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Loss of *ATF3* Promotes Akt Activation and Prostate Cancer Development in a *Pten* Knockout Mouse Model

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Abstract

Activating transcription factor 3 (ATF3) responds to diverse cellular stresses, and regulates oncogenic activities (*e.g.*, proliferation, survival and migration) through direct transcriptional regulation or protein-protein interactions. Although aberrant ATF3 expression is frequently found in human cancers, the role of ATF3 in tumorigenesis is poorly understood. Here we demonstrate that ATF3 suppresses the development of prostate cancer induced by knockout of the tumor suppressor Pten in mouse prostates. Where as the oncogenic stress elicited by *Pten* loss induced ATF3 expression in prostate epithelium, we found that *ATF3* deficiency increased cell proliferation and promoted cell survival, leading to early onset of mouse prostatic intraepithelial neoplasia and the progression of prostate lesions to invasive adenocarcinoma. Importantly, loss of ATF3 promoted activation of the oncogenic AKT signaling evidenced by high levels of phosphorylated AKT and S6 proteins in ATF3-null prostate lesions. In line with these *in vivo* results, knockdown of ATF3 expression in human prostate cancer cells by sgRNA-mediated targeting activated AKT and increased matrix metalloproteinase-9 expression. Our results thus

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Author Contributions

ZY and DX bred the mice, ZY carried out the experiments with the help of HD and JZ. JK performed statistical analyses of clinical data. TH provided the *ATF3*$^{-/-}$ mice and analyzed the data. HD, JZ, and TH edited the manuscript. CY conceived the study, analyzed the data, and wrote the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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link ATF3 to the AKT signaling, and suggest that ATF3 is a tumor suppressor for the major subset of prostate cancers harboring dysfunctional Pten.

Keywords

ATF3; Pten; AKT; prostate cancer; knock-out mouse

Introduction

As a disease caused by genetic alterations successively occurring during its course, prostate cancer remains as one of the leading causes of death from cancer, partly due to the poor understanding of genetic factors that control the development of this deadly disease. Inactivation of the phosphatase and tension homolog protein (Pten) by gene mutations or deletion occurs in about 30% of prostate tumors and up to 60% of metastatic prostate cancers, and is often considered the driving force for prostate tumorigenesis[1]. Pten in activation results in loss of the enzymatic activity that catalyzes dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate (PIP_3), thereby activating the oncogenic phosphoinositide 3-kinase (PI3K)/AKT signaling crucial for the growth, survival and distal dissemination of prostate cancer cells[2]. Moreover, Pten dysfunction confers advanced prostate cancer with resistance to conventional therapies that are mostly based on androgen deprivation. Indeed, deletion of *Pten* in mouse prostate epithelium not only recapitulates the progression of human disease from prostatic epithelial neoplasia (PIN) to invasive adenocarcinoma[3], but results in malignant lesions that are intrinsically resistant to androgen deprivation or castration[4,5]. Given that Pten inactivation is one of the most common genetic alterations in prostate cancer, it would be of interest to identify other genetic alterations that may act in concert with Pten dysfunction to drive the development of prostate cancer.

The immediate early gene activating transcription factor 3 (ATF3) is an ATF/CREB family member whose expression is rapidly induced by a wide range of cellular stresses including DNA damage, cellular injury and oxidative stress [6]. In response to cellular stresses, ATF3 regulates diverse cellular functions (*e.g.*, proliferation, survival, and migration) through binding to the ATF/CREB *cis*-regulatory element [6], or interacting with other proteins (*e.g.*, p53, and NF-κB) [7,8]. Accumulating evidence has linked ATF3 to several important cellular signaling pathways, including those mediated by p53, TGFβ, and Toll-like receptor 4 [7–9]. Activation of p53 by ATF3, for instance, can regulate cellular responses to genotoxic stresses and prevent cellular transformation induced by oncogenic Ras expression [7]. ATF3 can also interact with the NF-κB network to regulate cytokine expression thereby engaging in cellular immune responses [8]. Although recent studies have revealed that ATF3 contributes to many important human diseases including secondary infections during sepsis-associated immunosuppression [10] and skin cancer induced by immunosuppressants[11], the role of ATF3 in cancer, particularly prostate cancer, remains poorly understood [12]. Whereas ATF3 appears to be proapoptotic in prostate cancer cells [13,14], ATF3 also binds the androgen receptor (AR) and represses androgen signaling indispensable for sustaining prostate cancer cell proliferation and survival [1], indicating that ATF3 could be a putative tumor suppressor for prostate cancer. Indeed, several unbiased microarray results have revealed that ATF3

expression is downregulated in prostate cancers, particularly in metastatic prostate cancers [15,16]. In the similar vein, ATF3 has been shown to suppress tumor growth and metastasis in many other cancer types (*e.g.*, glioblastoma, colon, bladder and lung cancer) [17–20]. However, ATF3 can also promote lung metastasis of mouse melanoma cells and rat prostate cancer cells [21,22]. Moreover, a recent report demonstrates that ATF3 expressed by stromal cells promotes breast cancer cells to disseminate into lungs [23]. Therefore, the contributions of ATF3 to cancer remain elusive.

Here, we employed a *Pten* conditional knockout mouse model to determine the role of ATF3 in prostate cancer. Our results indicate that loss of *ATF3* promoted the development of prostate cancer through activating the AKT signaling. We thus provided the first genetic evidence arguing for that ATF3 is a tumor suppressor for the major subset of prostate cancer harboring *Pten* dysfunction.

Results

Loss of Pten induces ATF3 expression in prostate epithelium

We previously reported that ATF3-knockout mice developed prostatic hyperplasia due to increased AR activity, but ATF3 deficiency alone was not sufficient to induce mouse prostatic intraepithelial neoplasm (mPIN) or carcinoma [24]. To further explore the role of ATF3 in prostate cancer, we crossed *ATF3*$^{-/-}$, *Pten*$^{L/L}$and *PB-Cre4* mice (all in C57BL/6 background), and generated offspring with a genotype of *Pten*$^{L/L}$*; ATF3*$^{+/+}$, *Pten*$^{L/L}$*; ATF3*$^{-/-}$, *Pten*$^{L/L}$*; ATF3*$^{+/+}$*; Cre*$^{+}$, or *Pten*$^{L/L}$*; ATF3*$^{-/-}$*; Cre*$^{+}$, referred to as *WT*, Δ*ATF3*,Δ*Pten*, and Δ*ATF3*Δ*Pten*, respectively (Fig 1a). Loss of *Pten* expression in prostatic epithelial cells of *Pten*-knockout mice *(i.e.*, Δ*Pten* and Δ*ATF3*Δ*Pten*) was confirmed by immunofluorescence staining (Fig 1b). Interestingly, while ATF3 was weakly expressed in mouse prostatic epithelial cells, ATF3 staining was significantly increased in *Pten*-knockout prostates (Δ*Pten vs. WT*, Fig 1b and 1c), arguing for the notion that ATF3 is a stress-inducible gene in prostates and can be induced by the oncogenic stress caused by *Pten* deficiency. Such oncogenic stress also induced expression of the tumor suppressor p53 as reported (Fig 1b, Δ*Pten* vs. *WT*)[25]. However, p53 induction was diminished in ATF3-null prostates (Fig 1b and 1c, Δ*ATF3*Δ*Pten* vs.Δ*Pten*,) - a result consistent with our previous report that ATF3 stabilizes p53 under stressed conditions[7].

