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14. ABSTRACT

The Rb pathway suppresses tumorigenesis by constraining G1 cell cycle progression. Functional inactivation or reduction of this pathway is a common event found in many types of human tumors. To test how the Rb pathway interacts with other cellular pathways in tumor suppression, we characterized mice with combined mutations in the CDK inhibitor $p18^{Ink4c}$ and the lipid phosphatase *PTEN*, which regulates cell growth and survival. The $p18^{-/-}$*; PTEN*$^{+/-}$ double mutant mice develop prostate cancer in the anterior and dorsolateral lobes and thyroid C-cell tumors with nearly complete penetrance, and pituitary tumors in both the anterior and intermediate lobes. AKT/PKB, an oncoprotein and downstream substrate of PTEN, was activated and accumulated at the plasma membrane in *PTEN*$^{+/-}$ cells, and further activated and accumulated in the nucleus in $p18^{-/-}$*; PTEN*$^{+/-}$ tumor cells, suggesting a tissue- and genetic background-dependent haploinsufficiency of *Pten* in tumor suppression. *p18* deletion, CDK4 overexpression, or oncoviral inactivation of Rb family proteins caused activation of Akt/PKB that was recessive to the reduction of PTEN activity. We suggest that *p18* and *Pten* cooperate in tumor suppression by constraining a positive regulatory loop between cell growth and cell cycle control pathways

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Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer death in males. The *PTEN* (phosphatase and tensin homolog deleted from chromosome 10) tumor suppressor (Li et al., 1997), also known as MMAC1 (mutated in multiple advanced cancers) (Steck et al., 1997) or TEP1 (TGFβ-regulated and epithelial cell enriched phosphatase) (Li and Sun, 1997), is located on a genomic region that frequently suffers loss of heterozygocity (LOH) in different types of advanced human cancer, including prostate cancer (Amanatullah et al., 2001; Cantley and Neel, 1999; Di Cristofano and Pandolfi, 2000). Loss of function of the tumor suppressors PTEN as well as p18INK4c, a cyclin-dependent kinase inhibitor, is a frequent event in metastatic prostate cancer and PTEN+/- mice display hyperplastic/dysplastic features in the prostate at a young age. P18INK4c is an inhibitor of CDK4 and a homologue of p16INK4a, a tumor suppressor that is altered in an estimated 40% of human tumors of different types. Genetic analysis of *PTEN* mutant mice sustaining monoallelic or null mutations strongly support a key function of *PTEN* in suppressing prostate tumor suppression (Di Cristofano et al., 2001; Di Cristofano et al., 1998; Podsypanina, 1999; Stambolic et al., 2000; Wang et al., 2003). The biochemical mechanism underlying *PTEN*'s tumor suppression function is believed to lie in its phosphatase activity. Most missense mutations in *PTEN* detected in primary tumors and in established cell lines are confined to exon 5 encoding the phosphatase domain. The main *in vivo* substrate of PTEN phosphatase activity is the lipid second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Maehama and Dixon, 1998), placing PTEN into a previously defined signaling pathway in which the proto-oncogene serine/threonine kinase Akt is a major effector of PTEN (Amanatullah et al., 2001; Cantley and Neel, 1999; Di Cristofano and Pandolifi, 2000). The cellular function of *PTEN* was recently linked to cell growth control by the findings that TSC1/2, a heterodimeric complex consisting of TSC1 and TSC2 whose mutations predispose individuals to hamartomas in many tissues and inhibit mTOR-mediated protein synthesis, is a major downstream target of AKT (Cantley, 2002). We reason that simultaneous stimulation of cell growth, resulting from a reduction of PTEN activity, and the cell cycle, caused by the loss of function of p18, may more effectively promote tumor development than the alteration of either pathway alone. We have generated *PTEN*-p18 double mutant mice and found that PTEN and p18 have a cooperative role in tumor suppression in the prostate as well as in the pituitary, adrenal medullary, thyroid and lymph node.

Body

I. Background

Animal models for studying prostate cancer

Human prostate cancer is characterized by a long latency between the appearance of precursor lesions, known as prostate intraepithelial neoplasia (PIN), as early as in the twenties in men, and the manifestation of clinically detectable carcinomas late in life (sixties or older). One of the major goals of current prostate cancer research is to identify the genes and understand the molecular pathway(s) underlying the initiation and early steps of progression of prostate tumors which are nearly inaccessible in humans. Historically, a major limitation toward this goal has been the lack of suitable animal models that faithfully recapitulate different stages of prostate tumor progression, in large part because of anatomical differences and the rate of tumor development between the mouse and human prostate[17]. Over the years, various lines of gain-of-function oncogene transgenic mice and loss of function tumor suppressor knock-out mice strains have been developed in attempts to model human prostate tumors. The most extensively characterized transgenic mice, the transgenic adenocarcinoma mouse prostate (TRAMP) expressing from a minimal probasin promoter both SV40 large T and small t oncoproteins and the less aggressive line expressing only the large T (LADY) model, inactivate both p53 and Rb functions and develop high-grade PIN and/or prostate cancer within 12 weeks of birth[18-20]. While these two models display prostate-restricted disease and characteristic features of cancer progression, they differ from human prostate cancer in two key aspects: the rapid rate of progression and the prevalence of neuroendocrine (NE) tumors. In addition to SV40 oncoproteins, several other transgenic lines of mice have been generated that overexpress various oncogenes, including c-myc[21,22], IGF-1[23], and androgen receptor[24]. The prostate phenotypes of these transgenic models are significantly less severe than the SV40 model—prone to develop PIN, but not adenocarcinomas.

