



The essential role of WD repeat domain 77 in prostate tumor initiation induced by *Pten* loss

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Abstract

Prostate cancer is the most commonly diagnosed malignancy among men, but few genetic factors that drive prostate cancer initiation have been identified. The WD repeat domain 77 (Wdr77) protein is essential for cellular proliferation when localizes in the cytoplasm of epithelial cells at the early stage of prostate development. In the adult prostate, it is transported into the nucleus and functions as a co-regulator of the androgen receptor to promote cellular differentiation and prostate function. This developmental process is reversed during prostate tumorigenesis, i.e., Wdr77 is translocated from the nucleus into the cytoplasm to drive proliferation of prostate cancer cells. In this study, we used *in vivo* genetic studies to further investigate the role of Wdr77 in prostate tumorigenesis. We found that prostate-specific deletion of *Wdr77* abolished prostate tumor initiation induced by loss of the tumor suppressor *Pten*. Mechanistically, *Wdr77* ablation inhibited E2F3 activation and enhanced TGF β signaling, leading to attenuated cellular proliferation induced by loss of *Pten*. These findings establish a critical role of Wdr77 for prostate tumor initiation.

Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related death among men in the United States [1, 2]. Prostate cancer develops through multiple clinical stages, including prostatic intraepithelial neoplasia (PIN), adenocarcinoma, and metastasis [2]. A few genes that initiate prostate cancer or/and promote prostate cancer progression have been identified, including the tumor suppressor *Pten*, the gene rearrangement *TMPRSS2-ERG*, and *c-Myc* [3, 4]. Some genes (such as *Sox9*, *Hoxb13*, and *Nkx3.1*) and pathways (such as WNT signaling) involved in prostate development are found reactivated or inactivated in cancer initiation and progression [5]. However, there is still a need to determine

additional drivers of prostate cancer that could help to understand prostate tumorigenesis and become the targets of new therapies.

The WD repeat domain 77 (Wdr77) protein is composed of 342 amino acid residues and 7 putative WD-40 repeats and was identified as a component (MEP50) of the methylome complex [6], a subunit (WD45) of the SMN complex [7], and a novel androgen receptor (AR)-interacting protein (p44) [8, 9]. Wdr77 localizes in the cytoplasm of epithelial cells and is required for cell proliferation at the growth stage of prostate development [9–11]. In contrast, in the adult prostate, Wdr77 expression is decreased and the Wdr77 protein is translocated into the nucleus to function as an androgen receptor cofactor to establish and maintain luminal epithelia in a growth-arrested fully differentiated state (the G1/G0 cell cycle phase). Thus, Wdr77 provides for the integrated regulation of proliferation and differentiation of prostate epithelial cells through its distinct subcellular localization during prostate development. The increased *Wdr77* gene expression and Wdr77 protein translocation from the nucleus to the cytoplasm is associated with age-related prostatic intraepithelial hyperplasia and prostate tumorigenesis [9–11]. The cytoplasmic Wdr77 is also required for the proliferation of prostate cancer cells and growth of prostate tumor xenografts [9, 11, 12]. Therefore, the molecular event involving Wdr77 in prostate

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development is reversed during cancer tumorigenesis. Further studies indicated that *Wdr77* is required for cell proliferation via modulating the cell cycle progression [12] and expression of some cell growth regulators [13]. More recently, we found that *Wdr77* expression also causes the non-sensitivity of proliferating cells to the TGF β signaling, thereby contributing to cellular proliferation during tumorigenesis [14].

The tumor suppressor *Pten* gene encodes a lipid phosphatase that catalyzes the dephosphorylation of phosphatidylinositol-trisphosphate, resulting in the down-regulation of PI3K-AKT signaling pathway [15]. AKT, when activated, modulates a variety of downstream effectors involved in cell proliferation, apoptosis, cell growth, and metabolism [16]. Deletion or mutation of the *Pten* gene is one of the most frequent genetic alterations in many human cancers, including prostate cancer [17]. Loss of the *Pten* gene in prostate epithelial cells in the mouse results in the development of prostatic intraepithelial neoplasia (PIN) and prostate cancer with complete penetrance [18–20].

