sup>INK4a</sup> to enforce the arrest (Fig. 7C).

This result would be consistent with the idea that the total levels of INK4 proteins in the cell might determine the availability of CDK4 and CDK6 to associate with the D-type cyclins. Human fibroblasts contain substantially more CDK4 than CDK6 RNA, as judged by deep sequencing (unpublished results of Helen Pemberton and the authors), and we and others have previously demonstrated that only a small fraction of the total CDK6 is associated with D-type cyclins (5, 32). Excluding the high-molecular weight complexes with heat shock chaperone proteins, most of the CDK6 is bound by INK4 proteins in 1:1 complexes of around 50-60 kDa whereas the bulk of the CDK4 is in 150-170 kDa complexes with D-cyclins and CIP/KIP proteins (Supp. Fig. S5A). In proliferating cells, CDK6 is primarily associated with p18<sup>INK4c</sup> as judged by co-immunoprecipitation, but in senescent cells, virtually all of the CDK6 becomes bound to p16<sup>INK4a</sup> (Fig. 7D and Suppl. Fig. S5B). Although CDK4 can be found associated with p16<sup>INK4a</sup> and p18<sup>INK4c</sup> in early passage cells, its association with cyclin D1 is preserved at senescence despite the excess of p16<sup>INK4a</sup> (Fig 7D and (32)). In summary, the down-regulation of p18<sup>INK4c</sup> at senescence results in re-assortment of the D-cyclins, CDKs and CDK inhibitors to retain CDK6 in an apparently inactive state.

## Discussion

A motivation for this study was the evidence that p18<sup>INK4c</sup> acts as a tumor suppressor not only in mouse models but also in a number of human cancers. Remarkably, deletions of *INK4c* have been found in tumors that also have defects in *INK4a* (17, 21-23, 45) prompting questions about the selective rationale for concomitant inactivation of two members of the same family. As mutation or silencing of p16<sup>INK4a</sup> can enable incipient cancer cells to escape from oncogene-induced senescence, it was suggested that p18<sup>INK4c</sup> serves as a backup in these circumstances, causing pressure to escape from p18<sup>INK4c</sup>-mediated arrest (21, 23). The data we describe make this scenario unlikely.

In primary human fibroblasts undergoing senescence, whether in response to an activated oncogene, continuous passaging or CDK inhibitors, there is a marked decline in the expression of p18<sup>INK4c</sup> which is effectively a mirror image of the dramatic accumulation of p16<sup>INK4a</sup> in most of these settings. The down-regulation of *INK4c* occurs at the RNA level and has been previously documented in gene expression profiles of RAS-induced senescence (46-48), although these studies did not discuss the implications. We find that the effects on p18<sup>INK4c</sup> are not dependent on the p16<sup>INK4a</sup> status of the cell or on cell cycle arrest per se.

In seeking potential mechanisms, we considered known activators of *INK4c* such as menin (10-12) and E2F1 (21, 39, 40). Although H-RAS<sup>G12V</sup> caused reduced binding of menin at the *INK4c* locus and the levels of menin declined in all of the situations in which we documented down-regulation of p18<sup>INK4c</sup>, the effects were relatively modest and unlikely to account for the almost complete silencing of *INK4c*.



A more cogent case can be made for E2F1. In situations where we observed loss of p18<sup>INK4c</sup> expression, we invariably saw a substantial decline in the total levels of E2F1. Again, our findings agree with published evidence that E2F1 is down-regulated in replicative and H-RAS<sup>G12V</sup>-induced senescence (46, 49). However, the interpretation is complicated by the fact that agents that modulate E2F1 activity rather than its total levels, such as cell cycle arrest or inactivation of pRb, had little effect on p18<sup>INK4c</sup>. While it is clear that the response of E2F target genes can be context dependent, our data do not support the notion of a linear pathway through which loss of p16<sup>INK4a</sup> induces E2F-dependent expression of p18<sup>INK4c</sup> (21).

The concept of a linear pathway was based on evidence that shRNA-mediated knockdown of p16<sup>INK4a</sup> in primary astrocytes resulted in up-regulation of p18<sup>INK4c</sup> (21). However, this inverse relationship was not apparent in the logarithmically growing human fibroblasts studied here and, while there could be differences between cell types, our data suggest that the passage history of the cells can have an important bearing on the interpretation of such experiments. Agents that neutralize p16<sup>INK4a</sup>, such as PcG proteins, *INK4a* shRNAs, or ectopic expression of CDK4, have the potential to extend the replicative lifespan of cells and thereby delay the down-regulation of p18<sup>INK4c</sup>. Depending on how close the control cells are to senescence, this can give the false impression that p18<sup>INK4c</sup> is being up-regulated.