Two knock-out mouse models, the *Nkx3.1* homeobox gene and *Pten* phosphatase, display prostatic tumor phenotypes. The *Nkx3.1* gene is expressed restrictively in the prostate epithelium early during mouse embryogenesis and at all stages of subsequent prostate differentiation. Targeted disruption of the *Nkx3.1* gene resulted in an age- and allelic-dependent development of prostate epithelial hyperplasia and dysplasia[25-27], indicating a haploinsufficiency of *Nkx3.1* gene in prostate tumor suppression. No overt tumors developed in *Nkx3.1* mutant mice up to 2 years of age[25,26,28], suggesting that full activity of Nkx3.1-mediated prostate tumor suppression may involve a functional collaboration with other tumor suppressor(s). The *Pten* gene, on the other hand, is expressed broadly during development and in many adult tissues. Pten is essential for embryogenesis and heterozygous Pten mice develop cancers in multiple tissues including the prostate [4,6,29]. The prostate tumor phenotype developed slower in both *Nkx3.1* and *Pten* mutant mice than in TRAMP or LADY mice, occurred primarily at the dorsolateral prostate, and was not found in neuroendocrine cells, presenting these two mice as excellent models for studying mechanisms of prostate cancer initiation and progression.

It is emerging that cancer development not only involves collaboration between gain-of-function of oncogene(s) and loss-of-function of tumor

suppressor(s), but also between losses of function of different tumor suppressors. A functional collaboration between tumor suppressor genes in suppressing prostate tumors was observed between *Pten* and *Nkx3.1*30 and between *Pten* and CDK inhibitor *p275*. In both *Pten*$^{+/-}$;*Nkx3.1*$^{-/-}$ and *Pten*$^{+/-}$;*p27*$^{-/-}$ double mutant mice, the prostate tumor phenotype developed at an accelerated rate and was more aggressive. A major goal of this proposal is to test the hypothesis that Pten functionally collaborates with another tumor suppressor, CDK inhibitor p18^{Ink4c}, to suppress prostate tumor growth.

Tumor suppression by PTEN

The *PTEN* (phosphatase and tensin homolog deleted from chromosome 10) tumor suppressor1, also known as MMAC1 (mutated in multiple advanced cancers)[2] or TEP1 (TGFâ-regulated and epithelial cell enriched phosphatase)[31], is located on a genomic region that frequently suffers LOH in different types of advanced human cancer, including prostate cancer[32-34]. Genetic analysis of Pten mutant mice sustaining monoallelic or null mutation strongly supports a key function of PTEN in suppressing prostate tumor formation[4-7,29]. Aims I and III of this proposal describe experiments to further establish the genetic function of Pten in suppressing prostate tumor development, and Aim II includes experiments to determine the androgen-dependency of prostate tumor growth in mice deficient in Pten function.

Mammalian G1 control and tumor suppression by the Rb pathway

Inevitably, various cell growth control pathways such as mitogenic stimulation, tumor suppression, and cell differentiation must interact with the pathways that regulate progression through the G1 phase of the cell cycle. The eukaryotic cell cycle is primarily regulated by a family of serine/threonine protein kinases, consisting of a regulatory cyclin subunit and a catalytic CDK subunit (cyclin dependent kinase)[35,36]. In mammalian cells, G1 progression is controlled principally by two CDK enzymes: CDK4 or CDK6 in combination with D-type cyclins (D1, D2, and D3), and CDK2 in association with E-type cyclins (E1 and E2). The expression of the cyclin D genes and their associated CDK4/6 kinase activity, with resulting phosphorylation of pRB, is induced during the delayed early response to mitogenic stimulation[37-39], supporting the notion that CDK4/6-cyclin D functions to couple mitogenic signals to the cell cycle.

The major negative regulation of both G1 CDKs is provided by binding with members of two families of CDK inhibitors, the KIP/CIP family and the INK4 family (diagrammed in Fig. 1). In mammalian cells, there exist two distinct families of CDK inhibitors. The p21 family includes three related genes, p21$^{Cip1/Waf1}$, p27^{Kip1} and p57^{Kip2}, which evolved from an ancestor that predates the origin of *C. elegans* and plants. The p16 family comprises four closely related members, p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}, and evolved later after the emergence of vertebrates. The physiologic significance of evolving a separate family of CDK inhibitors and multiple members within each family in mammalian cells is presumed to meet increasing needs for integrating more intricate and multifaceted cell growth signals, both intracellular and extracellular, into a single cell cycle control machinery[40]. Ectopic overexpression of individual INK4 genes caused a G1 cell cycle with a correlative dependency on the intact Rb pathway[16], and loss of either Rb or a combination of p107 and p130 function effectively canceled the G1 arrest by INK4 overexpression[41-43]. These findings provide evidence that, at least in cultured cells, the function of INK4, and CDK4 and CDK6 by extension, in

controlling the G1-to-S transition is dependent on the presence of both intact Rb and p107-p130 functions.