To understand whether *Wdr77* is essential for prostate tumorigenesis in vivo, we have developed a mouse model in which prostate-specific deletion of both *Wdr77* and *Pten* genes is generated in the epithelium using Cre-loxP site-specific recombination. We report here that deletion of *Wdr77* abolished PIN and prostate tumor development initiated by homozygous loss of *Pten*, demonstrating the essential role of *Wdr77* in prostate tumor initiation. We further show that *Wdr77* is necessary for maintenance of cellular proliferation induced by loss of *Pten*. In addition, we demonstrated that *Wdr77* is critical for suppressing the TGF β signaling in vivo, suggesting that *Wdr77* plays a role in relieving growth suppression of the TGF β signaling.

Results

Generation of mice bearing prostate-specific deletion of *Pten* or *Pten* and *Wdr77* genes

Previous studies have demonstrated that *Wdr77* was essential for growth of normal prostate epithelial cells, as well as prostate cancer cells and prostate tumor xenografts [9, 11]. However, it has yet to be shown the role of *Wdr77* in prostate tumorigenesis in vivo. To explore this, we used the *Pten* gene knockout mouse model, which closely mimics human prostate cancer [11, 21]. To generate mice that were homozygous mutants for *Pten* (*Pten*^{pc-/-}) and both *Pten* and *Wdr77* genes (*Pten*^{pc-/-};*Wdr77*^{pc-/-}) in the prostate, we crossed *Pten*^{loxP/loxP} and *Pten*^{loxP/loxP};*Wdr77*^{loxP/loxP} mice with *PRR2Bi-Cre* mice, respectively. The prostate-specific deletion of *Pten* and *Wdr77* genes were confirmed by genomic typing (Supplementary Fig. 1a, lane 6).

Consistent with previously published reports [12, 18], *Pten* protein expression in the cytoplasm of the prostate luminal epithelia with sporadic nuclear staining (Supplementary Fig. 1b, panel A) and *Wdr77* protein expression in the nucleus (panel C) were observed. The majority of prostate epithelial cells of *Pten*^{pc-/-};*Wdr77*^{pc-/-} mice lost both *Pten* and *Wdr77* protein expression (Supplementary Fig. 1b, panels B and D), with a few cells (about 1–3%) still expressing *Pten* or *Wdr77* but at low levels (inserts, indicated by black arrows). As expected, loss of the *Pten* gene led to up-regulation of the phosphorylated AKT protein in prostate epithelial cells of both prostate epithelial cells of *Pten*^{pc-/-} and *Pten*^{pc-/-};*Wdr77*^{pc-/-} mice relative to the *WT* mouse (Supplementary Fig. 2a, panels B–E vs. panel A; Supplementary Fig. 2a, lanes 2 and 4 vs. lanes 1 and 3), indicating that *Wdr77* gene deletion did not affect AKT activation induced by *Pten* loss. Loss of the *Wdr77* gene did not affect expression of the *Pten* gene or reverse versa (Supplementary Fig. 2b, lanes 2 and 4 vs. lanes 1 and 3), suggesting no physiological regulation between *Pten* and *Wdr77*.

Cytoplasmic *Wdr77* is essential for prostate tumor initiation induced by *Pten* loss

Prostate glands were derived from *Pten*^{pc-/-} and *Pten*^{pc-/-};*Wdr77*^{pc-/-} mice at the ages of 1, 2, and 4 months (Table 1) and submitted to the analyses. Examined following Hematoxylin and Eosin (H&E) staining and consistent with previous observations [18, 22], *Pten*-null mice exhibited PIN lesions (PIN, circled) and tumors (T, circled) at the age of 2 months (Fig. 1a). The tumor incidence was increased as mice aged (Fig. 2b). Interestingly, examination of *Pten*^{pc-/-};*Wdr77*^{pc-/-} mouse prostate revealed a few PIN lesions (PIN, circled) and small tumors (T, circled) (Fig. 1b). The incidence of PIN and tumors in *Pten*^{pc-/-};*Wdr77*^{pc-/-} mice was drastically decreased in mice at the ages of both 2 and 4 months (Fig. 2). Gland sizes are significantly increased (Fig. 2c) and epithelial enfoldings are lacking (Fig. 2d) in the DLP prostate of the *Pten*^{pc-/-} mouse due to tumor growth. Loss of the *Wdr77* gene decreased gland sizes (Fig. 2c), as well as epithelial enfoldings (Fig. 2d), consistent with the critical role of *Wdr77* in growth and differentiation of