Although they are virtually indistinguishable biochemically, a substantial body of evidence implies that the INK4 family of proteins and their target kinases, CDK4 and CDK6, have distinct roles. Whereas p16<sup>INK4a</sup> is primarily involved in the implementation of senescence in response to diverse forms of stress, most studies link p18<sup>INK4c</sup> with differentiation and suggest that p18<sup>INK4c</sup> and CDK6 are partners in crime (3, 4, 6, 50-53). Thus, the over-expression of CDK6 or loss of p18<sup>INK4c</sup>, as observed in a variety of human cancers, would be expected to impair differentiation.

Interestingly, in terminally differentiated myoblasts, virtually all of the CDK6 is bound to p18<sup>INK4c</sup> (3) whereas in senescent fibroblasts almost all of the CDK6 is bound by p16<sup>INK4a</sup>. This suggests a fundamental difference between these states of cell cycle arrest. But what purpose is served by expressing p18<sup>INK4c</sup> and CDK6 if the major fraction of both proteins is locked in a futile embrace and why would the substitution of p18<sup>INK4c</sup> by p16<sup>INK4a</sup> at senescence make a difference? One possibility would be that senescence requires an INK4 protein that is more able to inhibit CDK4. Another is that while differentiation is reversible, as evident from the reprogramming of pluripotency in somatic cells, senescence is generally considered to be irreversible. Interestingly, *INK4c* levels decline when human fibroblasts are transduced with the OSKM reprogramming factors, consistent with reports that senescence acts as a barrier to reprogramming (A. Banito and J. Gil, personal communication). It will be interesting to explore the dynamics of p16<sup>INK4a</sup> and p18<sup>INK4c</sup> expression and their regulation by PcG complexes during this process.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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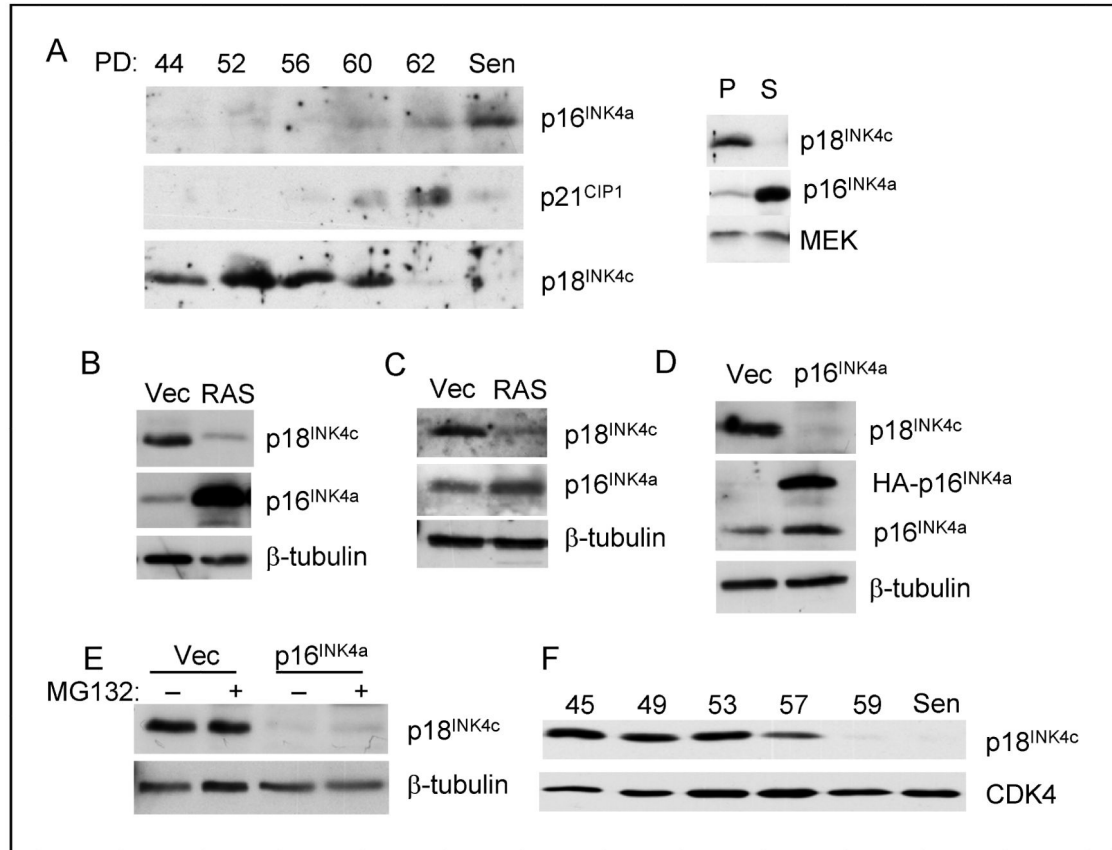
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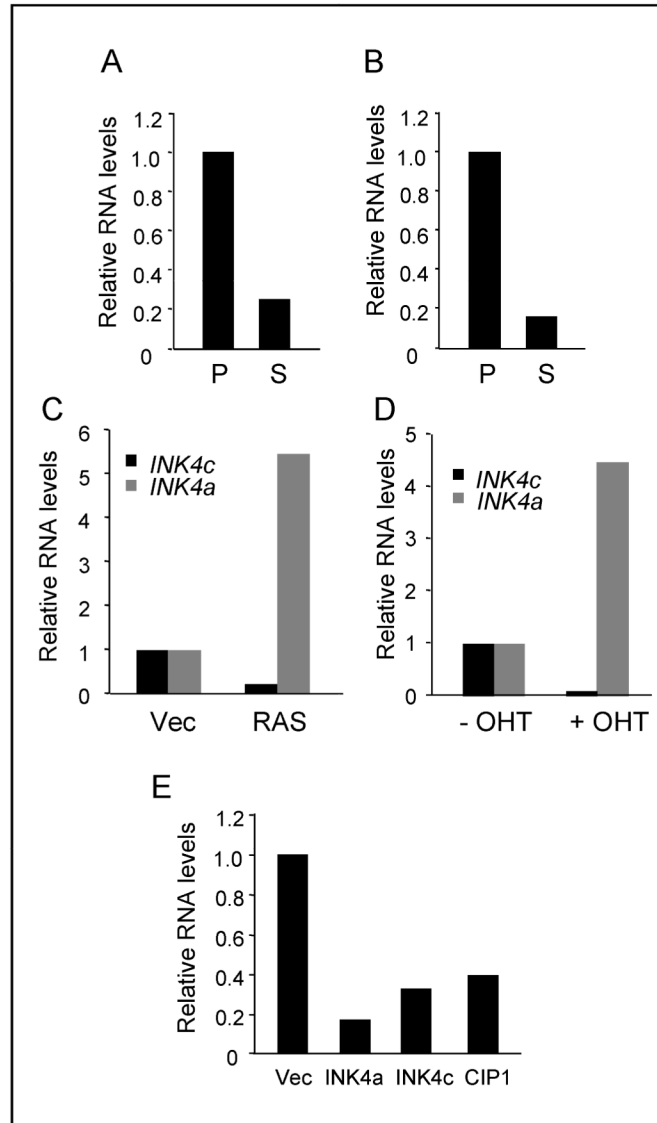
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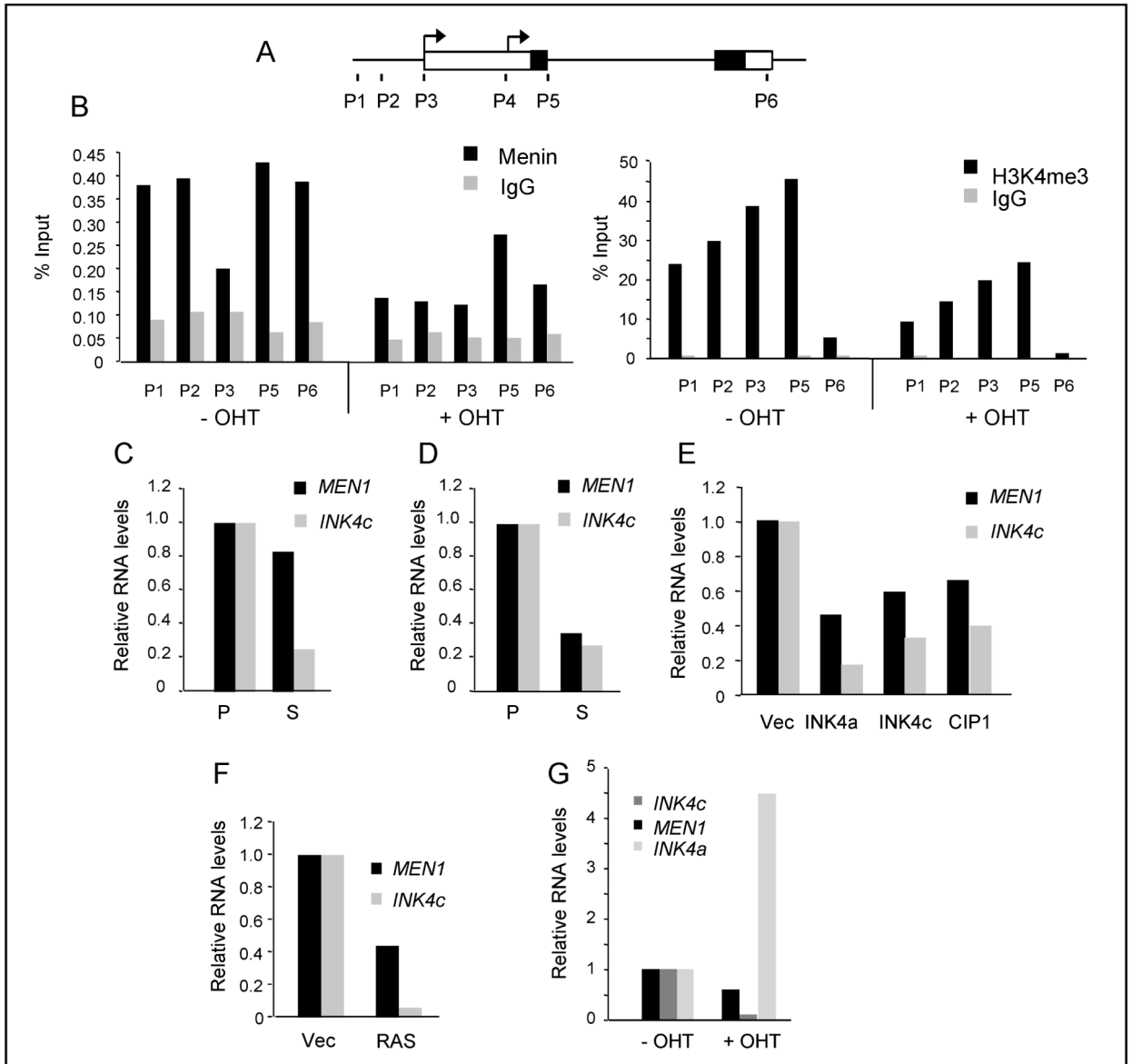
### Figure 1. Downregulation of p18<sup>INK4c</sup> at senescence