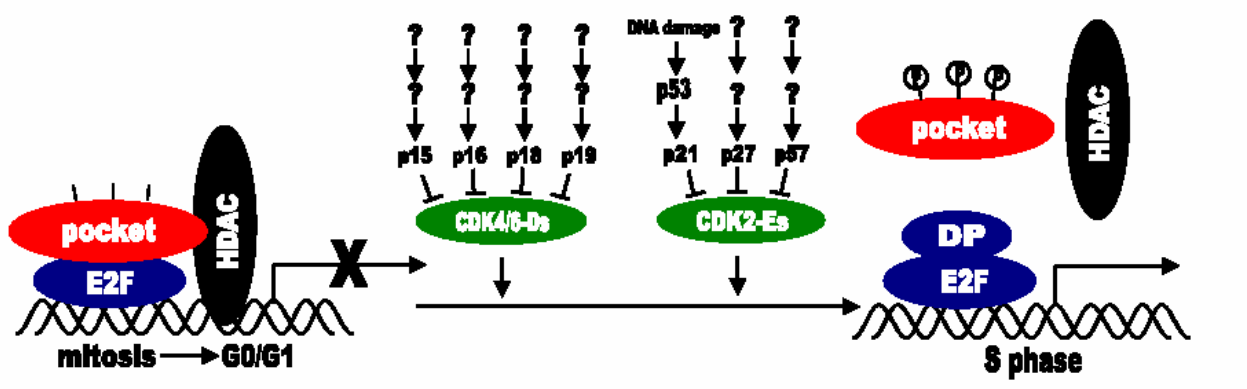


Fig.1. Mammalian G1 Cell Cycle by CDKs, Cyclins, and CDK Inhibitors. Emerging from mitosis, pocket proteins (Rb, p107 and p130) are hypophosphoryated and bind to and recruit histone deacetylase (HDAC) to repress E2F-responsive promoters. During G1, mitogenic signals induce the synthesis of D-type cyclins, leading to activation of cyclin D-dependent CDK4 and CDK6, which in collaboration with cyclin Es-CDK2 (not depicted in this figure) phosphorylate pocket proteins, de-repressing E2F to allow transcription of E2F target genes, thereby permitting S-phase entry. Members of two families of CDK inhibitors inhibit CDK4 and CDK6. Various genes involved in this pathway, Rb, p16, p18, p27, CDK4, cyclin D1 and E2F1, have been genetically linked with tumor suppression, by either mutations in human cancers or by the development of tumors in targeted mice.

Rb is the prototypical tumor suppressor gene whose germline mutation predisposes individuals to retinoblastoma in childhood and, to a lesser extent, osteosarcoma later in life. Sporadic mutations of the Rb gene have been found in a wide range of human cancers. In fact, genetic alterations of Rb and its upstream regulators, D-type cyclins, CDK4, and $p16^{INK4a}$, occur so frequently in human cancer that inactivation of this so-called “Rb pathway” may be necessary for the development of most tumors[44-46]. Genetic study in mice provides further support for a critical function of this pathway in tumor suppression. Mice heterozygous for *Rb* mutations spontaneously develop tumors in several neuroendocrine organs, including the characteristic intermediate lobe of the pituitary gland[47-51]. Transgenic mice engineered to overexpress cyclin D1 in mammary glands resulted in hyperplasia and carcinoma[52], and conversely ablation of cyclin D1 renders mice resistance to breast cancer development[53]. Mice carrying an INK4-insensitive mutation (R24C) in the *CDK4* locus develop a wide spectrum of tumors, including tumors in several neuroendocrine organs (Leydig cell of testis, granulosa cell of ovary, pancreatic islet cells, intermediate lobe of the pituitary gland, and thyroid) as well as lung adenomas/adenocarcinomas and hepatocellular tumors[54].

Function of CDK inhibitors in tumor suppression

Several observations suggest that $p18^{INK4c}$ may play an important and broad role in mediating the tumor suppression function of the Rb pathway. We have

previously generated and characterized mutant mice lacking *p18*8. *p18*-deficient mice are generally developmentally normal and fertile, but develop wide-spread hyperplasia, gigantism, intermediate lobe pituitary tumors with nearly complete penetrance by the age of 10 months, and less frequently (~25%) T-cell lymphomas at a later stage between 12 and 14 months. Mice lacking both *p18* and *p27*, like mice chimeric for *Rb*-deficiency, invariably died from pituitary adenomas by 3 months. In addition to the pituitary tumors, mice lacking both *p18* and *p27*, but not either alone, rapidly developed at least seven additional types of hyperplastic tissues and/or tumors in the adrenal glands, thyroid, parathyroids, testes, pancreas, duodenum and stomach[10]. Mice lacking three of the four *p18* and *p27* alleles displayed an intermediate incidence and rate of tumorigenesis in many tissues between the single and double null mice, indicating gene dosage-dependent tumor suppression by these two genes[10]. We have recently demonstrated that p18 is haploinsufficient in tumor suppression, and that loss and reduction of p18 function sensitizes mice to carcinogen-induced tumorigenesis[11].