Loss of ATF3 promotes the development of prostate cancer in mice

It was recently reported that deletion of *Pten* in prostate epithelium of albino (white) C57BL/6 mice, which contain a spontaneous mutation at the tyrosinase gene, leads to mouse prostatic intraepithelial neoplasia (mPIN), but does not cause adenocarcinoma[26]. Similar to that study, we found that loss of *Pten* in our mutant mice also resulted in progressively enlarged prostates (Supplementary Fig S1). However, in addition to cribiform-like mPIN lesions, loss of *Pten* in our black C57/BL6 mice resulted in apparent epithelial invasion into stromal tissues in anterior prostates (AP) and dorsal prostates (DP) (Fig 2a and supplementary Fig S2, arrows) evidenced by the lack of α-smooth muscle actin (α-SMA) staining in invasion regions (Fig 2b, arrows), suggesting the development of adenocarcinoma in these mice. Microinvasion was first seen in 6-week-old DP and 9-week-

old AP, and 100% of mice older than 12 weeks developed carcinoma (Fig 2c). In contrast, only low-grade mPIN was seen in ventral prostates (VP) while no lesion other than hyperplasia was found in lateral prostates (LP) of ΔPten mice (Supplementary Fig S2). The cancerous cells were originated from luminal epithelial cells as they were positive for AR staining but negative for p63 expression (Supplementary Fig S3). Thus, loss of *Pten* led to rapid development of adenocarcinoma in our mouse model. Interestingly, whereas ATF3 expression was initially induced by *Pten* loss (Fig 1b and Supplementary Fig S4b), the ATF3 expression level was decreased along with the progression of prostate lesions from mPIN to adenocarcinoma in Δ*Pten* mice (Supplementary Fig S4b and S4c), suggesting that loss or downregulation of ATF3 expression appeared to be required for the development of *Pten*-null prostate cancer.

Indeed, we found that loss of *ATF3* promoted the development of prostate cancer in *Pten*-knockout mice. In contrast to Δ*Pten* mice, which developed mPIN at 6 weeks of age in 4 out of 9 mice, 10 out of 11 Δ*ATF3*Δ*Pten* mice developed mPIN at the same age ($p < 0.05$, Fisher's Exact test) (Fig 2c). Similarly, adenocarcinoma was found in 8 out of 9 Δ*ATF3*Δ*Pten* mice as compared to 4 out of 11 Δ*Pten* mice at 9 weeks ($p < 0.05$, Fisher's Exact test) (Fig 2c). Moreover, mPIN in Δ*ATF3*Δ*Pten* prostates was often high-grade, and more prostate lesions in these compound-mutant mice were invasive (Fig 2a and Supplementary Fig 2a, arrows). Staining the prostates for α-SMA expression (Fig 2b, arrows) confirmed that Δ*ATF3*Δ*Pten* mice had a significantly larger number of invasive adenocarcinoma in both AP (Fig 2d) and DP (Fig 2e). Taken together, these results indicate that loss of *ATF3* promoted the development of prostate cancer induced by *Pten* deletion.

Loss of ATF3 increases proliferation but decreased apoptosis of Pten-loss-induced tumor cells

To understand the mechanism by which *ATF3* deficiency promoted the development of prostate cancer, we tested whether ATF3 affects proliferation and survival of prostate epithelial cells under the *Pten*-knockout condition. Towards this end, we stained the prostates for Ki67 expression (a proliferation marker) and cleaved caspase 3 expression (a apoptosis marker), and counted positively-stained cells. As expected, the oncogenic stress conferred by *Pten* deletion promoted proliferation (Fig 3a) while inducing apoptosis of prostate cancer cells (Fig 3c). Importantly, the number of Ki67-positive cells was significantly increased in Δ*ATF3*Δ*Pten*lesions than Δ*Pten* lesions in mice at 6 weeks and 9 weeks of age (Fig 3a and 3b). Conversely, Δ*ATF3*Δ*Pten*lesions contained a significantly lower number of apoptotic cells as compared to Δ*Pten* prostates at all ages (Fig 3c and 3d). The decrease in the apoptotic cell number in Δ*ATF3*Δ*Pten*lesions at 12-week of age (Fig 3d) is noteworthy given that *ATF3* deficiency did not promote proliferation under the same condition (Fig 3b). These results thus indicate that the loss of *ATF3* likely promoted the development of prostate cancer by increasing cell proliferation while inhibiting apoptosis.

Loss of ATF3 enhances AKT signaling in mouse prostatic epithelial cells

A major consequence of *Pten* inactivation is AKT phosphorylation, which in turn triggers a cascade of events that drive cell proliferation, sustain cell survival, and also promote cell invasion [2]. To gain a further insight of the mechanism by which *ATF3* deficiency promoted

cell proliferation, survival, and subsequent development of prostate cancer, we examined AKT activation in prostate lesions by staining prostates for AKT phosphorylation. Indeed, loss of *Pten* resulted in phosphorylation of AKT at S473 and T308 (Fig 4b, Δ*Pten*), which otherwise was undetectable in *Pten*-wildtype prostates (Fig 4a). Loss of *ATF3* alone was not sufficient to induce AKT phosphorylation (Fig 4a, Δ*ATF3*). However, we found that *ATF3* deficiency dramatically elevated the levels of S473- and T308-phosphorylated AKT under the *Pten*-knockout condition (Fig 4b and 4d, Δ*ATF3*Δ*Pten* vs. Δ*Pten*). Not only the overall AKT phosphorylation level, but the level of membrane-bound phosphorylated AKT was largely increased in Δ*ATF3*Δ*Pten* lesions as compared to Δ*Pten* tumors (Fig 4b). Moreover, *ATF3* deficiency also dramatically increased the phosphorylation level of S6 protein (Fig 4c and 4d) - a downstream effector of the mTOR pathway which is activated by AKT and also required for prostate tumorigenesis induced by *Pten* loss[27]. Neither the total AKT level nor the total S6 level was altered by loss of *ATF3* (Fig 4b, 4c, and 4d). These results indicate that loss of *ATF3* enhanced the AKT signaling in prostate cancer induced by *Pten* deficiency.