A. HDFs (TIG3) were passaged continuously until they reached replicative senescence (Sen). Cell lysates prepared at the indicated population doublings (PD) were fractionated by SDS-PAGE and immunoblotted for p16<sup>INK4a</sup>, p21<sup>CIP1</sup> and p18<sup>INK4c</sup>. Right panel shows a direct comparison between proliferating (P) and senescent (S) cells. B. HDFs (TIG3) were infected with a retrovirus encoding H-RAS<sup>G12V</sup> or the empty vector control (Vec). Following drug selection, the levels of p18<sup>INK4c</sup> and p16<sup>INK4a</sup> were assessed by immunoblotting. C. Primary MEFs were infected with a retrovirus encoding H-RAS<sup>G12V</sup> or the empty vector control (Vec). The levels of p18<sup>INK4c</sup> and p16<sup>INK4a</sup> were assessed by immunoblotting. D. HDFs (Hs68) were infected with a retrovirus encoding HA-tagged p16<sup>INK4a</sup>. The levels of p18<sup>INK4c</sup> and p16<sup>INK4a</sup> were assessed by immunoblotting. E. MG132 had no effect on p18<sup>INK4c</sup> levels in cells transduced with HA-p16<sup>INK4a</sup> or empty vector. F. The Leiden strain of p16<sup>INK4a</sup>-deficient fibroblasts were passaged until they reached senescence. Samples taken at the indicated PDs were immunoblotted for p18<sup>INK4c</sup>, with CDK4 as a loading control.



**Figure 2. Downregulation of *INK4c* RNA at senescence**

A and B. The Hs68 (A) and Leiden (B) strains of HDF were passaged to senescence and the relative levels of *INK4c* RNA in proliferating (P) and senescent (S) cells were assessed by reverse transcription and quantitative real-time PCR (qRT-PCR). C. Relative levels of *INK4c* and *INK4a* RNAs in HDFs (Hs68) infected with a retrovirus encoding H-RAS<sup>G12V</sup> or empty vector control (Vec). D. Relative levels of *INK4c* and *INK4a* RNAs following addition of tamoxifen (OHT) to HDFs (BF) expressing ER:H-RAS<sup>G12V</sup>. E. Relative levels of *INK4c* RNA in HDFs (Hs68) transduced with retroviral vectors encoding HA-tagged versions of p16<sup>INK4a</sup>, p18<sup>INK4c</sup> or p21<sup>CIP1</sup>. Note that endogenous *INK4c* RNA was measured using primer sets that do not detect the ectopically expressed *INK4c* cDNA.