Table 1 Ages, genomic types, and numbers of male mice analyzed

| Age (month) | Genomic type | | | |
|-------------|---------------------------|-----------------------|---|---|
| | PTEN ^{loxP/loxP} | PTEN ^{pc-/-} | PTEN ^{loxP/loxP} ; WDR77 ^{loxP/loxP} | PTEN ^{pc-/-} ; WDR77 ^{pc-/-} |
| 1 | 2 | 5 | 2 | 5 |
| 2 | 2 | 5 | 2 | 5 |
| 4 | 2 | 5 | 2 | 7 |

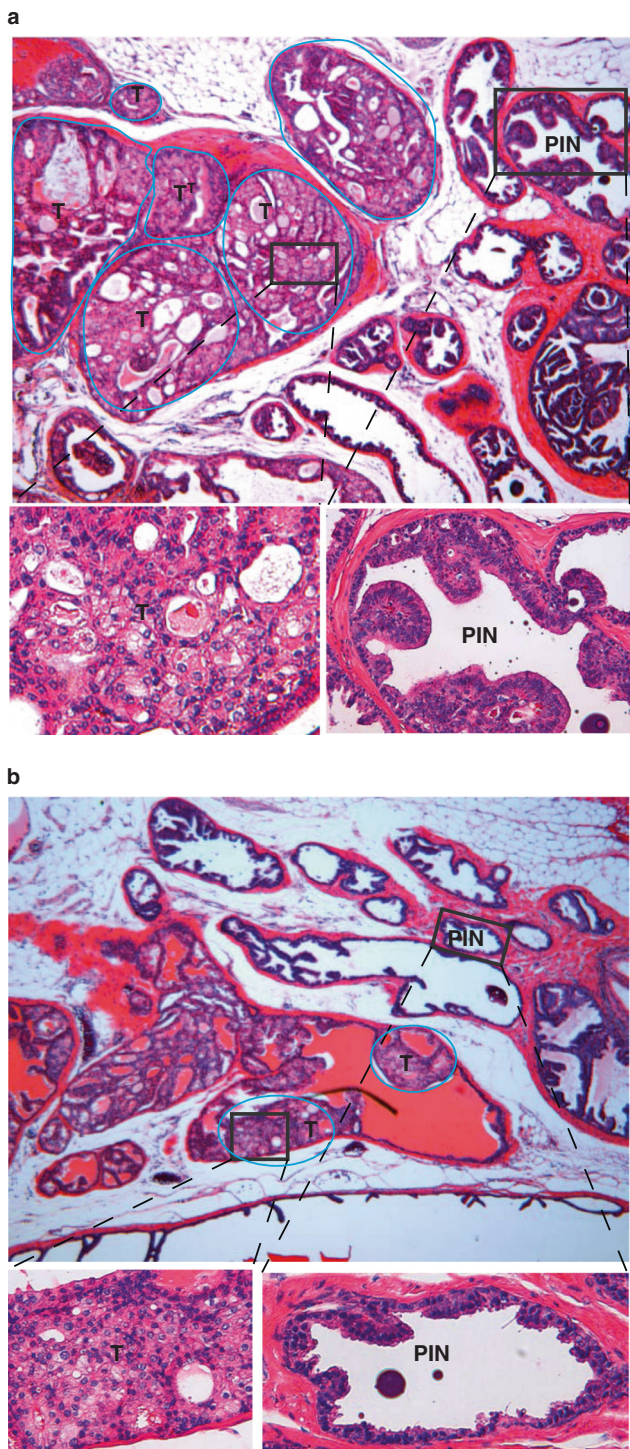


Fig. 1 Loss of *Wdr77* inhibited prostate tumorigenesis induced by *Pten* gene deletion. The prostate tissue was derived from the *Pten*^{pc-/-} (a) or *Pten*^{pc-/-};*Wdr77*^{pc-/-} (b) mouse at the age of 2 months and stained with H&E. Tumor (T) regions are circled (top panels). The bottom panels show amplification of selected tumor (left) and PIN (right) lesions

prostate epithelial cells. But *Pten* gene deletion could not restore grand size and enfolding numbers decreased by *Wdr77* deletion (Fig. 2c, d).