Knockdown of ATF3 expression in prostate cancer cells activates AKT signaling

To corroborate the important finding that loss of *ATF3* promoted AKT signaling, we employed the emerging CRISPR-Cas9-based technology [28] to knock down ATF3 expression in human prostate cancer cells (LNCaP, PC3 and DU145). This gene targeting technology takes advantage of a short, single guided RNA (sgRNA)that specifically binds to a target genomic region (the region spanning the ATF3 start codon in our case, Fig 5a) and recruits a nuclease Cas9 to cleave DNA (Fig 5a, vertical arrow). The generated double-strand DNA break would then be repaired by the error-prone homology-directed repair mechanism, resulting in a deletion or insertion in a region immediate downstream of the start codon of the *ATF3* gene thereby disrupting*ATF3* gene expression. Employing this approach, we isolated several clones that expressed a significantly lower level of ATF3 (Fig 5c, 5d and 5e). The Surveyor-based mutagenesis detection assay confirmed that these clones carried at least one mutant *ATF3* allele (Fig 5b). Western blotting showed dramatic elevation of the phosphorylation levels of AKT and S6 in these ATF3-low expressing prostate cancer cells, although the expression level of total AKT and S6 was not altered (Fig 5c, 5d and 5e). These results thus demonstrate that down-regulation of ATF3 expression in human prostate cancer cells enhances AKT signaling as well.

ATF3 deficiency/downregulation increases the NF-κB signaling and MMP-2/-9 expression

The AKT signaling can promote cancer progression by regulating expression of matrix metalloproteinases (MMPs)[29–33]. To understand the mechanism by which loss of ATF3 promotes invasion of prostate cancer cells (Fig 2b), we carried out qRT-PCR assays to examine expression of a range of MMPs in sgATF3-expressingand control DU145 cells. Whereas MMP-2, -8 and -12 expression were undetectable, downregulation of ATF3 significantly increased expression of MMP-9, -10, -13, -14 and -15 in DU145 cells (Fig 6a). Using zymography, we confirmed the induction of MMP-9 expression in LNCaP, DU145 and PC3 cells (Fig 6b). Interestingly, sgATF3 also induced MMP-2 expression in LNCaP cells although the parental LNCaP cells did not express detectable MMP-2 (Fig 6b). As IKK phosphorylation by AKT can activate NF-κB [29,30] and the latter is well known to regulate

MMP-9 transcription[30,31], we explored a possibility that enhanced AKT signaling in ATF3-downregulated prostate cancer cells transactivates the MMP-9 promoter by activating the NF-κB signaling. Indeed, whereas the MMP-9 promoter activity was significantly higher in sgATF3-expressing prostate cancer cells, mutating the κB *cis*-regulatory element in the MMP-9 promoter [31] completely abolished the sgATF3-mediated transactivation of the promoter. Consistent with these results, the NF-κB activity was increased in the ATF3-downregulating cells as the activity of a luciferase reporter driven by tandem repeats of κB elements (κB-luc), but not mutated κB elements (mκB-luc), was significantly higher in the sgATF3-expressing cells than that in control DU145 cells (Fig 6d). Moreover, the IKK and IκB phosphorylation levels as well as the nuclear p65 level were increased in sgATF3 cells (Fig 6e). Collectively, these results are in line with the notion that AKT activates the NF-κB signaling, leading to induction of MMP-9 expression under ATF3 down-regulated or deficient conditions. To determine whether ATF3 affects MMP expression in mouse prostate lesions as well, we stained the prostate sections for MMP-2 and MMP-9 expression. We found that loss of *ATF3* indeed dramatically promoted MMP-2 and MMP-9 expression in *Pten*-knockout prostate lesions (Fig 6f and 6g, Δ*ATF3*Δ*Pten* vs. Δ*Pten*). Therefore, loss of *ATF3* likely enhances the AKT signaling, leading to increased MMP expression thereby promoting invasion of *Pten*-null prostate cancer. Of note, ATF3 might directly repress MMP-2 transcription as previously reported[34–36]. We thus conclude that loss/downregulation of ATF3 expression likely contributes to the development of *Pten*-deficient prostate cancer by activating AKT and consequently promoting its downstream events including cell proliferation, resistance to apoptosis, and expression of pro-invasive genes (Fig 6h).

Discussion

In line with an early report that ATF3 expression can be induced by oncogenic stresses elicited by oncogene expression (*e.g.*, Ras)[37], we showed here that the oncogenic stress caused by *Pten* loss could also induce ATF3 expression in prostate epithelium (Fig 1b). This result suggests that ATF3 could serve as an anti-cancer barrier that functions to eliminate oncogenic stresses thereby preventing the development of prostate cancer. Indeed, whereas ATF3 expression was downregulated in *Pten*-null adenocarcinoma in Δ*Pten* mice (Supplementary Fig S4), we have demonstrated that lack of ATF3 induction in double-knockout mice promoted the development of prostate cancer. Thus, downregulation of ATF3 expression, frequently found in human prostate cancer [15,16], might promote the progression of the major subset of prostate cancer harboring dysfunctional *Pten* (Fig 6e). We therefore identified an additional genetic alteration that may act in concert with *Pten* inactivation to promote the development of human prostate cancer.

The tumor suppressor role of ATF3 in prostate cancer is also supported by the observations that ATF3 is a proapoptotic molecule in prostate cancer cells and that ATF3 represses androgen receptor signaling required for sustaining the growth and survival of prostate cancer cells[1,13,14]. However, ATF3 expression was also shown to promote invasion of a human prostate cancer cell line and lung colonization of a rat prostate cancer cell line[22,38]. These seemingly controversial results well represent the fact that ATF3 often regulates gene expression in a context-dependent manner. For instance, ATF3 induces expression of the

metastasis suppressor KAI1 when it interacts with JunB, but represses expression of the same gene when co-expressed with NF-κB [38]. Nevertheless, our current study provides the first genetic evidence supporting that ATF3 suppresses prostate cancer harboring *Pten* dysfunction. Whereas it remains unclear whether ATF3 also suppresses prostate cancer carrying wild-type *Pten*, we recently showed that ATF3 can counteract the oncogenic activities of mutant p53 proteins thereby suppressing metastasis of *TP53*-mutated cancer [39]. Given that *TP53* mutation is a common genetic alteration in late-stage prostate cancer [1], ATF3 might function as a tumor suppressor for advanced prostate cancer in general. Indeed, downregulation of ATF3 expression is more often seen in metastatic prostate cancers (Supplementary Fig S5) [40]. It is worth noting that several other reports found elevated ATF3 expression in prostate cancer as compared to normal prostate tissues[40,41]. Such an elevation might be the result of acute ATF3 induction by oncogenic stresses in the early stage of tumorigenesis as we observed in this study (Fig 1b), and the ATF3 expression level in normal prostate tissues adjacent to tumor is indeed often higher than that in their counterparts obtained from organ donors free of prostate cancer (Supplementary Fig S5b). Elevated ATF3 expression might also be the result of upregulated expression of ATF3 splice variants, some of which can function as natural antagonists for the full-length ATF3 [42]. Alternatively, it might also be that downregulation of ATF3 expression is only required for the development of a subset of prostate cancer harboring *Pten* dysfunction. Therefore, it might be necessary to stratify prostate cancer patients for a better understanding of the relevance of ATF3 expression to clinical outcomes.