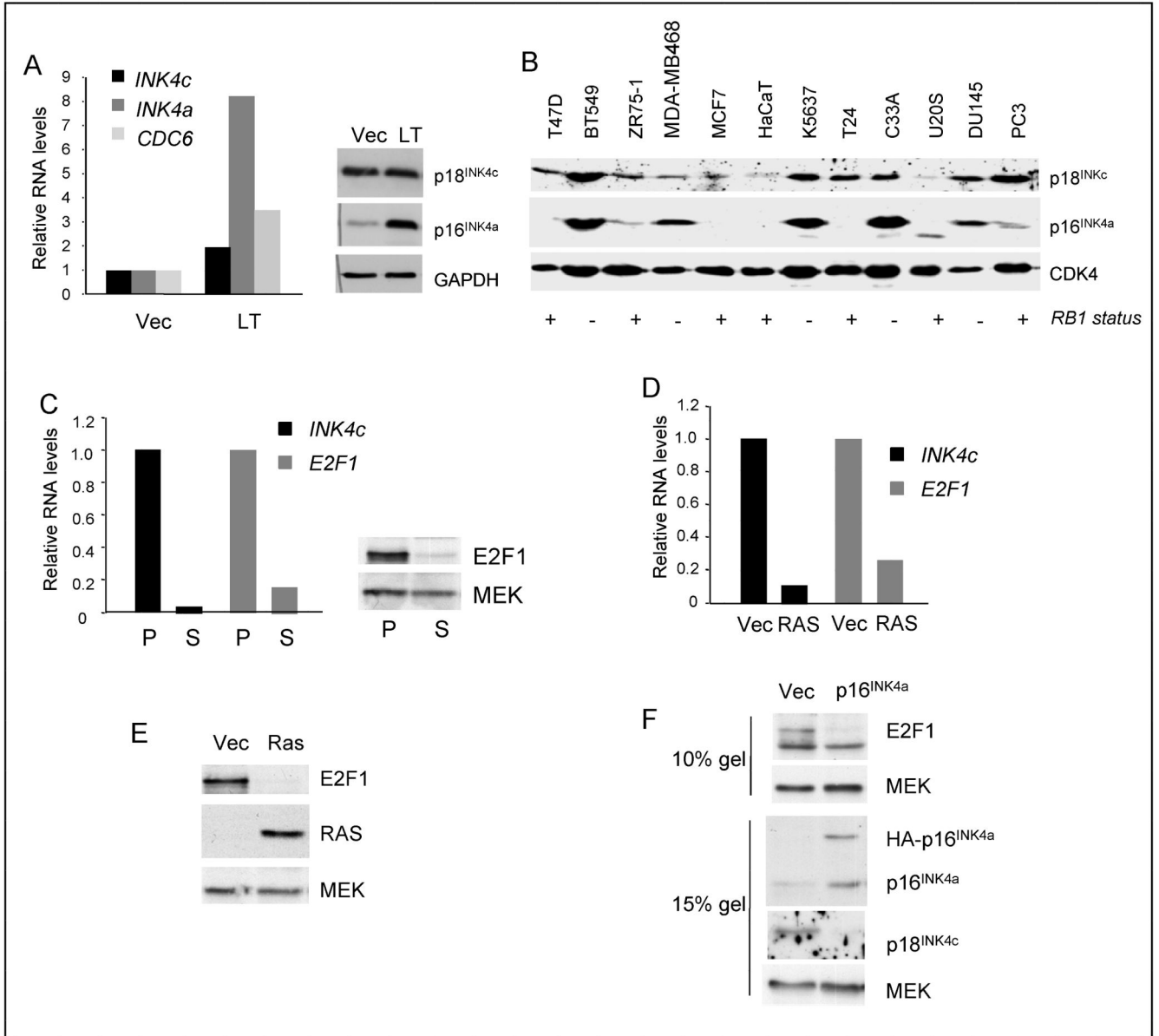


**Figure 3. Role of menin in the down-regulation of *INK4c***

A. Diagram of the human *INK4c* locus with exons depicted as boxes and coding domains shaded. The location of the transcriptional start sites (arrows) and primer sets P1-P6 used for ChIP analyses are indicated. B. ChIP of menin and H3K4me3 at the *INK4c* locus before and after treatment of IMR90 ER:RAS cells with OHT. Enrichment for each primer set was assessed by qPCR relative to a non-specific IgG and expressed as percentage of input. C and D. Comparison of *MEN1* and *INK4c* RNA levels in proliferating (P) and senescent (S) populations of Hs68 (C) and Leiden (D) fibroblasts (same samples as in Fig. 2A and B). E. Relative levels of *MEN1* and *INK4c* RNA in cells transduced with retroviral vectors encoding HA-tagged versions of p16<sup>INK4a</sup>, p18<sup>INK4c</sup> or p21<sup>CIP1</sup>, as in Fig. 2C. F. Relative levels of *INK4c*, *MEN1* and *INK4a* RNAs in Hs68 cells infected with a retrovirus encoding H-RAS<sup>G12V</sup> or empty vector control (Vec), as in Fig. 2D. G. Relative levels of *INK4c*,