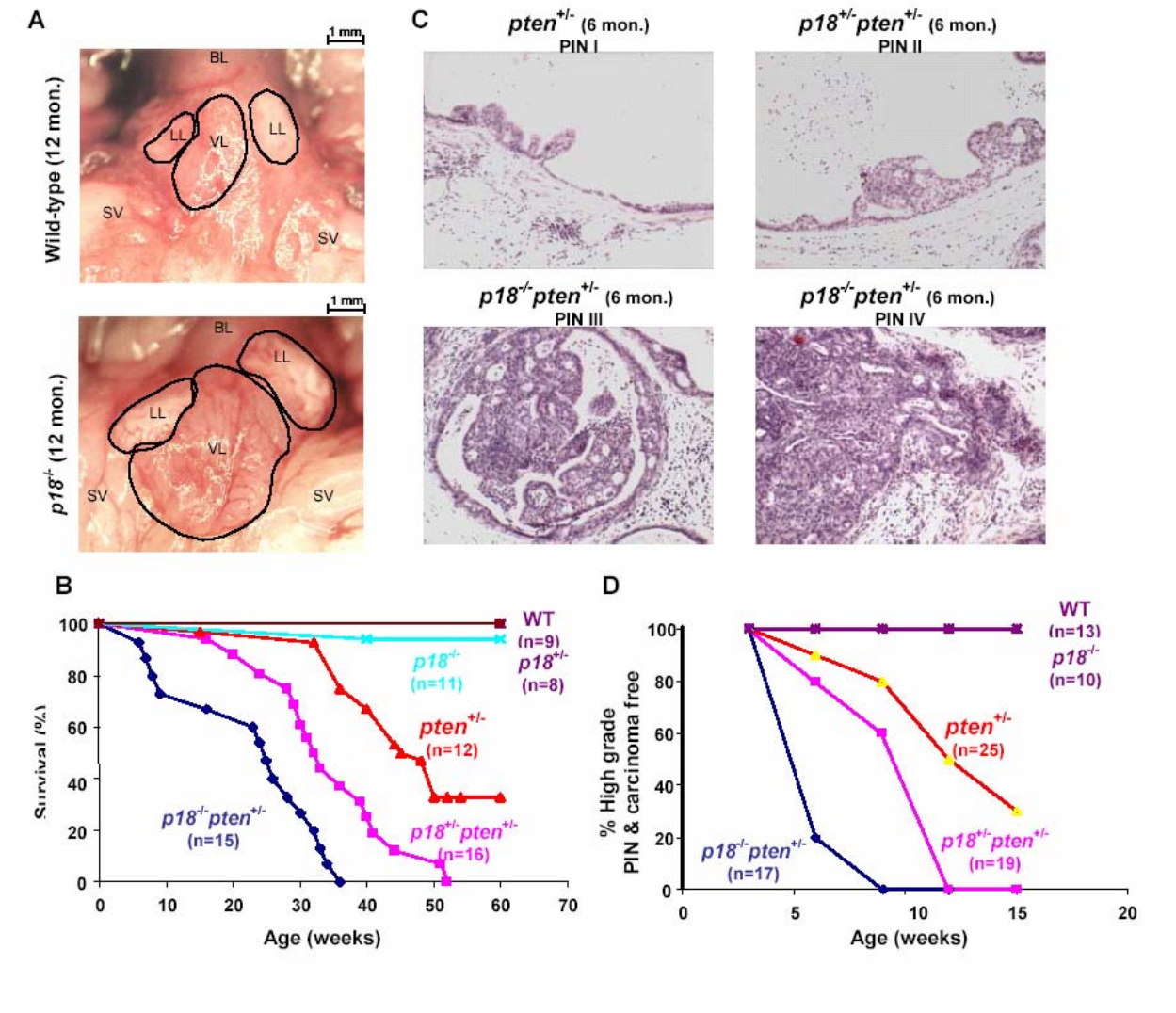


Fig. 2. Prostate tumorigenesis in p18-Pten double mutant mice. (**A**) Age-matched wild type and *p18*$^{-/-}$ mice (12 months) are shown. Significant

enlargement of ventral lobes (VL) and lateral lobes (LL) were seen in *p18*$^{-/-}$ mice. (BL) Bladder; (SV) Seminal Vesicle. Both pictures were reproduced with the same magnification. (**B**) Survival curve of *p18* and *Pten* mutant mice. *p18-Pten* double mutant mice also developed additional phenotypes and the exact cause of death has not been determined, but unlikely to be due to prostate tumors. (**C**) Development of PIN in *p18* and *Pten* mutant mice at age of six months. PIN was categorized into four different grades according to Park et al[55]; PIN I containing lesions in one or two layers of atypical cells, PIN II with two or more layers of atypical cells, PIN III with normal duct outline, but lumen filled by cells with large and pleomorphic nuclei, and PIN IV containing lumen filled with atypical cells and invading into surrounding tissues. (**D**)Development of high grade PIN (PIN III and PIN IV) and carcinoma in *p18* and *Pten* mutant mice. A cohort of a minimum of 3 mice of each genotype was dissected at 3, 6 and 9 month of age. Mice free of high grade PIN or carcinoma was plotted against age.

