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Differential p53-independent Outcomes of p19Arf Loss in Oncogenesis

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Abstract

One reported function of the tumor suppressor p19^{Arf} is to stabilize p53, providing a critical checkpoint in the response to oncogenic insults. Acute loss of Pten leads to an increase in the abundance of p19^{Arf}, p53, and p21 proteins as part of a fail-safe senescence response. Here, we report that loss of p19^{Arf} in prostate epithelium does not accelerate—but rather partially inhibits—the prostate cancer phenotype of Pten-deficient mice. Moreover, cellular senescence and a further decrease in the number of pre-neoplastic glands were observed in prostates of the Pten-p19^{Arf} double-mutant mice. In both prostate epithelium and primary mouse embryo fibroblasts (MEFs), the increase in p53 protein abundance found upon loss of Pten was unaffected by the simultaneous loss of p19^{Arf}. However, in contrast to that in the prostate epithelium, p19^{Arf} deficiency in MEFs lacking Pten abolished cell senescence and promoted hyperproliferation and transformation despite the unabated increase in p53 abundance. Consistent with the effect of p19^{Arf} loss in Pten-

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One Sentence Summary: p19^{Arf} signals independently of p53 in prostate cancer associated with the loss of PTEN.

Editor's Summary In most cases, cancer progression depends on multiple genetic insults. For example, in prostate, the complete loss of the gene that encodes the tumor suppressor Pten (phosphatase and tensin homolog deleted from chromosome 10) elicits a compensatory increase in the abundance of the tumor suppressor p53, triggers a cellular senescence response that inhibits cancer progression. Thus, in mouse prostate, loss of Pten alone leads to a non-lethal form of prostate cancer, whereas concomitant inactivation of the genes encoding both Pten and p53 leads to a rapidly lethal form of the disease. The tumor suppressor p19^{Arf} is known for its role in inhibiting p53 degradation. Chen *et al.* found that, unexpectedly, loss of p19^{Arf} failed to phenocopy the effect of p53-loss on the progression of prostate cancer associated with loss of Pten. Rather, compound *Pten-p19^{Arf}* double-null mutant mice retained a robust senescence response and exhibited a decrease in pre-neoplastic lesions. Moreover, the increase in p53 elicited by loss of Pten was unaffected by loss of p19^{Arf} both in the mouse prostate and in mouse embryonic fibroblasts. Thus, the authors conclude that p19^{Arf} signals independently of p53 in prostate cancer associated with loss of Pten and does not play a tumor suppressor role in the prostate epithelium.

deficient mouse prostate, we found that in human prostate cancers, loss of PTEN was not associated with loss of p14ARF (the human equivalent of mouse p19^{Arf}). Collectively, these data reveal differential consequences of p19^{Arf} inactivation in prostate cancer and MEFs upon Pten loss that are independent of the p53 pathway.

Introduction

ARF (alternative reading frame), one of two products of the *INK4a/ARF* locus, plays a critical role in suppressing tumor initiation and progression, and accumulates in response to such oncogenic events as loss of *Pten*, or activation of Ras or Myc (2), (3,4). *p19^{Arf}*-deficient mice are predisposed to development of sarcomas, lymphomas, and pulmonary and mammary adenocarcinomas (1), but not prostate cancer. ARF (p14^{ARF} in human and p19^{Arf} in mouse) is functionally coupled to p53 through its inhibition of Mdm2-mediated p53 degradation, and overexpression of p19^{Arf} leads to p53-dependent growth arrest and cellular senescence in vitro and in vivo in some tissues (3,5,6). The possibility that ARF might act independently of p53 has been suggested based on binding of ARF to proteins other than MDM2 (murine double minute protein 2), although its p53-independent functions remain controversial and poorly understood (6).

PTEN (phosphatase and tensin homolog deleted from chromosome 10) was identified as a tumor suppressor gene that is frequently mutated or deleted in human cancers, including prostate cancer, in which up to 70% of cases show loss or mutation of one allele of *PTEN* at presentation (7,8,9). Complete germ line deletion of *Pten* in mice results in embryonic lethality at embryonic days 6.5 to 9.5 (E6.5–9.5) (10), highlighting the importance of Pten during mouse embryonic development. Mice lacking a single *Pten* allele survive and develop various tumors in adulthood (10). Reduction of PTEN protein leads to activation of phosphatidylinositol-3-OH kinase (PI3K) and thereby to hyper-activation of the AKT-mTOR (mammalian target of rapamycin) signaling pathway (11–13). Surprisingly, however, acute inactivation of both alleles of *Pten* triggers a cellular senescence response that suppresses cancer progression, through activation of the ARF-p53 pathway (2,14,15). Bypassing senescence through concomitant inactivation of both *Pten* and *p53* leads to a lethal prostate cancer phenotype in mice (2). In agreement with the mouse models, *PTEN*-loss-induced senescence also occurs in human cells (16). Overall, these findings suggest that p19^{Arf}, through its inhibition of Mdm2-mediated p53 degradation, plays a critical role in opposing prostate tumorigenesis in response to loss of *Pten*.