We previously showed that ATF3 can activate p53 [7], the tumor suppressor which has been shown to block the development of prostate adenocarcinoma in *Pten*-knockout mice [25]. In line with this, we found that p53 induced by *Pten* deletion appeared to depend on ATF3 expression (Fig 1b). However, the ATF3-mediated activation of p53 does not appear to be sufficient to cause the suppression of prostate cancer development in Δ*Pten* mice as prostate lesions in these mice rapidly progressed to adenocarcinoma. Likewise, although ATF3 is an AR repressor [24], it is unlikely that ATF3 suppressed prostate cancer development in Δ*Pten* mice through repressing androgen signaling, as it has been shown that *Pten*-null prostate cancer does not require AR signaling for their growth [4,5]. Indeed, castration of ΔPten mice at 7-week of age when mPIN emerges did not affect the prostate cancer progression, nor cause apparent tumor regression (Supplementary Fig S6).

A striking finding from this study is the link between ATF3 and the AKT signaling. Previously, ATF3 was shown to activate AKT in neurons[43] and promote cytokine-induced AKT phosphorylation in mouse master cells [44]. In this study, we found that ATF3 rather inhibited the oncogenic AKT signaling in *Pten*-null prostate lesions. Whereas knockdown of ATF3 expression in *Pten*-null (LNCaP and PC3) or -defective (DU145) human prostate cancer cells also activated AKT, it remains elusive whether *Pten* dysfunction, the transformation state, or the cell/tissue type, is the prerequisite for ATF3-mediated AKT inhibition. Given that ATF3 mainly localizes in the nuclei of prostate epithelial cells, it is more likely that ATF3 suppresses AKT activation through regulating transcription of other AKT regulators. Expression of CTMP, an AKT activator in breast cancer [45], for instance, can be repressed by ATF3 [46]. Regardless of which mechanism(s) may involve, activation of

AKT in *ATF3*-null or down-regulated prostate cancer cells would promote proliferation, prevent death, and lead to a cascade of molecular events contributing to the development of cancer (Fig 6h). One such downstream event was the induction of pro-invasive MMP-9 expression, a likely consequence of transactivation of the MMP-9 promoter by the transcription factor NF-κB[31] activated by AKT [47]. Although ATF3 itself is a transcription factor, we previously showed that ATF3 does not directly regulate MMP-9 expression [34].

It is well established that the genetic background of mouse strain determines the effect of *Pten* deletion on mouse prostate tumorigenesis[48]. Whereas studies on mice with mixed 129 backgrounds often report highly-penetrate invasive adenocarcinoma in all of the 4 prostate lobes[3,49], knockout of *Pten* in a pure C57BL/6 background generated mixed results. Although monoallelic *Pten* knockout led to fully-penetrant adenocarcinoma in anterior prostates [27], it was a surprise that biallelic *Pten*-knockout mice only developed mPIN in a recent report[26]. It is important to note that the latter study employed an albino strain that contains a mutation at the tyrosinase gene. This strain appears to be different from the black C57BL/6 mice used in our study as we found that 100% of *Pten*-null mice developed invasive adenocarcinoma in AP and DP at 12 weeks of age. Whether other genetic alteration(s) accompanied by the tyrosinase gene mutation in the albino mice prevents the development of prostate adenocarcinoma is an intriguing question and worthy of further investigation. It is important to note that monoallelic knockout of *Pten* (*Pten*$^{pc(+/-)}$) in the prostates of our C57BL/6 mice only led to low-gradem PIN lesions even when the mice were older than 11 months (Supplementary Fig S7). Therefore, partial loss of *Pten* does not appear to be sufficient to cause prostate cancer in our mouse model.

Materials and Methods

Animals and genotyping

Animal experiments were carried out according to protocols approved by the Institutional Committee of Animal Care and Use (ICACU) of the Albany Medical College and the ICACU of Georgia Regents University. *ATF3* knockout (*ATF3*$^{-/-}$) mice (in C57BL/6 background) were described previously [24,50], and we obtained B6.129S4-Pten^{tm1Hwu}/J (*Pten*$^{loxP/loxP}$, or *Pten*$^{L/L}$) and PB-Cre4 (*Cre*$^{+}$) mice from the Jackson Laboratory and the NCI Mouse Repository, respectively. To generate compound mutants, *ATF3*$^{-/-}$ mice were first crossed with *Pten*$^{L/L}$and *Cre*$^{+}$ mice, yielding *Pten*$^{L/L}$*; ATF3*$^{-/-}$ and *ATF3*$^{-/-}$*; Cre*$^{+}$ mice, respectively. The cross of the latter two strains generated male *Pten*$^{L/+}$*; ATF3*$^{-/-}$*; Cre*$^{+}$ mice, which were further bred with female *Pten*$^{L/L}$*; ATF3*$^{-/-}$ mice to generate male *Pten*$^{L/L}$*; ATF3*$^{-/-}$*; Cre*$^{+}$ (Δ*ATF3*Δ*Pten*) and *Pten*$^{L/L}$*; ATF3*$^{-/-}$(Δ*ATF3*) for histolopathological examinations. Male *Pten*$^{L/L}$*; ATF3*$^{+/+}$*; Cre*$^{+}$ (Δ*Pten*) and *Pten*$^{L/L}$*; ATF3*$^{+/+}$(*WT*) mice were generated similarly. For genotyping, mouse tails were lysed in PBND buffer supplemented with 0.2 μg/ml Proteinase K at 55°C overnight, and lysates were directly subjected to PCR following the protocols provided by the Jackson Laboratory. For castration, testes of 7-week-old male mice were surgically removed, and the mice were sacrificed at 12 week of age for histopathological examinations.

Immunohistochemistry (IHC) and immunofluorescence staining

Prostate lobes were separated by microdissection, and embedded in paraffin for sectioning [24]. After antigen retrieval in hot citrate buffer, sections were blocked in 5% of normal horse serum and 1% of normal goat serum, and subjected to immunohistochemistry staining using the ABC Elite Kit and the DAB Kit (Vector) according to the manufacturers' recommendations. The following antibodies were used: Ki-67 (ab15580, 1:600) from Abcam; ATF3 (Santa Cruz sc-188, 1:200), AR (sc-816, 1:200), and p63 (sc-8430, 1:200) from Santa Cruz; p-AKT S473 (#4060, 1:200), p-AKT T308 (#2965,1:200), AKT (#4691, 1:200), p-S6 (#2211, 1:400), S6 (#2217, 1:200), cleaved caspase-3 (#9661, 1:300), and Pten (#9188, 1:100)from Cell Signaling; MMP2 (NB200-193, 1:200) from Novus; and MMP-9 (ab137867, 1:1000) from Abcam. For α-smooth muscle actin (α-SMA) staining, sections were incubated with alkaline phosphatase (AP)-conjugated anti-α-smooth muscle actin antibody (Sigma, 1:600) followed by detection of AP activity using the SIGMAFAST Fast Red TR/Naphthol AS-MX tablets (F4523, Sigma) according to the supplier's protocol. For quantifying IHC staining intensity, random microscopic fields were captured and digitized by a CCD camera (Olympus). Signal intensity was determined using the Image-Pro Plus software and presented as integrated optical density (IOD).