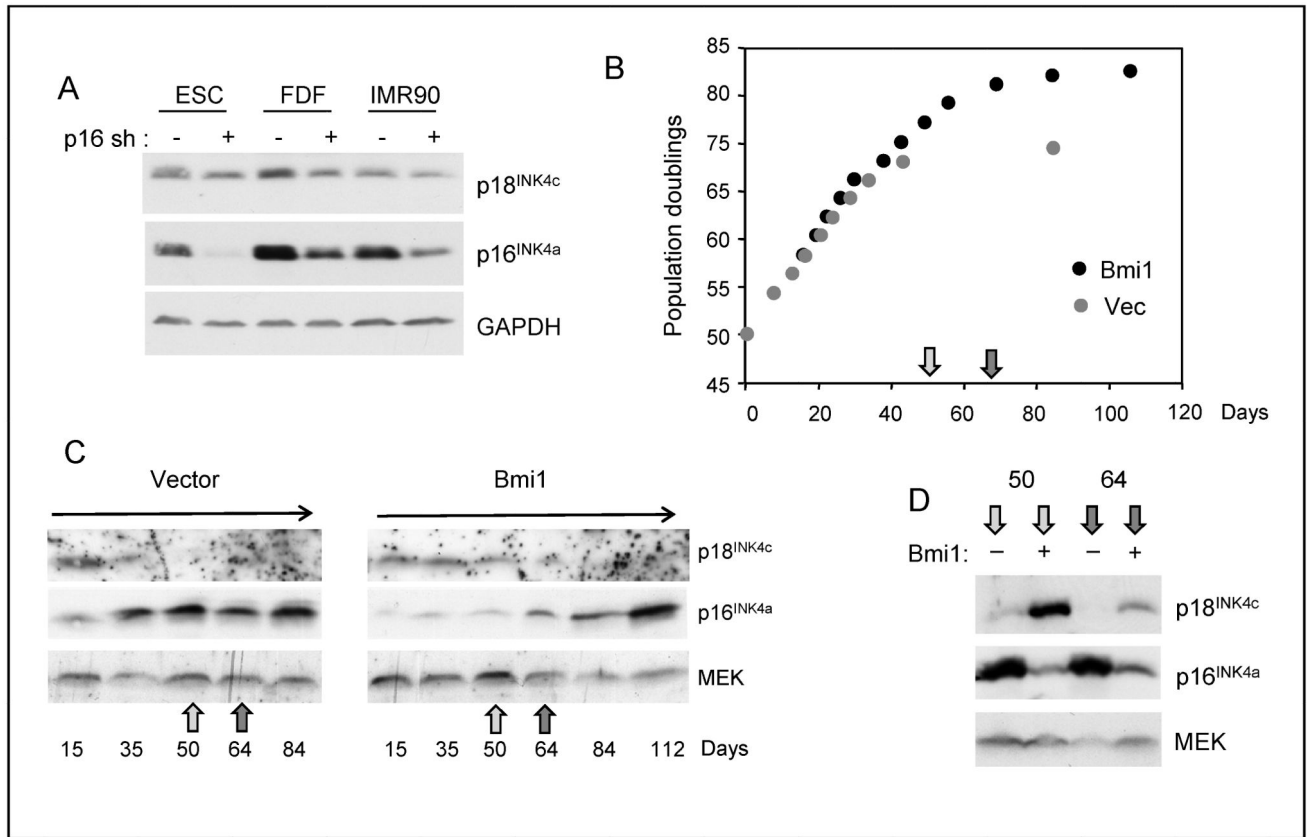
*MEN1* and *INK4a* RNAs following addition of tamoxifen (OHT) to BF cells expressing ER:H-RAS<sup>G12V</sup> (as in Fig. 2E).