Here, we identify unexpected roles for ARF in cellular senescence, transformation, and cancer initiation that are uncoupled from the p53 pathway.

Results

Loss of *p19^{Arf}* does not accelerate prostate tumorigenesis

We previously showed that concomitant inactivation of *Pten* and *Trp53* accelerates tumor progression in *Pten*-deficient mice by overcoming *Pten*-loss-induced cellular senescence and, as a result, double mutant mice with specific inactivation of *Pten* and *Trp53* in prostate epithelium invariably die of prostate cancer at 7 months of age (2). p19^{Arf} accumulates upon acute loss of *Pten* both in mouse embryo fibroblasts (MEFs) and in early prostate intraepithelial neoplasia (PIN) lesions, in which the cellular senescence program is active (2). This fact, together with the notion that p19^{Arf} promotes the stabilization of p53, prompted us to examine the role of p19^{Arf} in prostate tumorigenesis driven by loss of *Pten*. We generated prostate-specific [Probasin (PB)-Cre4-driven] *Pten-p19^{Arf}* double mutant mice following the same strategy described previously (2). Briefly, we crossed *Pten^{loxP/+}*;

PB-Cre4 transgenic mice with *p19^{Arf}^{-/-}* mice to obtain the *p19^{Arf}^{-/-}; Pten^{loxP/loxP}*; *Pb-Cre4* mice, hereafter referred to as *p19^{Arf}^{-/-}; Pten^{pc-/-}*. To minimize the effects of variations in genetic background among individual genotypes, a cohort of wild type (*Wt*), *p19^{Arf}^{-/-}*, *Pten^{pc-/-}* single mutant and *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-null mice were generated from F2 offspring and intercrossed for more than three generations (fig. S1A).

Because *p19^{Arf}* and *p53* are functionally coupled (6,17), we reasoned that *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-mutant mice would, at least in part, phenocopy the cancer acceleration observed in *Pten-Trp53* double mutant mice. We followed a cohort of 77 animals over a period of 17 months (Fig. 1A), subjecting them to bi-weekly Magnetic Resonance Imaging (MRI) analysis as described previously (2). Unlike the lethal prostate cancer phenotype of *Pten-Trp53* double-null mice, concomitant loss of *Pten* and *p19^{Arf}* did not affect disease survival relative to that in *p19^{Arf}^{-/-}* mice (Fig. 1A, non significant differences between *p19^{Arf}^{-/-}* and *p19^{Arf}^{-/-}; Pten^{pc-/-}* compound mutant mice, $P > 0.05$, Kaplan-Meier survival test). Indeed, all *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-mutant mice died by 13 months of age of tumors typically found in *p19^{Arf}* mutant mice, rather than of prostate cancer. Overt tumor masses in the prostate region were detected by 6 months of age in both *Pten^{pc-/-}* mutant mice and *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-mutant mice, but not in *p19^{Arf}^{-/-}* or *Wt* cohort (Fig. 1B, C). The prostate lobes of *Pten^{pc-/-}* mutant mice and *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-mutant mice were visibly enlarged to a similar extent. Prostate tumors from *p19^{Arf}^{-/-}; Pten^{pc-/-}* double mutant mice were comparable in size to those from *Pten^{pc-/-}* mutant mice by MRI visualization (fig. S1B) and after biopsy (anterior prostates, Fig. 1B). The average tumor weight of *p19^{Arf}^{-/-}; Pten^{pc-/-}* double mutant mice was indistinguishable from that in *Pten^{pc-/-}* mutant mice (Fig. 1C; 339 ± 52 mg, compared with 336 ± 87 mg; $n=10$, $P = 0.91 > 0.05$, *t*-test). These results show that *p19^{Arf}* does not play a major tumor suppressive role in *Pten*-loss driven prostate tumorigenesis and highlight a phenotypic discordance between *Trp53* loss and *p19^{Arf}* loss in prostate cancer progression in *Pten*-null mice.

***p19^{Arf}*-loss constrains prostate cancer driven by *Pten* deficiency**

To investigate the functional consequences of *p19^{Arf}* loss in *Pten*-loss driven prostate tumorigenesis, prostates were collected from 11 to 16 week-old double mutant mice and histopathological analysis (H&E staining) was performed on all three prostatic lobes [anterior prostate (AP), ventral prostate (VP), and dorsolateral prostate (DLP)]. Prostates from *p19^{Arf}^{-/-}* mice, like those from *Trp53* mutant mice, failed to show pathological alterations such as hyperplasia or prostatic intraepithelial neoplasia (PIN); glandular architectures of *p19^{Arf}^{-/-}* prostates were indistinguishable from those of age-matched *Wt* mice ($n = 10$; Fig. 2A). Indeed, no visible alterations were observed in the prostate morphology of *p19^{Arf}^{-/-}* mice over a period of one year.