Immunostaining of the prostate derived from *Pten*^{pc-/-};*Wdr77*^{pc-/-} mouse with anti-*Wdr77* antibody revealed that cells in PIN and tumor regions still expressed *Wdr77* (Fig. 3a). In contrast, *Wdr77* was absent in the benign epithelial cells (Fig. 3a, circled by red lines). These results suggest that PIN and tumors were derived from cells in which *Wdr77* was not deleted. Thus, *Wdr77* expression is essential for prostate PIN and tumors driven by the *Pten* gene deletion.

We have previously reported that at the early stage of prostate development, *Wdr77* is localized in the cytoplasm to drive cellular proliferation [21]. In contrast, it is transported into the nucleus in adult prostate to drive cellular differentiation. During prostate tumorigenesis, this developmental process is reversed, i.e., *Wdr77* is transported from the nucleus into the cytoplasm to initiate cellular proliferation [21]. We investigated whether a similar event happens during prostate tumorigenesis driven by the *Pten* deletion. Similar to that observed during prostate tumorigenesis [11], *Wdr77* is localized in the nucleus of normal prostate epithelial cells (Fig. 3b, top panels) and in contrast, *Wdr77* is transported into the cytoplasm in hyperplasia (middle panels) and tumor (bottom panels) lesions. Cells in which *Wdr77* was not deleted but localized to the nucleus were also observed in normal luminal epithelia (Fig. 3a, indicated by red arrows). We previously demonstrated that *WDR77* in the cytoplasm is essential and sufficient to drive cellular proliferation and, in contrast, when localized in the nucleus, it inhibited cell growth and promoted cellular differentiation [23]. Thus, *WDR77* cytoplasmic translocation may be also required for prostate tumorigenesis driven by *Pten* gene deletion.

Loss of *Wdr77* inhibited cellular proliferation induced by the *Pten* deletion via E2F transcriptional factor

We previously demonstrated that cytoplasmic *Wdr77* is essential for proliferation of prostate epithelial cells, as well as prostate cancer cells [11, 21]. Quantification of proliferation was accomplished by Ki-67 immunostaining (brown, indicated by black arrows) on *WT*, *Wdr77*^{fl/fl}, *Wdr77*^{pc-/-}, *Pten*^{pc-/-} and *Pten*^{pc-/-};*Wdr77*^{pc-/-} prostate sections (Fig. 4a). In *WT* prostate, about 4% of epithelial cells were Ki-67-positive (Fig. 4b). However, the deletion of the *Pten* gene significantly enhanced the proliferation rate of epithelial cells in PIN (5.5-fold) and tumor (7.7-fold) regions (Fig. 4b, right panel) but no effect on the proliferation of benign prostate epithelial cells (left panel). Simultaneous deletion of *Wdr77* or both *Pten* and *Wdr77* genes resulted in the proliferation rate of benign prostate epithelial cells lower than that observed in the *WT* mouse (Fig. 4b, left panel). However, the proliferation rate of