#### Figure 4. Regulation of p18<sup>INK4c</sup> by E2F1

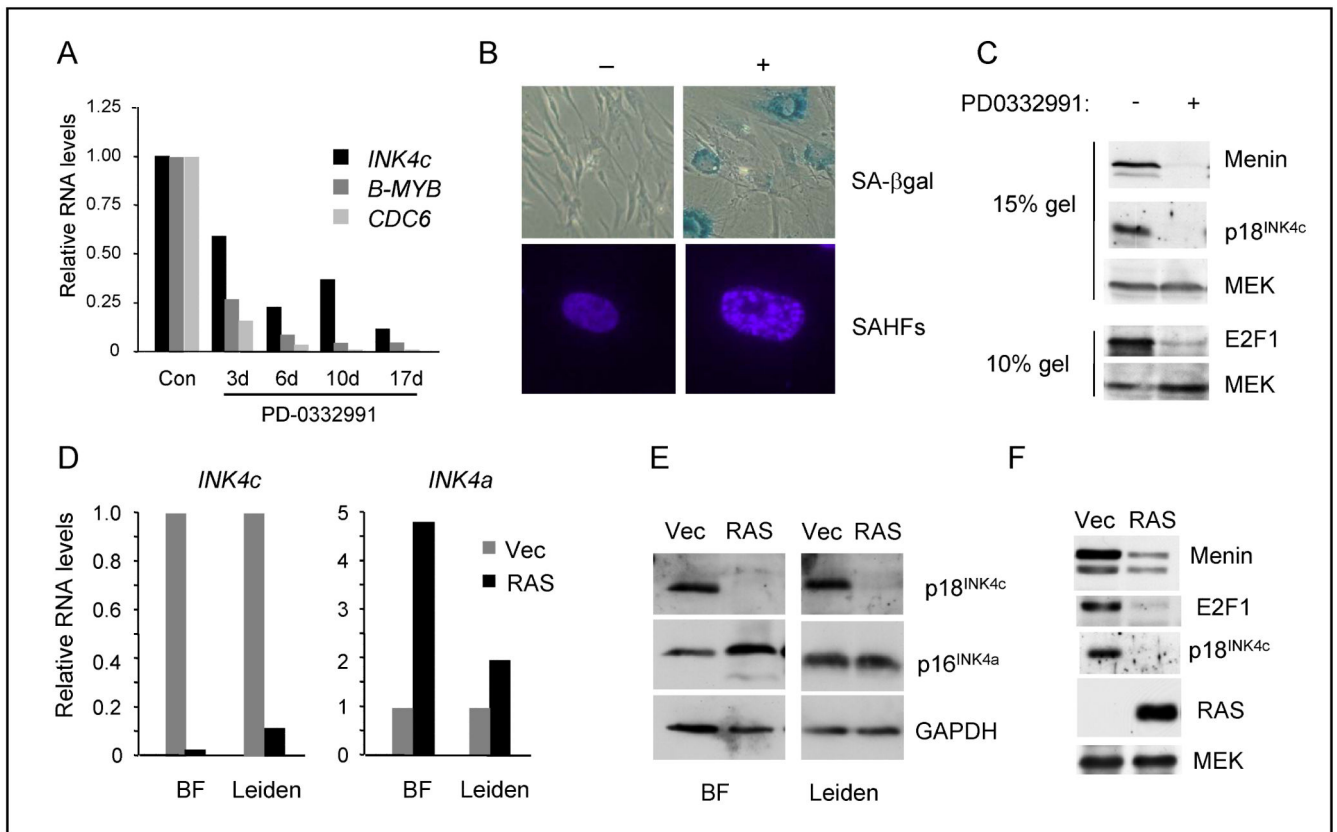
A. Relative levels of *INK4c*, *INK4a* and *CDC6* RNAs in Hs68 cells infected with a retrovirus encoding SV40 large T-Ag (LT) or empty vector control (Vec) (left panel). Lysates from the same cells were fractionated by SDS-PAGE and immunoblotted for p18<sup>INK4c</sup> and p16<sup>INK4a</sup> as indicated (right). B. Survey of p18<sup>INK4c</sup> and p16<sup>INK4a</sup> expression in a series of human cancer cell lines from different anatomical sites: breast (T47D, BT5499, ZR75-1 MDA-MB468 and MCF7), keratinocyte (HaCaT), bladder (K5637 and T24), cervix (C33A), bone (U2OS) and prostate (DU145 and PC3). The +/- below each lane refers to the functional status of the RB1 gene in these cell lines, based on published literature. CDK4 was used as a loading control. C. Comparison of E2F1 RNA (left) and protein (right) levels in proliferating (P) and senescent (S) HDFs (TIG3). D. Comparison of *INK4c* and *E2F1* RNA expression in HDFs (IMR90) infected with a retrovirus encoding H-RAS<sup>G12V</sup> (Ras) or

empty vector (Vec). E. Down-regulation of E2F1 protein in HDFs (TIG3) cells transduced with H-RAS<sup>G12V</sup>. F. Down-regulation of E2F1 in HDFs infected with a retrovirus encoding HA-p16<sup>INK4a</sup>.



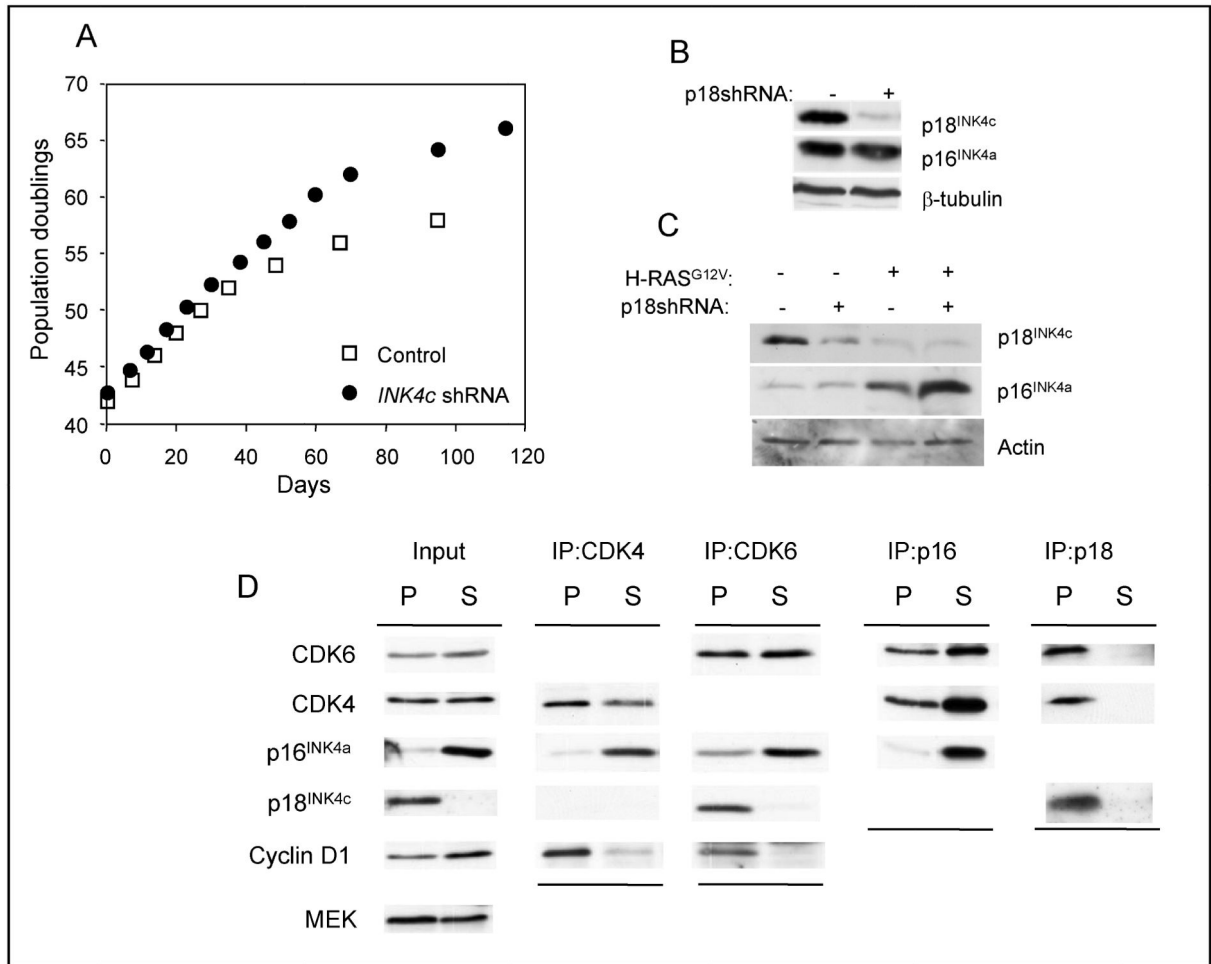
**Figure 5. Distinction between activation and delayed repression**