Analysis of the *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-null mutant mice revealed a diminution of the *Pten^{pc-/-}* phenotype (Fig. 2A, B). The VP of double-null mutant mice (but not the anterior and dorsolateral lobes) showed a decreased incidence of high-grade PIN (HG-PIN) in many individual glands compared with those of *Pten^{pc-/-}* mice (Fig. 2A, right panels). Many glands still showed *Wt*-like glandular features with a single layer of epithelial cells (Fig. 2A and insets), as compared to multicellular layered or cribriform architecture of epithelial cells in glands with HG-PIN. Quantification revealed a significant decrease in the percent of glands with HG-PIN in VP of *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-null mice compared with those from *Pten^{pc-/-}* mice (Fig. 2B; 93% HG-PIN compared with 49% HG-PIN; $P = 0.0024 < 0.005$, *t*-test), whereas no marked difference was found in AP and DLP between these two genotypes. Similarly, *p19^{Arf}^{-/-}; Pten^{pc-/-}* double-null mice showed a reduced epithelial component in tumors compared to *Pten*-null mutant mice at 6 months of age (fig. S1C). Immunohistochemical analysis indicated that *p19^{Arf}*-loss did not alter *Pten*-PI3K pathway signaling, as determined by measuring Akt activation (phospho-Akt) and its

membrane recruitment in $p19^{Arf^{-/-}}; Pten^{pc^{-/-}}$ double-null mutant mice (Fig. 2C). Indeed, epithelial cells positive for phospho-Akt were detected as part of monolayers from $p19^{Arf^{-/-}}; Pten^{pc^{-/-}}$ double-null prostates, indicating that progression to PIN was impaired in $p19^{Arf^{-/-}}; Pten^{pc^{-/-}}$ compound mutant mice (Fig. 2B) despite Akt activation consequent to the complete loss of *Pten*. Additionally, cell proliferation as assayed by KI-67 immunostaining was markedly decreased in glands in which PIN diminution was observed (Fig. 2D). Together, these results support the notion that loss of $p19^{Arf}$ fails to promote prostate cancer driven by loss of *Pten*.

Loss of $p19^{Arf}$ does not affect accumulation of p53 protein and cellular senescence in mouse *Pten*-null prostates

Next, we investigated the effect of $p19^{Arf}$ loss on increased p53 abundance and the senescence response in *Pten*-null prostates. To do so, prostates were collected from $p19^{Arf^{-/-}}; Pten^{pc^{-/-}}$ double mutant mice at 11 weeks of age to evaluate the activity of senescence-associated β -galactosidase (SA- β -gal). $Pten^{pc^{-/-}}$ mutants showed a strong senescence response as determined by SA- β -gal activity (Fig. 3A). $p19^{Arf^{-/-}}; Pten^{pc^{-/-}}$ double-null mutant prostates retained a senescence response comparable to that of $Pten^{pc^{-/-}}$ mutants (Fig. 3A and right panels for a representative example from anterior prostate lobes, arrows denote senescent cells), whereas prostates from age-matched *Wt* and $p19^{Arf^{-/-}}$ mice showed low to undetectable SA- β -gal activity (Fig. 3A). Indeed, the percentage of senescent cells in $p19^{Arf^{-/-}}; Pten^{pc^{-/-}}$ double-null mutant mice prostates was indistinguishable from that in $Pten^{pc^{-/-}}$ (Fig. 3B; $19.6 \pm 5.3\%$ compared with $17.8 \pm 4.3\%$; $P = 0.33 > 0.05$, *t*-test), both of which had nearly 15 times the number of senescent cells as did *Wt* prostates ($1.3 \pm 0.87\%$). The increase in p53 protein and percentage of p53-positive cells was unaffected in prostatic epithelium of $p19^{Arf^{-/-}}; Pten^{pc^{-/-}}$ double-null mutant mice compared to $Pten^{pc^{-/-}}$ mice (Fig. 3C, 3D). Moreover, p53 function was intact in compound mutant prostates, as determined by an increase in the abundance of p21, a transcriptional target of p53 (fig. S2).

Our results therefore demonstrate that, following loss of *Pten*, p53 accumulation and execution of senescence are independent of $p19^{Arf}$, suggesting that, in prostate cancer, the functions of $p19^{Arf}$ are unrelated to the p53 pathway.