Close examination of old *p18*$^{-/-}$ male mice revealed an evident enlargement of their prostate gland that was not previously noted in an early study (Fig. 2A). Of four *p18*$^{-/-}$ male mice analyzed (aged 12-14 months), the prostate glands were approximately 80% larger than age-matched control wild type mice of same genetic background (mixed 129sv/B6D2). Prompted by this phenotype and the report that double mutant mice lacking *p27* and heterozygous for *Pten* (*p27*$^{-/-}$;*Pten*$^{+/-}$) developed prostate cancer at early age and with high penetrance5, we have generated and characterized *p18-Pten* mice of various genotypes. While 10 out of 11 *p18*$^{-/-}$ mice remained alive and healthy by 15 months and 3 out of 12 *Pten*$^{+/-}$ mice survived beyond 15 months, no *p18*$^{+/-}$;*Pten*$^{+/-}$ mice (N=16) survived beyond 13 months and no *p18*$^{-/-}$;*Pten*$^{+/-}$ mice (N=15) survived beyond 9.5 months (Fig. 2B). A clear correlation between the progression of PIN with loss of one or two p18 alleles in combination with Pten heterozygosity was observed (Fig. 2C and 2D). At 3 months of age, prostates from mice of all genotypes are free of high grade PIN. No PIN III was found at this age. At 6 months, 80% of *p18*$^{-/-}$;*Pten*$^{+/-}$ mice developed high grade PIN or carcinoma. In comparison, 20% of *p18*$^{+/-}$;*Pten*$^{+/-}$ and 12% of *Pten*$^{+/-}$ mice developed high grade PIN, respectively. By 9 months of age, all *p18*$^{-/-}$;*Pten*$^{+/-}$ mice developed high grade PIN, while only 40% of *p18*$^{+/-}$;*Pten*$^{+/-}$ and 20% of *Pten*$^{+/-}$ mice developed high grade PIN, respectively. At 12 month, while all of *p18*$^{+/-}$;*Pten*$^{+/-}$ mice developed high grade PIN, there were still 50% of *Pten*$^{+/-}$ mice free of high grade PIN. Importantly, the prostate lesions were primarily observed in the dorsolateral lobe, but also in the anterior lobe and seminal vesicles (Fig. 2C). These results suggest that Pten and p18 have an important and cooperative role in suppression of prostate tumors, and that various stages of prostate tumor phenotype developed in *p18-Pten* double mutant mice in a gene dosage-dependent manner. This proposal is aimed at: (i) determining the molecular and cellular mechanisms underlying the development of prostate tumor in the *p18-Pten* mutant mice, and (ii) exploring the utility of the *p18-Pten* mutant mice in modeling human prostate cancer.

II. Hypothesis

Mutation of the protein/lipid phosphatase PTEN gene is associated with the development of several types of human tumors, including prostate cancer. Loss-of-function of the CDK inhibitor gene p18^{Ink4c} in mice resulted in

hyperplastic cell growth in various tissues including the prostate gland. *p18-Pten* double mutant mice developed various stages of prostate tumor phenotype in a gene dosage-dependent manner and with a high-degree of penetrance. Prostate tumor phenotypes that developed in the *p18-Pten* double mutant mice occurred primarily in the dorsolateral prostate, a lobe analogous to the peripheral zone in the human prostate where 80% of human prostate cancers arise. Two major goals of this proposal are to test the hypothesis that Pten phosphatase and $p18^{Ink4c}$ CDK inhibitor functionally collaborate to suppress prostate tumor growth, and to determine the molecular and cellular mechanisms underlying prostate tumor growth using the *p18-Pten* double mutant mice as a model.

III. Objectives

Aim 1: Determine the cellular and molecular mechanisms of p18 and Pten in prostate tumor suppression.
Aim 2: Genetic analysis of *p18* null mice with prostate-specific deletion of *Pten.*

IV. METHODS

Aim I. Cellular and molecular mechanisms of p18 and Pten in prostate tumor suppression

1. Does p18 regulate Akt activation and localization?

Reduction of PTEN activity in $Pten^{+/-}$ prostate, as expected, resulted in an increase of phosphorylated Akt as determined by direct immunoblotting (Fig. 3A). Unexpectedly, while the level of phosphorylated (activated) Akt was not detectably changed in the prostate of mice having lost one or both p18 alleles, it was significantly increased in prostates of $p18^{-/-}$;$Pten^{+/-}$ double mutant mice compared with $Pten^{+/-}$ alone. To confirm this finding, we carried out immunohistochemical analysis of prostate tissues. A substantial increase of activated Akt was seen in $p18^{-/-}$;$Pten^{+/-}$ prostates (Fig. 3B, and immunohistochemistry data for other genotypes not shown). A close examination indicated that not only the level of activated Akt was increased in $p18^{-/-}Pten^{+/-}$ prostates than in $Pten^{+/-}$ prostates, but also subcellular localization was changed. While activated Akt was localized predominantly near the plasma membranes of $Pten^{+/-}$ prostate cells, there was clear accumulation of activated Akt in the nucleus of $p18^{-/-}$;$Pten^{+/-}$ prostate cells (Fig. 3C). These findings suggest a previously unrecognized function of p18, most likely through its primary effector CDK4 and CDK6, as a negative regulator of Akt activation and nuclear accumulation. We will carry out experiments in cultured cells to confirm and determine the mechanism underlying Akt regulation by p18.