Cell culture and sgRNA-mediated knockdown of ATF3 expression

LNCaP, DU145, and PC3 cells were cultured in DMEM, RPMI1640, and T-medium supplemented with 5–10% fetal bovine serum, respectively. The CRISPR-Cas9 system was used to generate ATF3 low-expressing cells as described [28]. Essentially, a plasmid expressing a single guided RNA (sgRNA) targeting a region spanning the ATF3 start codon (5′-AAAATGATGCTTCAACACCCAGG-3′; the start codon was underlined)was constructed and co-transfected into cells with a hCas9-expressing plasmid [28]. Single cells were plated into 96-well plates, and derived clones screened for ATF3 down-regulation using Western blotting. To confirm that obtained clones were derived from sgRNA-guided knockdown, genomic DNA was prepared and subjected to Surveyor mutation detection using a kit purchased from Transgenomic.

Western blotting and quantitative reverse transcription-PCR (qRT-PCR)

These were carried out as described previously [7,51]. Briefly, cells were lysed in RIPA buffer (50 mMTris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 mMNaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche)), and subjected to SDS-polyacrylamide electrophoresis for Western blotting. We purchased the following antibodies from Cell Signaling: IKKα (#11930), phospho-IKKα/β (#2697), IκBα (#4814), phospho-IκBα (#2859), and NF-κB p65 (#8242). For qRT-PCR assays, total RNA was extracted from cells using Trizol reagent (Invitrogen), and then reverse transcribed, followed by real-time PCR assays for MMP expression [51]. Theprimer sequences are available upon request.

Zymography

Zymography was performed as described previously [34]. Briefly, condition media were collected, concentrated, and loaded on a polyacrylamide gel containing 1 mg/ml gelatin after

normalization to equal cell number. The gel was rinsed with 2.5% Triton X-100 at room temperature for 1 h, and then incubated in a buffer containing 50 mMTris-HCl, pH 7.5, 10 mM $CaCl_2$, and 150 mMNaCl at 37 °C overnight. The gel was stained with 0.2% Coomassie Blue, and MMPs were detected as transparent proteolytic bands against a dark blue background.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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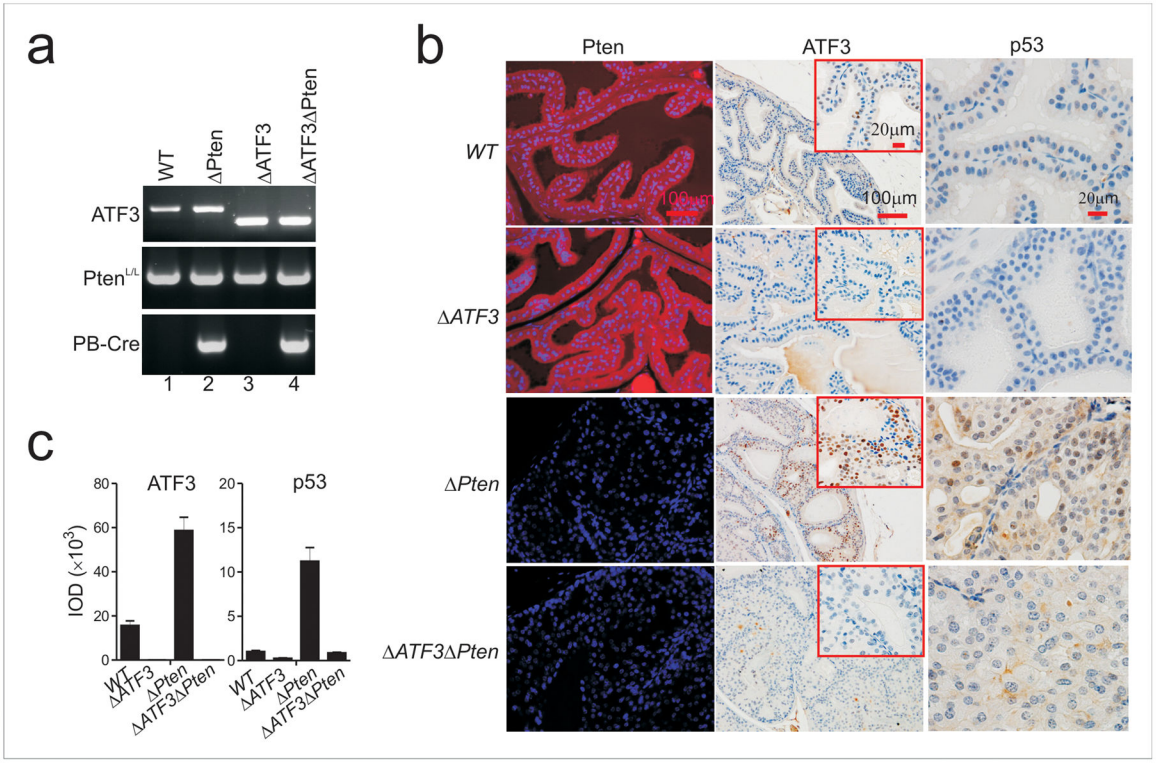

Fig 1. ATF3 expression is induced by loss of *Pten* in mouse prostate epithelium

(**a**) Representative genotyping results of four groups of mice used in this study. (**b, c**) ATF3 and p53 levels were elevated in Δ*Pten* mice that lost *Pten* expression. Anterior prostates were dissected from indicated mice (9 weeks of age), and subjected to immunofluorescence or IHC staining for Pten, ATF3 and p53 expression. Staining intensity was quantified and presented as IOD (c).