$p19^{Arf}$ -loss bypasses senescence and promotes transformation independently of p53 in *Pten*- null MEFs

We used primary MEFs to investigate the independence of $p19^{Arf}$ - and p53- signaling and the biological consequences of this uncoupling. First, we determined whether the increased abundance of p53 protein and senescence response that occur with *Pten* loss were affected by $p19^{Arf}$ -loss in MEFs. Following the same strategy reported previously (2), we prepared a series of conditional $p19^{Arf}$ -*Pten* ($p19^{Arf^{-/-}}; Pten^{\Delta/\Delta}$) MEFs and acutely deleted *Pten* through retroviral delivery of Cre-PURO-IRES-GFP (Cre-puromycin resistant-internal ribosome entry site- green fluorescent protein). In the $p19^{Arf}$ -deficient context, virus-mediated expression of *Cre* recombinase led to efficient excision of *LoxP* alleles of conditional *Pten* and subsequently an increase in Akt phosphorylation following *Pten* inactivation in MEFs (Fig. 4A). Consistent with the in vivo prostate data, increased abundance in p53 and p21 proteins following loss of *Pten* was unaffected in $p19^{Arf^{-/-}}; Pten^{\Delta/\Delta}$ MEFs (Fig. 4A, C), strengthening the notion that $p19^{Arf}$ is not required for the increase in p53 that follows loss of *Pten*. Quantification revealed that p53 protein abundance in $p19^{Arf^{-/-}}; Pten^{\Delta/\Delta}$ MEFs was similar to that in $Pten^{\Delta/\Delta}$ MEFs, around 2.5-times that of *Wt* (Fig. 4B). Moreover, the half-life of p53 in $p19^{Arf^{-/-}}; Pten^{\Delta/\Delta}$ cells was similar to that in *Wt* cells (Fig. 4D), further supporting the notion that p53 increase induced by *Pten* deletion is independent from the inhibition of Mdm2-mediated p53 degradation by $p19^{Arf}$. The half-life

of p21, downstream target of p53, was also comparable to that in *Wt* cells (fig. S3A), suggesting that p53 signaling is unaffected by loss of p19^{Arf}.

Despite a sharp increase in p53 abundance, loss of p19^{Arf} resulted in inhibition of the senescence response (Fig. 4E); SA- β -gal activity was undetectable in p19^{Arf}^{-/-}; *Pten* ^{Δ/Δ} cells (Fig. 4E and fig. S3B). In addition, combined loss of *Pten* and p19^{Arf} led to a significant increase in cell proliferation (Fig. 4F, $P = 0.042 < 0.05$, *t*-test) and cell transformation as determined by soft agar transformation assay (Fig. 4G), as compared to p19^{Arf} null cells. As p16^{Ink4a}-RB/E2F pathway plays critical roles in senescence and cell proliferation, the abundance of their proteins was evaluated in MEFs. Increased phospho-RB (pRB, ser 780) and p16^{Ink4a} protein levels were observed in both p19^{Arf}^{-/-}; *Pten* ^{Δ/Δ} and *Pten* ^{Δ/Δ} MEFs (Fig. 4A and Fig. 4B, lower panel). The increase in RB phosphorylation at ser 780 was also observed in p19^{Arf}^{-/-}; *Pten* ^{$\Delta/+$} cells but not in p19^{Arf}^{-/-} cells, indicating that phosphorylation of RB is triggered even in cells lacking a single copy of *Pten*. (Fig. 4C). E2F-1 and its transcriptional target, proliferating cell nuclear antigen (PCNA), were increased in p19^{Arf}^{-/-}; *Pten* ^{Δ/Δ} MEFs compared to p19^{Arf}^{-/-} and *Pten* ^{Δ/Δ} single mutant cells (Fig. 4A and lower right panel). The increase in E2F-1 abundance was restricted to the compound mutant cells, p19^{Arf}^{-/-}; *Pten* ^{Δ/Δ} and p19^{Arf}^{-/-}; *Pten* ^{$\Delta/+$} , suggesting that loss of p19^{Arf} cooperates with loss of *Pten* to promote cell proliferation in association with the increase in E2F-1 (Fig. 4C).

Next, we analyzed the status of E2F-1 and PCNA in mouse prostate epithelium. Immunostaining of the ventral prostate of 11 week-old mice revealed a marked increase of E2F-1 in the *Pten*^{pc-/-} mutant mice, which was attenuated in prostates from p19^{Arf}^{-/-}; *Pten*^{pc-/-} double mutant mice (Fig. 5A). These results correlated with the decrease in PIN observed in these glands. Additionally, PCNA staining, commonly employed as a marker for proliferation, showed a similar pattern as Ki-67 (Fig. 2D). PCNA was decreased in non-PIN areas of prostate epithelium from p19^{Arf}^{-/-}; *Pten*^{pc-/-} double mutant mice, as compared to PIN lesions in *Pten*^{pc-/-} mice and PIN regions of p19^{Arf}^{-/-}; *Pten*^{pc-/-} double mutant mice (Fig. 5B, lower right panel and inset). These data suggest that the opposite effects of p19^{Arf}-loss on cell proliferation in MEFs and in the prostate epithelium correlate with modulation of E2F-1 abundance.