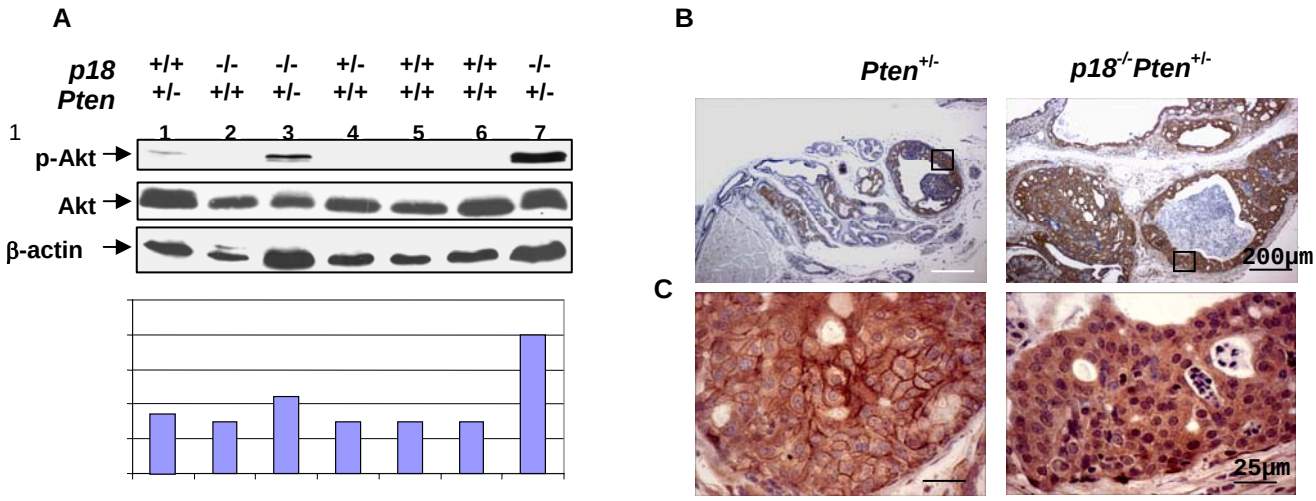


Fig. 3. Regulation of Akt activation and localization by p18. (**A**) Total cellular proteins were extracted from prostate tissues of mice with different genotypes, resolved by SDS-PAGE and immunoblotted with indicated antibodies. Expression of P-Akt was quantified by NIH image sofeware (**B,C**) Sections of *Pten*$^{+/-}$ and *p18*$^{-/-}$;*Pten*$^{+/-}$ prostates from age-matched mice (8 months) were stained with antibody specific to Ser-473 phosphorylated Akt.

Methods. Two separate experiments—one in mouse embryo fibroblasts (MEFs) and one in human prostate tumorderived cells—will be carried out to determine whether a change of kinase activity of CDK4, the primary target of p18, would affect the level and localization of activated Akt. (i) Because it is difficult to derive prostate cells ***in vitro*** from mice, we have recently prepared wild-type and five lines of mutant MEFs (*Cdk4*$^{-/-}$, *p18*$^{-/-}$, *Pten*$^{+/-}$, *p18*$^{+/-}$;*Pten*$^{+/-}$ and *p18*$^{-/-}$;*Pten*$^{+/-}$). We will derive *Pten*$^{-/-}$ MEFs from the conditional PtenloxP/lox;PPB-Cre4 mice we recently obtained from Dr. Wu as well as *p18*$^{-/-}$;*Pten*$^{-/-}$ MEFs in the future when this strain of mouse is generated (see below Aim III). These cells will be infected with retroviruses expressing mouse CDK4, catalytic inactive CDK4K35M, INK4-resistant CDK4R24C and p18. The level and subcellular localization of activated Akt in both un-infected and virally infected MEFs will be examined by three assays: direct immunoblotting, indirect immunofluorescence, and immunoblotting of cytoplasmic, nuclear and chromatin-associated fractions of cell lysate. (ii) We have also obtained three human prostate tumor cell lines whose Pten status has been determined: LNcap (PTEN deficient), PC-3 (PTEN deficient) and DU145 (PTEN positive). The level and subcellular localization of activated Akt in these cells as well as in cells infected with retroviruses expressing human CDK4, CDK4^{K35M}, CDK4^{R24C} and p18 will be examined.