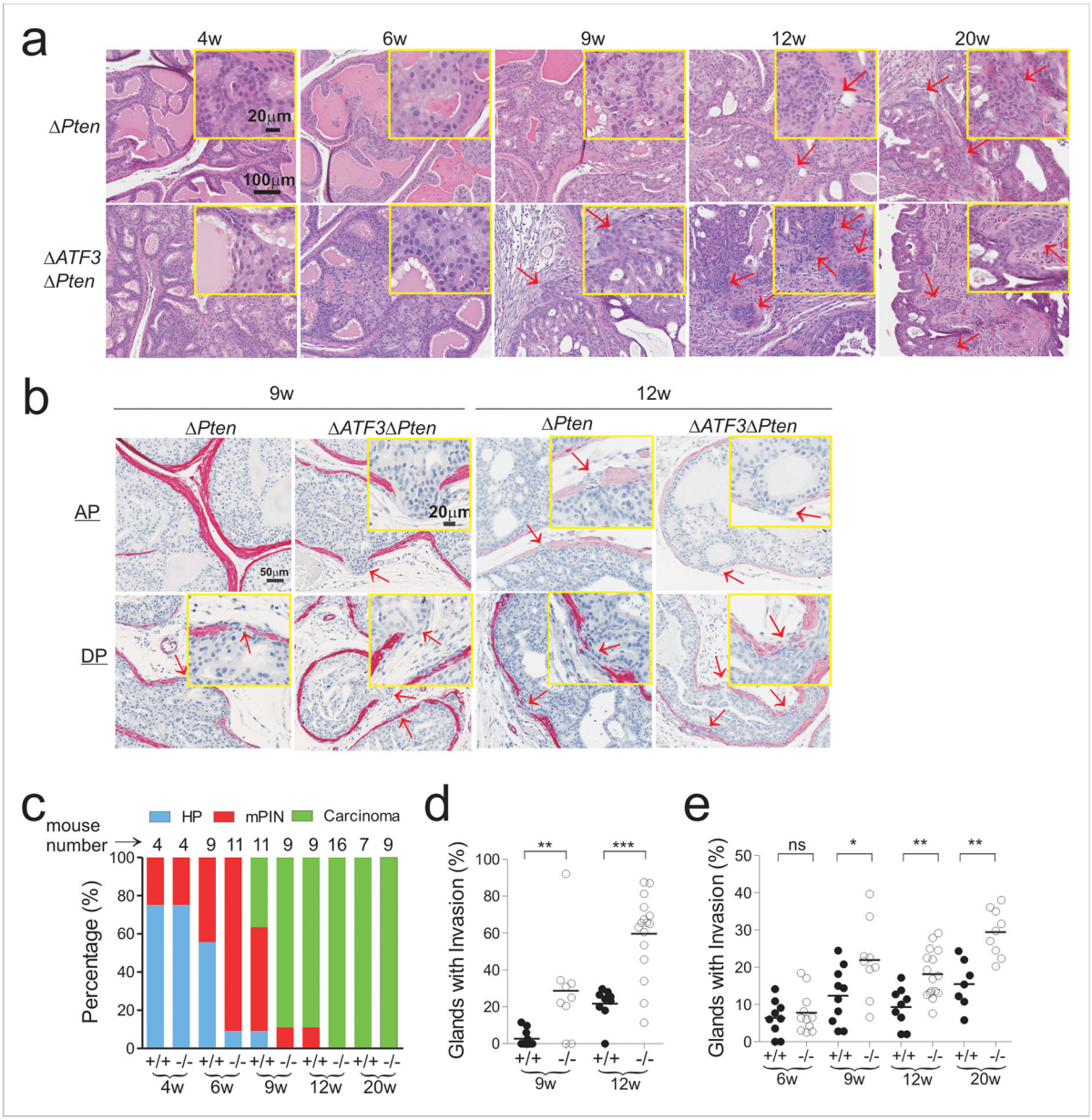

Fig 2. Loss of ATF3 promotes the development of prostate cancer

(**a**) H&E stained sections of anterior prostates from indicated mice at 4, 6, 9, 12 and 20 weeks of age. Arrows indicate invasion. (**b**)α-SMA stained sections of anterior prostates (AP) and dorsal prostates (DP) at 9 and 12 weeks show more invasive lesions in ΔATF3ΔPten mice. Arrows indicate invasion sites marked by discontinuous α-SMA staining. (**c**) Percentages of mice with hyperplasia, mPIN or adenocarcinoma in their anterior prostates. (**d, e**)Percentages of lesions with invasion are shown for AP (d) and DP (e). Invasive lesions were counted based on α-SMA staining. All glands in each section (average 42 AP and 79 DP) were counted. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no significant difference; Mann-Whitney U-test.

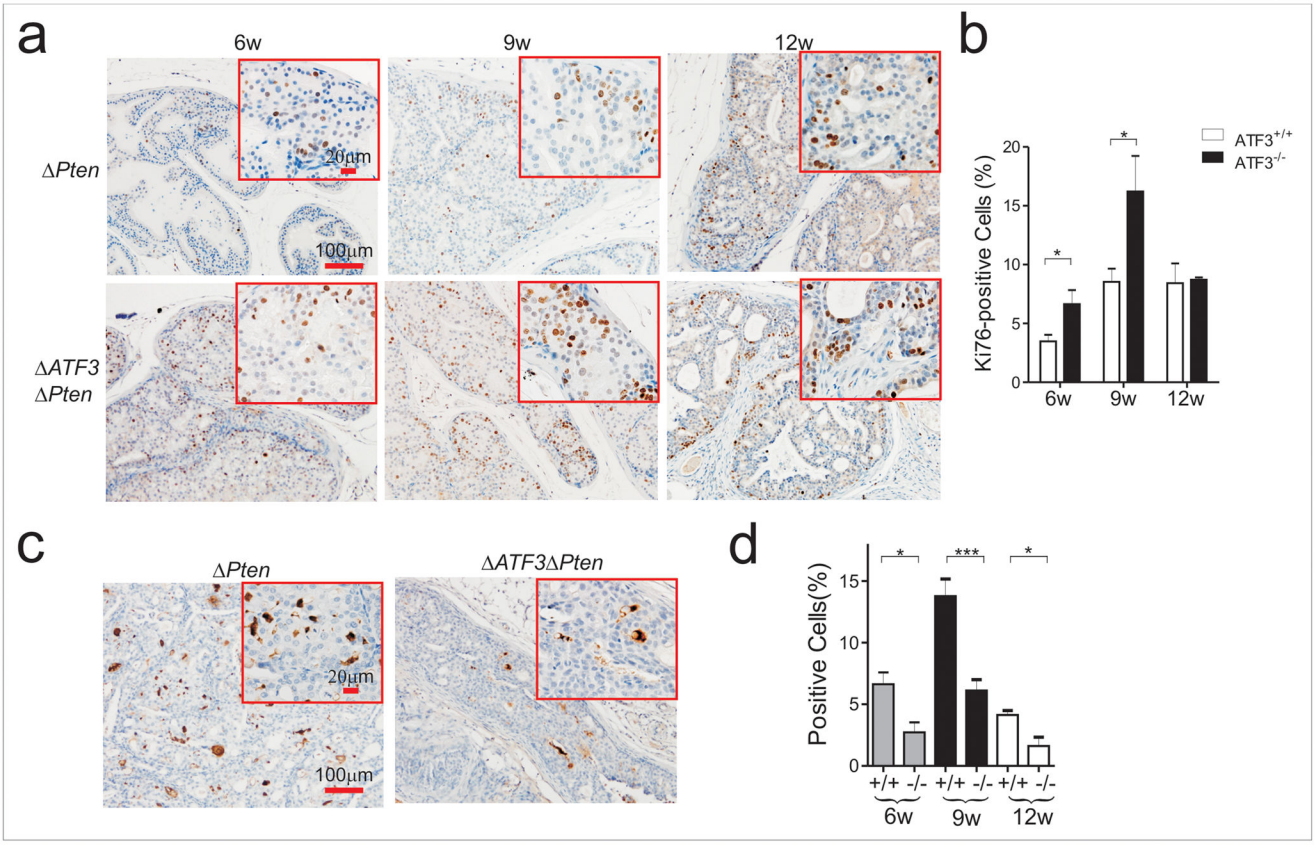

Fig 3. Loss of ATF3 promotes cell proliferation and survival in *Pten*-null prostate lesions