Human prostate cancer biopsies with loss of *PTEN* retain p14^{ARF} expression

Based on these data we hypothesized that p14^{ARF}-loss would not be selected in human prostate cancer in the context of *PTEN*-loss. To test this hypothesis, we analyzed the status of *PTEN* and p14^{ARF} in TMAs (tissue microarrays) from human prostate specimens. We found that complete loss of p14^{ARF} was extremely rare in human prostate cancer (Fig. 6A). In contrast to other types of cancer (18), increased p14^{ARF} abundance correlated with disease aggressiveness [Fig. 6A and Ref. (14)]. Furthermore, we found a correlation between *PTEN*-loss and p14^{ARF} overexpression in prostate cancers (Fig. 6B, N=129, $P < 0.000001$, Chi-square test). Loss of p53 function has been linked to accumulation of ARF as a feedback mechanism (19), which is thought to depend on the negative transcriptional regulation of ARF by p53 (20). Analysis of p53 abundance by immunohistochemical (IHC) staining did not show a significant correlation between p53 immunoreactivity and p14^{ARF} accumulation (Chi-square $p = 0.16$, fig. S4A). However, abundance of p53 by IHC analysis does not provide information about its genomic or mutational status. Therefore, we performed correlation analysis between genomic status of *TP53* and mRNA levels of p14^{ARF} (*CDKN2A*). In the subset of prostate cancer biopsies analyzed (N=103) genomic loss of *TP53* did not correlate with p14^{ARF} mRNA levels ($p = 0.6$, fig S4B). Taken together, our results suggest that, in prostate cancer, the loss of p14^{ARF} is selected against, and lead us to speculate that prostate tumors lacking *PTEN* might in fact for the retention or even the enhancement of p14^{ARF} function.

Discussion

The pathogenesis of prostate cancer (PCa), the second leading cause of cancer-related deaths in Western men (21,22), is a complex process involving alterations in the activity of multiple oncogenes and tumor suppressor genes (23–25). Of these, loss of *PTEN* function together with TMPRSS2-ERG (Transmembrane serine protease 22–Ets-related gene) translocation represent the most frequent events (8,12,26). Following complete loss of *Pten*, p53-dependent cellular senescence provides a fail-safe mechanism to oppose cancer progression; concomitant loss of p53 and *Pten* abolishes this senescence response and results in lethal prostate cancer (2). In general, inhibition of Mdm2 by the tumor suppressor ARF represents one of the most important molecular mechanisms for the upregulation of p53. (27) (28) (29) (30). In line with a tumor-suppressive role for ARF, *p19^{Arf}*-deficient mice develop lymphomas, sarcomas, and adenocarcinomas (1), dying by one year of age. The observation that *p19^{Arf}*-loss did not phenocopy the loss of *Trp53* in the *Pten*-null prostate, with no abrogation of senescence, but rather a decrease in the initiation of prostate cancer is consistent with the notion that ARF could affect in vivo tumor growth in a tissue-specific manner (31). These results provide a plausible explanation for the observation that increased p14^{ARF} abundance correlates with loss of *PTEN* and prostate cancer aggressiveness, in turn suggesting that prostate cancer development may, in fact, select against loss of ARF function.

Both in prostate epithelium and in MEFs, loss of *p19^{Arf}* did not affect the marked increase in p53 abundance observed in the context of *Pten* deficiency. Several groups have identified p53-independent functions of ARF through its interaction with various different proteins (6). Our results suggest that ARF may exert distinct functions in a tissue-specific and p53-independent manner, possibly due to interactions with different targets in different cell types. In line with this hypothesis, we studied the localization and abundance of the ARF-interacting protein NPM (nucleophosmin, also called B23), and did not detect any changes upon compound *Pten-p19^{Arf}* loss (fig. S5), suggesting NPM is not involved in p53 pathway in vitro and in vivo upon *Pten* loss.

In MEFs, however, the concomitant loss of *Pten* and *p19^{Arf}* resulted in the abrogation of senescence and in hyper-proliferation and transformation. The differential requirement of ARF with *Pten* loss could involve different wiring of Mdm2-p53 in MEFs and prostate epithelium. However, Mdm2 inhibitors (Nutlin-3) promote p53 upregulation in both experimental settings (32), confirming that in both MEFs and in prostate epithelial cells p53 is under the control of Mdm2. Further, in both settings p53 is markedly increased even in the absence of ARF. This suggests that, with *Pten* loss, ARF exerts different functions in MEFs and prostate epithelium.