A. A validated shRNA was used to knockdown p16<sup>INK4a</sup> in several strains of logarithmically growing HDFs and its effects on p16<sup>INK4a</sup> and p18<sup>INK4c</sup> were assessed by immunoblotting. B. Lifespan extension in TIG3 cells infected with a retrovirus encoding mouse Bmi1 compared to empty vector (Vec). C. The cells described in panel B were harvested at the indicated days post-infection and the levels of p16<sup>INK4a</sup> and p18<sup>INK4c</sup> were assessed by immunoblotting. D. Direct comparison of lysates prepared at days 50 and 64 (indicated by the arrows in panel B) from cells infected with Bmi1 virus (+) or empty vector (-).



**Figure 6. Senescence-dependent and -independent downregulation of p18<sup>INK4c</sup>**

A. HDFs (Hs68) treated with the CDK4/6 inhibitor PD0332991 were harvested at the indicated intervals and levels of *INK4c*, *B-MYB* and *CDC6* RNA were assessed by qRT-PCR and normalized to the untreated control. B. HDFs (TIG3) treated with and without PD0332991 for 14d were stained for SA-β-galactosidase activity and with DAPI to visualize SAHFs. C. Lysates from the cells described in panel B were immunoblotted for p18<sup>INK4c</sup>, p16<sup>INK4a</sup>, menin, and E2F1 with MEK as a loading control. D. HDFs (BF and Leiden) were infected with a retrovirus encoding H-RAS<sup>G12V</sup> or empty vector and the relative levels of *INK4c* and *INK4a* RNA were assessed by qRT-PCR and normalized to the vector only control. E. Lysates from the cells described in panel D were immunoblotted for p18<sup>INK4c</sup> and p16<sup>INK4a</sup>. F. Lysates from Leiden cells infected with a retrovirus encoding H-RAS<sup>G12V</sup> or empty vector were immunoblotted for menin, E2F1, p18<sup>INK4c</sup> and RAS.



**Figure 7. p18<sup>INK4c</sup> is not required for replicative or oncogene induced senescence**

A. Hs68 cells infected with a retrovirus encoding either shRNA against p18<sup>INK4c</sup> or a non-specific shRNA (Con) were passaged until they reached M1 senescence. B. Immunoblotting for p18<sup>INK4c</sup> and p16<sup>INK4a</sup> in cells infected with p18<sup>INK4c</sup> shRNA. C. Expression of H-RAS<sup>G12V</sup> in cells transduced with p18<sup>INK4c</sup> shRNA caused oncogene-induced senescence accompanied by a further reduction of p18<sup>INK4c</sup> levels and enhanced expression of p16<sup>INK4a</sup>. D. Lysates from proliferating (P) and senescent (S) fibroblasts were immunoprecipitated with rabbit antibodies against CDK6, CDK4, p18<sup>INK4c</sup> and p16<sup>INK4a</sup>. The proteins were fractionated by SDS-PAGE and immunoblotted with mouse monoclonal antibodies against the indicated proteins.