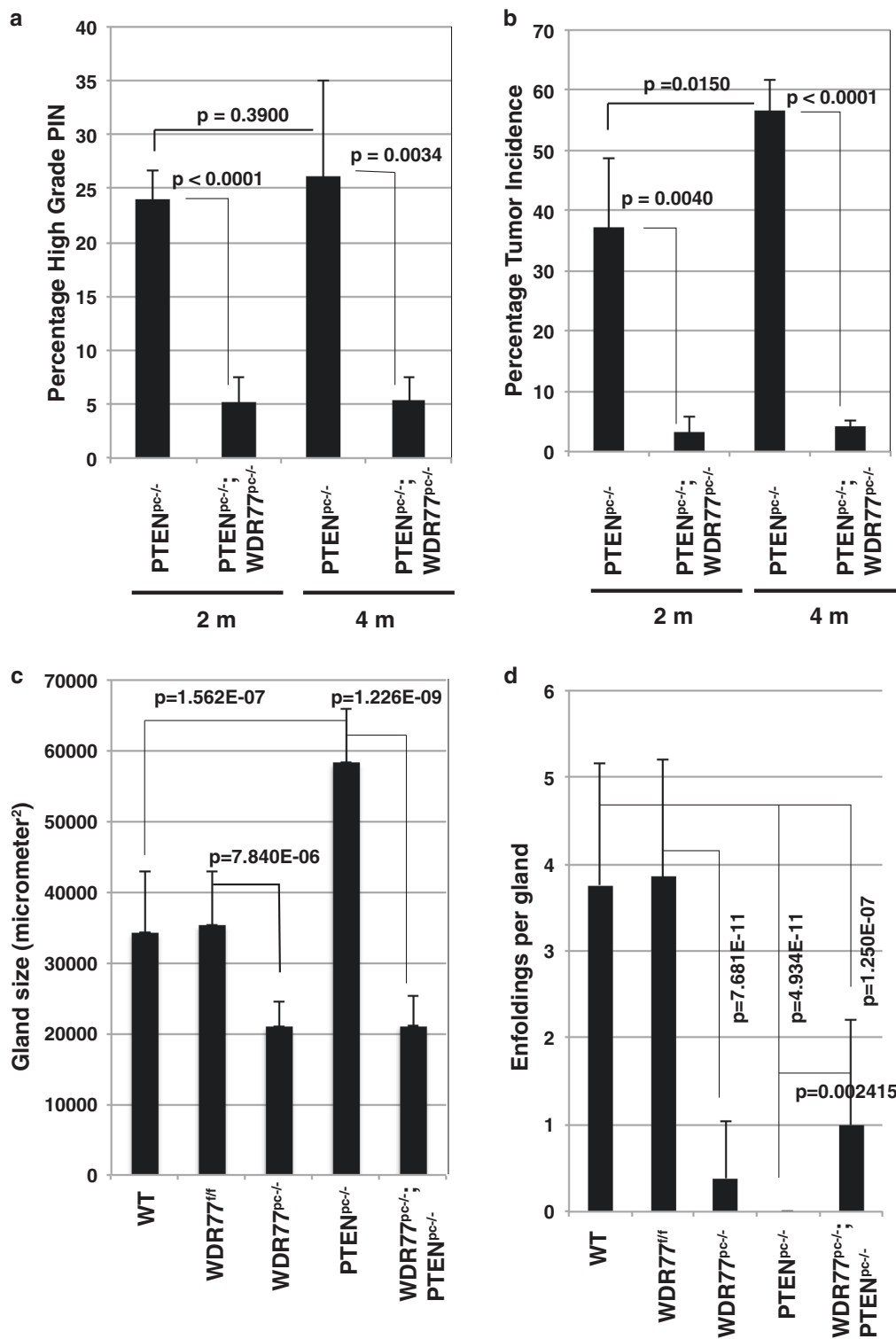