In conclusion, these findings establish cell context-specific functions for ARF in transformation, senescence, and cancer initiation that are independent of the Mdm2-p53 pathway. They also suggest that the role of ARF in cancer may vary, depending on tumor genetic milieu and tissue-cell type. Our findings also underscore the need for a careful reevaluation of the status and role of p14^{ARF} in human cancer. A deeper understanding of the p53-independent functions regulated by ARF may provide novel and critical therapeutic targets for cancer treatment.

Materials and Methods

Generation of *p19^{Arf}* and *Pten* double mutant mice

Pten^{loxP/loxP}, *PB-Cre4* and *p19^{Arf}^{-/-}* mice were maintained as described (1,2,33). Male *Pten^{loxP/+}*; *PB-Cre4* mutant mice were crossed with female *p19^{Arf}^{-/-}* mice to produce

mouse colonies. Then F1 male $p19^{Arf-/+}$; $Pten^{loxP/+}$; $PB-Cre4$ mice were mated with F1 female $p19^{Arf-/+}$; $Pten^{loxP/+}$ mice to obtain F2 offspring, which were used to generate the following genotypes: Wild type (Wt), $p19^{Arf}$ deficient mice ($p19^{Arf-/-}$), prostate-specific $Pten$ mutant ($Pten^{loxP/loxP}$; $PB-Cre4$, referred to as $Pten^{pc-/-}$), and prostate-specific $p19^{Arf}$ - $Pten$ double-mutant ($p19^{Arf-/-}$; $Pten^{loxP/loxP}$; $PB-Cre4$, referred to as $p19^{Arf-/-}$; $Pten^{pc-/-}$) mice. For genotyping, DNA extracted from mouse tails was subjected to PCR analysis using the same PCR primers and conditions as described (2). All experimental animals were kept in a mixed genetic background of C57BL/6J X129/Sv. Animal experiments were performed in accordance with the guidelines of Institutional Animal Care and Use Committee.

MRI and histopathology

Individual mice were subjected to Magnetic Resonance Imaging (MRI) assessment for the detection of prostate tumors as described (2). Tissues were fixed in 10% neutral-buffered formalin (Sigma) overnight, rinsed with PBS, and stored at 4°C in 70% ethanol. Tissues were processed for ethanol dehydration and embedded in paraffin by Histoserv Inc (Gaithersburg, MD) according to standard protocols. 5 µm tissue sections were prepared for hematoxylin and eosin (H&E) staining and antibody detection.

Cell proliferation, transformation, and senescence assays

MEFs were prepared from individual embryos of various genotypes, and early passage MEF cultures (P1-P2) as described (33) and were then infected with retroviruses expressing Cre-PURO-IRES-GFP or empty vector (without Cre) (2). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, and 100U/ml penicillin and streptomycin (GIBCO). To achieve a high titer of retroviral particles, 2×10^6 Phoenix cells were seeded per 10 cm Poly-D-Lysine-Coated culture dish (BD) followed by overnight culture in an incubator at 37 °C with 5% CO₂. pMSCV-Cre-PURO-IRES-GFP or empty vector was transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Forty-eight hours after transfection, retroviral supernatants were collected and filtered through a 0.45 µm filter. For retroviral infection, MEFs at passage 1 or 2 were plated at a density of $3-4 \times 10^5$ cells per 10-cm culture dish and incubated with 10ml of freshly-made viral supernatant containing 5µg/ml polybrene (Calbiochem). To increase infection efficiency, MEFs were exposed to viral supernatants a second time, followed by replacement with fresh medium after 8 hrs. After the second infection, MEFs were cultured for an additional 48 hours and selected with 2 µg/ml puromycin in 10ml of fresh medium (Sigma) for 2 days. Selected MEFs at passage 5 were used for growth curves, Western Blot, and cellular senescence analysis. To evaluate cell proliferation, MEFs were plated at 2×10^4 cells per well in 12-well plates in triplicate, and cells were counted with a hemacytometer at 2, 4, 6, 8 days. To determine senescence, MEFs were plated at 1×10^4 cells per well of a 6-well plate in triplicate, and after 4 days senescence-associated β-galactosidase (SA β-gal) was detected with the senescence detection kit (Calbiochem) and quantified (more than 200 cells per sample). For prostate tissue, frozen sections at 6 µm thickness were stained for β-gal as above. For transformation assay, selected MEFs (3×10^4) at passage 5 were suspended in medium containing 0.3% agar onto solidified 0.6% agar per well of a 6-well plate. The ability to grow in soft agar was assessed by size and number of colonies counted after 21 days.