(**a**)Anterior prostates were stained for Ki67 expression. Representative images are shown. (**b**) Ki67-positive cells were counted from random microscopic fields, and used to calculate positivity. (**c**)Anterior prostates from 9-week-old mice were stained for cleaved caspase 3 expression. Representative images are shown. *D*, Cells expressing cleaved caspase 3 were counted from random fields, and use to calculate positivity. *, $p < 0.05$; ***, $p < 0.001$; Student t-test.

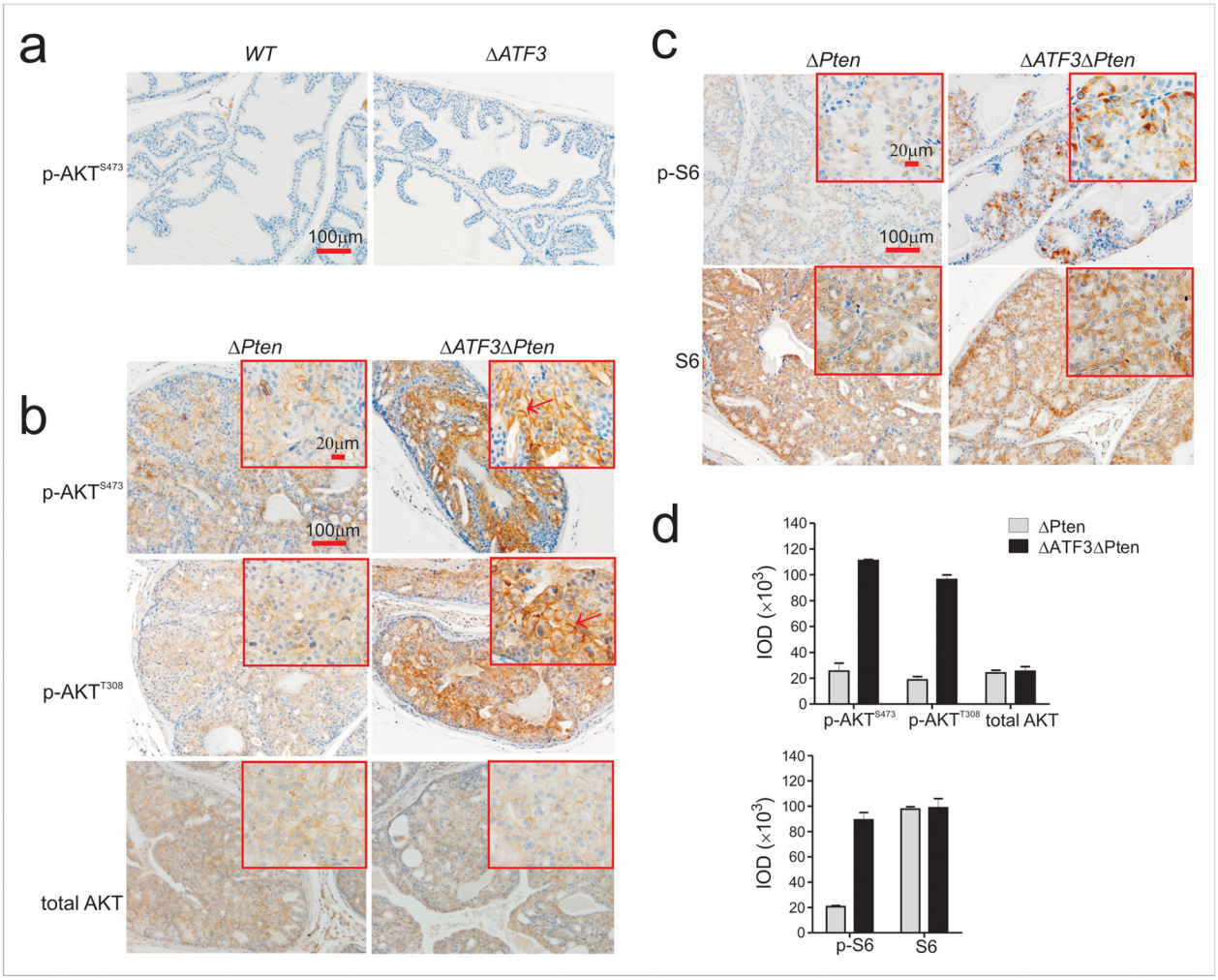

Fig 4. Loss ofATF3 promotes AKT activation and enhances AKT signaling in *Pten*-null prostate lesions

Prostate sections from indicated mice (9 weeks of age) were subjected to IHC staining for expression of indicated proteins (**a**, **b**, and **c**). Staining intensity of each protein was quantified using the Image-Pro Plus software, and presented as the integrated optical density (IOD) (**d**).