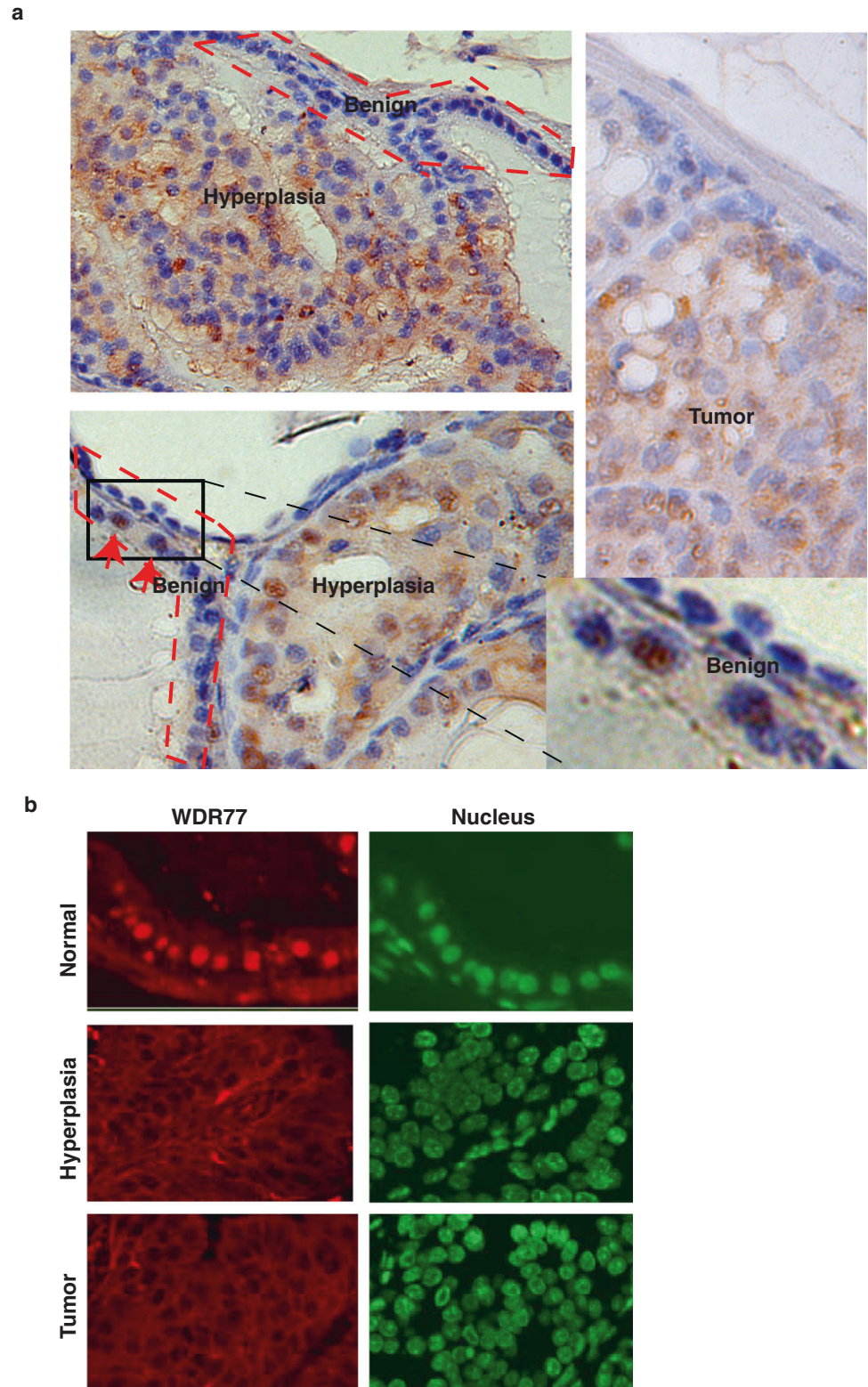