Data analysis & future experiments. Loss of Pten function, resulting from either genetic targeting in *Pten*$^{+/-}$ or *Pten*$^{-/-}$ MEFs or tumor-associated mutation in LNcap or PC-3 cells, as expected, increased the levels of activated Akt as determined by direct immunoblotting (our confirmatory results not shown). An increase of overall and nuclear level of activated Akt in *p18*$^{-/-}$ MEFs or in cells ectopically expressing either CDK4 or CDK4^{R24C}, and conversely a decrease of overall and nuclear activated Akt in *Cdk4*$^{-/-}$ or

in p18 infected WT cells, would provide a direct support for a regulation of Akt activation and localization by CDK4 and thus p18. Comparison of Akt level and localization between CDK4 and CDK4K33M virus-infected cells would allow us to determine whether the regulation of Akt by CDK4 is dependent on CDK4 kinase activity. Together, these experiments will help us to elucidate the molecular mechanism for the functional collaboration between Pten and p18 in suppression of prostate tumor development. Future experiments will determine: (i) if other INK4 proteins, especially p16 which is frequently mutated in human cancers including prostate tumors, similarly regulate Akt as p18, (ii) whether CDK4 regulates Akt in the cytoplasm or in the nucleus, (iii) whether members of p21 family CDK inhibitors, in particular $p27^{Kip1}$ which has been shown to collaborate with Pten in suppression of prostate tumors[5] and as a substrate of Akt[56-58], also regulate Akt as INK4 proteins, and (iv) whether the G1 kinase CDK2 is involved or is required for this regulation.

Aim 2: Genetic analysis of p18 null mice with prostate-specific deletion of Pten

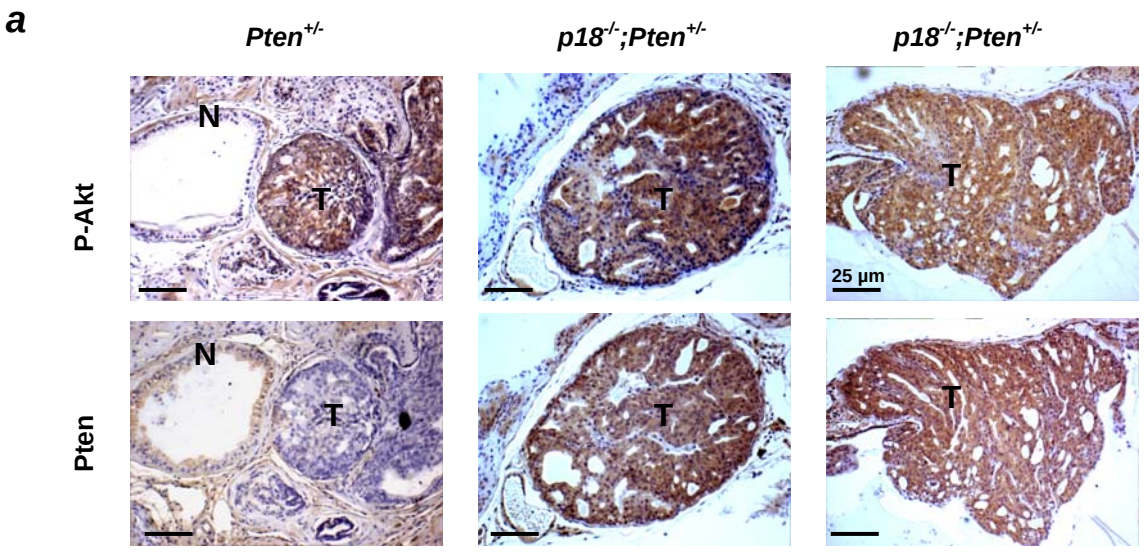
***a. Pten* haploinsufficiency in tumor suppression is tissue specific**

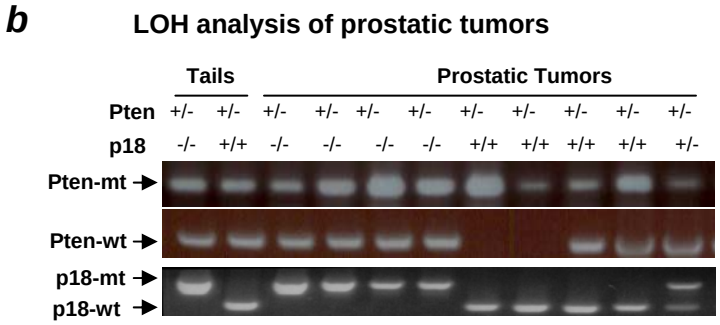
Development of tumors from Pten heterozygotes offered an opportunity to address the issue of Pten haploinsufficiency in tumor suppression. We took two approaches to this end: immunohistochemistry (IHC) of Pten and activated Akt, and loss-of-heterozygosity (LOH) analysis of the remaining *Pten* wild-type alleles. *Pten* was expressed in normal epithelium but not in tumor cells in $Pten^{+/-}$ prostates (Fig. 4a). Consecutive sections of the same tumor were stained with an antibody to Ser473-phosphorylated Akt, and demonstrated a substantially higher expression of activated Akt in the tumor cells than in the normal epithelium (Fig. 4a). These results suggest that the remaining wild-type *Pten* allele was lost in the prostate tumor cells. As determined by IHC, Pten protein expression was lost in 9 out of 11 prostate tumors from $Pten^{+/-}$ mice. In contrast, loss of Pten protein expression was not detected in any tumors from the pituitary (n=5), thyroid (n=12), and adrenal glands (n=9). Activated Akt was readily detected in all these tumors (Fig. 4a). These results indicate a tissue-specific haploinsufficiency of the *Pten* gene in tumor suppression.