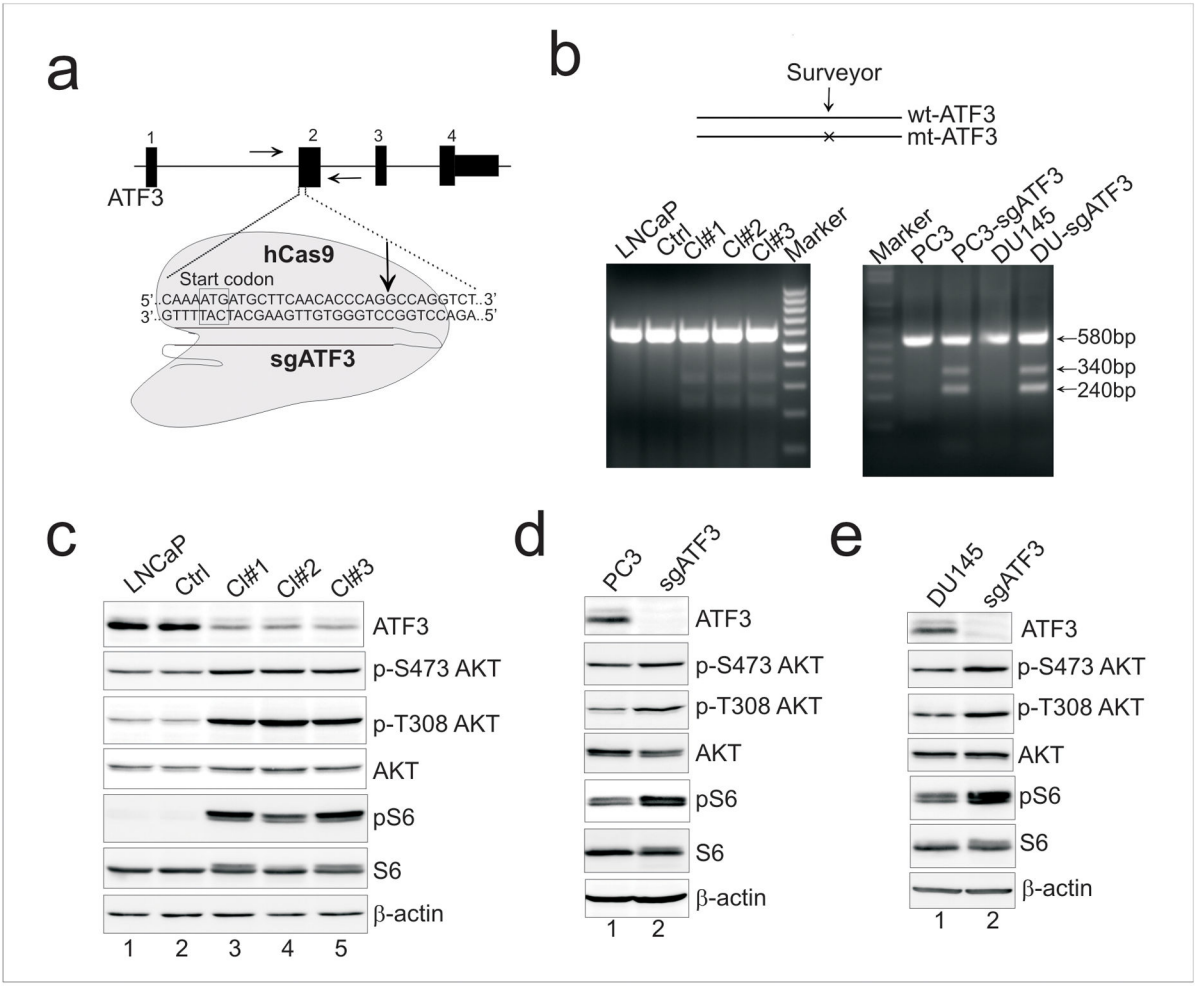

Fig 5. Downregulation of ATF3 expression by sgRNA-mediated targeting enhances AKT signaling in human prostate cancer cells

(**a**)Diagram depicts that the nuclease hCas9 recruited by a single guide RNA (sgATF3) specifically recognizing a region spanning the *ATF3* start codon cleaves the *ATF3* gene. The vertical arrow shows the potential cleavage site. (**b**) The Surveyor nuclease cleaves the mismatch site of a heteroduplex DNA (580 bp) formed by the wild-type and mutated ATF3 gene fragments amplified by PCR using the primers indicated by arrows in **a**, which generates two cleaved products (340 bp and 240 bp). Genomic DNA from indicated cells was used as templates for PCR.(**c, d, e**)Western blotting results show elevated AKT signaling in sgATF3-targeting cells.

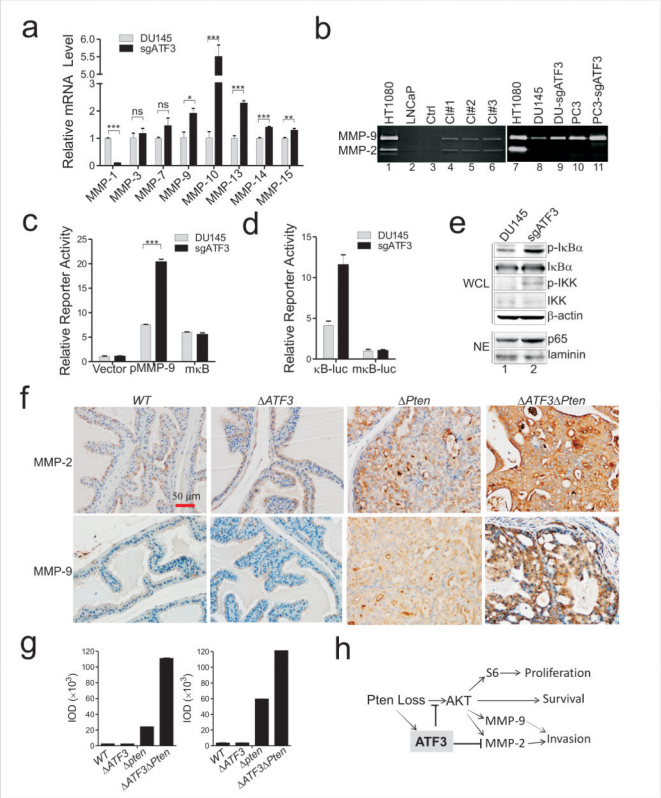

Fig 6. Loss ofATF3 increases MMP-2 and MMP-9 expression in prostate cancer cells

(**a**)MMP expression was increased in ATF3-downregulated DU145 cells. Cells were subjected to qRT-PCR assays to measure MMP expression levels. (**b**) MMP-9 and MMP-2 expression was increased in ATF3-downregulated human prostate cancer cells. Conditional media from indicated cells were subjected to zymography after normalized to equal cell number. HT1080 cells expressing high levels of MMP-2 and MMP-9 were used as positive controls. (**c**)The MMP-9 promoter activity was increased in ATF3-downregulated cells. A luciferase reporter driven by a human MMP-9 promoter (−670 ~ +34) or a κB-mutated (−600) promoter was transfected into DU145 or sgATF3-expressing cells along with pRL-CMV for dual-luciferase activity assays. (**d**) ATF3-downregulated cells harbored increased NF-κB reporter activity. A reporter driven by tandem repeats of κBcis-regulatory elements (κB-luc) or mutated κB elements (mκB-luc) were transfected into DU145 or sgATF3 cells for dual luciferase activity assays. (**e**) The NF-κB signaling was increased in sgATF3-expressing cells as detected by Western blotting. WCL, whole cell lysate; NE, nuclear extract. (**f, g**) Loss of *ATF3* increases MMP-2/-9 expression in *Pten*-null prostate lesions. Prostate sections from 9-week-old mice were subjected to IHC staining for MMP-2 and MMP-9 expression. Staining intensity was quantified and presented as IOD (d). (**h**) A model indicates that ATF3 induced by *Pten* loss could block AKT activation, leading to decreased proliferation, survival and invasiveness of prostate cancer cells. ATF3 could also directly inhibit MMP-2 expression thereby preventing prostate cancer cells from invasion.