Western Blot and immunohistochemistry

MEF lysates were prepared with RIPA buffer [1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Roche)]. The following antibodies were used for Western Blot: Mouse monoclonal antibody directed against Pten [anti-Pten (6H2.1, Cascade BioScience)], rabbit polyclonal anti-p53 (CM5, Novocastra), rabbit polyclonal anti-Akt and phospho-Serine 473 of Akt (Cell Signaling), rabbit polyclonal

phospho-RB (ser780, Cell Signaling), rabbit polyclonal anti-p19^{Arf} (C-19, NeoMarkers), rabbit polyclonal anti-p21 (C-19, Santa Cruz), rabbit polyclonal anti-p16 (M-156, Santa Cruz), rabbit polyclonal anti-E2F-1 (C-20, Santa Cruz), mouse monoclonal anti-PCNA (SC-56, Santa Cruz), rabbit polyclonal anti-NPM (3542, Cell signaling) and mouse monoclonal anti- β -actin (AC-74, Sigma). To determine the half-life of p53 and p21 proteins, MEFs (*Pten*^{+/+}-*Cre* and *p19*^{Arf}^{-/-}-*Pten* ^{Δ/Δ} -*Cre*, at 80% confluence and equal passage number) were treated with 30 μ g ml⁻¹ cycloheximide (Sigma) and harvested at the indicated times for Western analysis. For immunohistochemistry (IHC), sections were stained for phospho-Akt (Ser 473) (mouse monoclonal antibody, Cell Signaling), p53 (FL-393, rabbit polyclonal antibody Santa Cruz), p21 (F-5, mouse monoclonal antibody, Santa Cruz), rabbit polyclonal anti-E2F-1 (C-20, Santa Cruz), Ki-67 (Novacastra), rabbit polyclonal anti-NPM (3542, Cell signaling) and mouse monoclonal anti-PCNA (SC-56, Santa Cruz).

Tumor tissue microarrays (TMAs)

Prostate tumor tissue microarrays were constructed using a fully automated Beecher Instrument (ATA-27). The study cohort comprised prostate tumors biopsies from Memorial Sloan-Kettering Cancer Center (MSKCC). All biopsies were evaluated at MSKCC, and the histological diagnosis and Gleason score was based on established standard criteria. Use of tissues was approved by Institutional Review Board (IRB) waivers and by the Human Biospecimen Utilization Committee (HBUC). TMAs were stained with antibody directed against p14^{ARF} (clone 4C6/4, #2407, mouse monoclonal, Cell Signaling, 1:250 dilution), and scored using the following criteria: 0= tumor cells negative; 1= tumor cells focally positive; 2= tumor cells diffusely positive. PTEN staining (clone 6H2.1, mouse monoclonal, Cascade Bioscience, 1:75 dilution) and scoring were performed as previously published (34). Secondary antibody: biotinylated horse anti-mouse (1:500) and Avidin-Biotin (1:25) (Vector Lab).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Loss of p19^{Arf} does not accelerate prostate tumorigenesis

(A) Cumulative survival analysis (Kaplan-Meier plot) for *p19^{Arf-/-}* (blue line), *Pten^{pc-/-}* (black line) and *p19^{Arf-/-}; Pten^{pc-/-}* (red line). (B) Actual sizes of representative tumors from anterior prostates (AP) of *Wt*, *p19^{Arf-/-}*, *Pten^{pc-/-}*, and *p19^{Arf-/-}; Pten^{pc-/-}* double-mutant mice at 6 months of age. (C) Comparison of AP tumor masses from *Pten^{pc-/-}* and *p19^{Arf-/-}; Pten^{pc-/-}* mice at 6 months of age indicates that p19^{Arf}-deficiency does not accelerate the tumorigenesis in *Pten*-deficient mice (N=10 for each group, $P = 0.91 > 0.05$, *t*-test).

Fig. 2. Loss of p19^{Arf} constrains prostate cancer progression

(A) Histopathology analysis (haematoxylin/eosin staining) of ventral prostates in *Wt*, *p19^{Arf}^{-/-}*, *Pten^{pc}^{-/-}*, and *p19^{Arf}^{-/-}; Pten^{pc}^{-/-}* double mutant mice at 11–16 weeks of age. (B) Quantification of high-grade prostatic intraepithelial neoplasia (HG-PIN) in *Wt*, *p19^{Arf}^{-/-}*, *Pten^{pc}^{-/-}* and *p19^{Arf}^{-/-}; Pten^{pc}^{-/-}* double-mutant mice at 11–16 weeks of age (*N*=4 for each group). (C) Immunohistochemistry staining of pAkt (indicated by arrows) in adjacent sections from ventral prostates of (A). Insets indicate the lower magnification. Error bars in (B) represent S.D. for a representative experiment performed in triplicate. (D) Ki-67 in adjacent sections from ventral prostates of (A), and an inset showing the Ki-67 positivity in PIN lesions in *p19^{Arf}^{-/-}; Pten^{pc}^{-/-}* double mutant mice prostate.