b. Loss of the remaining wild-type *Pten* allele is protected by *p18* loss in prostate tumors

We next examined the Pten and phospho-Akt expression patterns in *p18-Pten* compound mice. Surprisingly, most $p18^{-/-};Pten^{+/-}$ prostate tumor cells retained *Pten* expression, and yet were positive for phospho-Akt (Fig. 4a). Only 2 out of 13 of $p18^{-/-};Pten^{+/-}$, and 3 out of 9 of $p18^{+/-}$-$Pten^{+/-}$ prostate tumors lost *Pten* expression as determined by IHC. These results revealed that loss of wild-type *Pten* allele in $p18^{-/-};Pten^{+/-}$ mice is protected by *p18* loss. To further confirm this, we conducted laser-capture microdissection and genomic PCR. DNA was extracted from the tumor tissues under the microscope, and PCR-based LOH analysis was performed. 5 out of 12 prostate tumors from $Pten^{+/-}$ mice exhibited loss of the wild type *Pten* allele and retention of the

null allele, whereas, none of the tumors derived from *p18;Pten* compound mutant mice (n=15 for $p18^{-/-};Pten^{+/-}$ and 9 for $p18^{+/-};Pten^{+/-}$) showed loss of the wild-type *Pten* allele (Fig. 4b), further confirming that LOH is not required for tumor development in these compound mutant mice. In addition to prostate tumors we also performed LOH analysis for tumors derived from pituitary, thyroid, and adrenal glands and found no LOH in those tumors from either *Pten* alone or *p18;Pten* compound mutant mice (Fig. 4c), suggesting that LOH occurs in a tissue-specific way in prostate tumor development. Taken together, our results demonstrated that loss of wild-type *Pten* allele in $Pten^{+/-}$ mice is prostate-specific and is protected by *p18* loss.





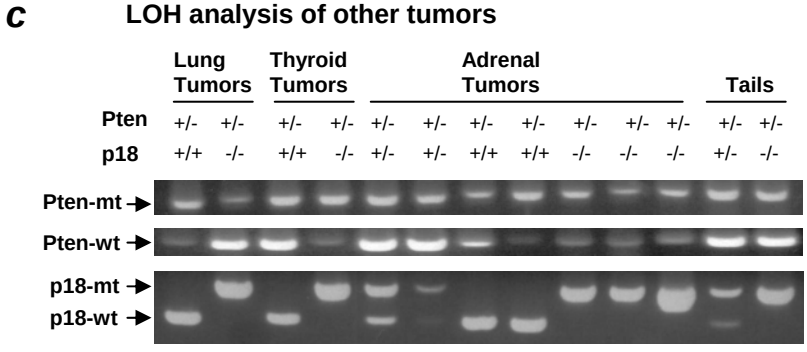


Figure 4. Loss of the remaining wild-type *Pten* allele is tissue-specific and is protected by p18 loss

a, Serial sections of prostate tumors from $Pten^{+/-}$ and $p18^{-/-};Pten^{+/-}$ mice were immunostained with activated Akt and Pten. Note the strong p-Akt staining in the tumor (T) cells and very faint (negative) p-Akt staining in the normal (N) epithelium. In $Pten^{+/-}$ prostates, there is a mutually exclusive staining pattern between *Pten* and p-Akt expression in the tumor (T) cells and normal (N) epithelium. In $p18^{-/-};Pten^{+/-}$ prostates most tumor cells retained *Pten* expression. **b**, Presence of the wild-type *Pten* allele in prostate tumors of $p18^{-/-};Pten^{+/-}$ and $p18^{+/-};Pten^{+/-}$ mice, and absence of the wild-type *Pten* allele in half of the prostate tumors of $Pten^{+/-}$ mice. DNA extracted from the microdissected samples of different genotype mice was amplified by PCR to detect wild-type (wt) and mutant (mt) alleles of *Pten* and p18, respectively. **c**, Presence of the wild-type *Pten* allele in other tumors of $p18^{-/-};Pten^{+/-}$, $p18^{-/-};Pten^{+/-}$ and $Pten^{+/-}$ mice. DNA extracted from the microdissected samples of different genotype mice was amplified by PCR to detect *Pten* and *p18* alleles.

Key Research Accomplishments

1. *p18-Pten* double mutant mice developed various stages of prostate tumor phenotype in a gene dosage dependent manner and with a high-degree of penetrance.
2. *PTEN* haploinsufficiency in tumor suppression is tissue specific.
3. Loss of remaining wild-type *Pten* allele is protected by *p18* loss in prostate tumors.

Reportable Outcomes

Manuscripts- $p18^{Ink4c}$ and *PTEN* cooperate in tumor suppression by controlling Akt activation and localization in a tissue-specific manner- submitted to C*ancer Cell.*

Conclusions

These results provide further support for functional interaction between *Pten* and *p18* and suggest that the haploinsufficiency of *Pten* in tumor suppression is tissue-specific and cooperate in tumor suppression by constraining a positive regulatory loop between cell growth and cell cycle control pathways.

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