Fig. 3. In vivo p19^{Arf} p53 uncoupling and cellular senescence contribute to cancer suppression in compound mutant mice

(A) Histopathology and senescence analysis of 11-week-old prostates, stained as indicated for H&E, and senescence associated-β-galactosidase (β-gal) in anterior prostates (AP). (B) Quantification (percentage of cells) of the β-gal staining seen on (A) sections from 11-week-old mice ($19.6 \pm 5.3\%$ for *p19^{Arf}^{-/-}; Pten^{pc}^{-/-}* double mutant mice compared with $17.8 \pm 4.3\%$ for *Pten^{pc}^{-/-}* mice; $P = 0.33 > 0.05$, *t*-test). (C) p53 staining on AP sections from 11-week-old mice, arrows denote the positive staining of p53 in nucleus of epithelial cells. (D) Quantification of the p53 staining seen on (C) sections from 11-week-old mice. Senescence and histopathology analysis of 11-week-old prostates. Representative sections from three

mice were counted for each genotype. Error bars in (B) and (D) represent S.D. for a representative experiment performed in triplicate.

Fig. 4. p19^{Arf}-p53 uncoupling in primary mouse embryonic fibroblasts (MEFs)
(A) Western Blot of lysates of primary MEFs with indicated antibodies in *Wt*, *Pten* Δ/Δ , and *p19*^{Arf} $^{-/-}$ -*Pten* Δ/Δ double-null cells. β -actin is used as a loading control. **(B)** Quantification of protein abundance in primary MEFs for p53, p16 and pRb from (A). **(C)** Western blotting of cellular lysates of primary MEFs with indicated antibodies in *p19*^{Arf} $^{-/-}$, *p19*^{Arf} $^{-/-}$ -*Pten* Δ/Δ and *p19*^{Arf} $^{-/-}$ -*Pten* Δ/Δ double-null cells. **(D)** Inhibition of protein synthesis by cycloheximide (CHX) combined with Western blot at the indicated times (minutes) shows that the half-life of p53 in *p19*^{Arf} $^{-/-}$ -*Pten* Δ/Δ double-null MEFs is comparable to that in *Wt* MEFs. Right panel: quantification of p53 half-life from the Western blots in left panel normalized to β -actin. Blue circles, *Wt*-Cre; red squares, *p19*^{Arf} $^{-/-}$ -*Pten* Δ/Δ Cre. **(E)** Cellular senescence assay of *Wt*, *p19*^{Arf} $^{-/-}$, *Pten* Δ/Δ , *p19*^{Arf} $^{-/-}$ -*Pten* Δ/Δ double-null cells. **(F)** Growth curves of primary MEFs, infected with retroviral Cre (under puromycin selection) followed over a 6 day period: *p19*^{Arf} $^{-/-}$ -*Pten* Δ/Δ double-null (red squares), *Pten* Δ/Δ (black squares), *p19*^{Arf} $^{-/-}$ (black circles) and *Wt* cells (blue triangles). **(G)** Transformation assay as determined by colony formation in soft agar from (E), and *Pten* Δ/Δ ; *p53* Δ/Δ double-null cells served as positive control. Error bars for (B, E, F and G) indicate S.D. for a representative experiment performed in triplicate.

Fig. 5. Loss of *p19^{Arf}* in *Pten* null prostate mutant mice results in decreased E2F-1 and PCNA upregulation
 Immunohistochemical (IHC) staining for E2F-1 (**A**) and PCNA (**B**) of ventral prostates in *Wt*, *p19^{Arf}^{-/-}*, *Pten^{pc}^{-/-}* and *p19^{Arf}^{-/-}; Pten^{pc}^{-/-}* double mutant mice at 11 weeks of age. PIN lesions in *p19^{Arf}^{-/-}; Pten^{pc}^{-/-}* double mutant mice prostate in (B) are represented in an inset.

Fig. 6. Overexpression of p14^{ARF} in human prostate cancer specimens correlate with PTEN-loss (A) p14^{ARF} protein abundance in prostate cancer biopsies. Right: Representative images for p14^{ARF} staining in specimens at low and high severity grades. Left: graph showing the correlation of disease aggressiveness and p14^{ARF} abundance; 0= tumor cells negative for p14^{ARF}; 1= tumor cells focally positive for p14^{ARF}; 2= tumor cells diffusely positive for p14^{ARF}. (B) Correlation of PTEN and p14^{ARF} protein abundance in human prostate specimens by immunohistochemistry (IHC). Representative images showing p14^{ARF} staining in specimens with normal PTEN abundance (1) and PTEN loss (0). Yellow indicates abundance 2, orange abundance 1 and red abundance 0. (C) Schematic representation of the findings highlighted in this study.