Fig. 2 The incidence of PIN (**a**) and prostate tumor (**b**) in *Pten*^{pc-/-} and *Pten*^{pc-/-}; *Wdr77*^{pc-/-} mice. Prostate glands were derived from mice at the ages of 2 ($n = 5$) and 4 ($n = 7$) months and PIN and tumor lesions were quantified for each prostate. Tumor or PIN incidence = numbers of glands with tumor or PIN per 100 glands. Data are presented as the means of 5 (at the age of 2 m) or 7 (at the age of 7 m)

prostates. Sizes (**c**) and enfoldings (**d**) per gland in DLP of WT, *Wdr77*^{pc-/-}, *Pten*^{pc-/-}, and *Pten*^{pc-/-}; *Wdr77*^{pc-/-} mice. Prostates of each genotype were derived from mice ($n = 5$) at the age of 2 months. Sizes or enfoldings of 10–30 glands in DLP of each mouse were measured and data are presented as mean of data obtained from all mice

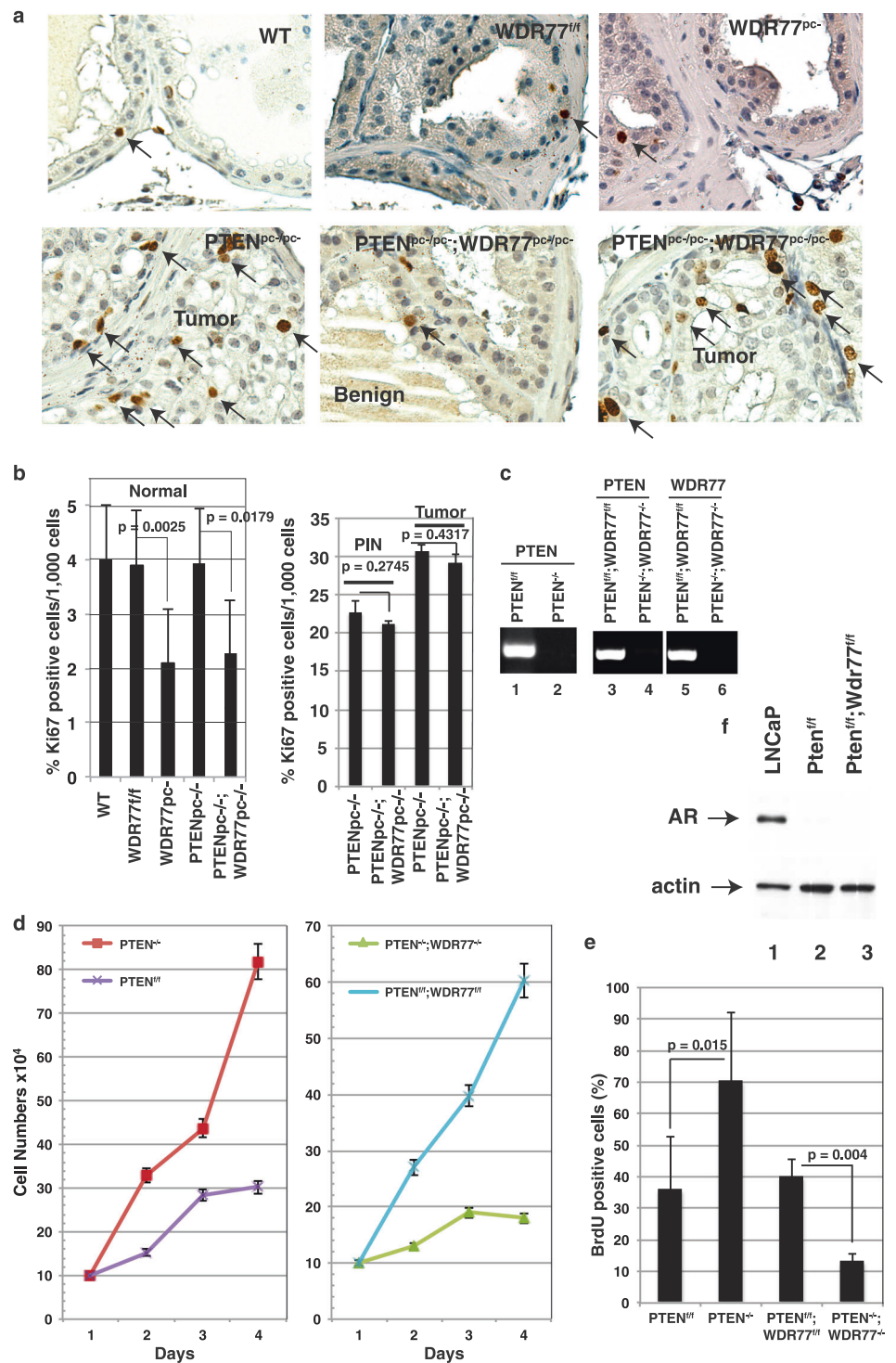
Fig. 3 Cytoplasmic Wdr77 is essential for prostate initiation induced by *Pten* gene deletion. **a** Immunostaining of Wdr77 (brown) in the prostate tissue derived from the *Pten^{pc-/-}; Wdr77^{pc-/-}* mouse at the age of 2 months. Benign regions are circled by red lines (top panel). Red arrows indicate benign epithelial cells expressing Wdr77 in the nucleus. **b** Wdr77 cytoplasm translocation is associated with prostate tumorigenesis induced by *Pten* gene deletion. The prostate tissue was derived from the *Pten^{pc-/-}* mouse at the age of 2 months and immunostained for Wdr77 (red, left panels). The nucleus was stained with SYTOX green (right panels)



epithelial cells in PIN and tumor regions of *Pten^{pc-/-}; Wdr77^{pc-/-}* prostate is indistinguishable with that observed in the *Pten^{pc-/-}* prostate, consistent with the fact that *Wdr77* was not deleted in these lesions (Fig. 3a).

Prostate epithelial cells were isolated from *Pten^{loxP/loxP}* and *Pten^{loxP/loxP}; Wdr77^{loxP/loxP}* mice and infected with adenovirus harboring the Cre recombinase to delete *Pten* (Fig. 4c, lane 2) and *Pten* plus *Wdr77* genes (lanes 4 and 6). Of

Fig. 4 Loss of *Wdr77* inhibited cellular proliferation induced by *Pten* gene deletion. **a** The prostate tissue were derived from the WT, *Wdr77^{fl/fl}*, *Wdr77^{pc/-}*, *Pten^{pc/-/-}*, and *Pten^{pc/-/-};Wdr77^{pc/-/-}* mice at the age of 2 months and stained for Ki-67 (brown). **b** The percentage of Ki-67 positive cells in WT ($n = 2$), *Wdr77^{fl/fl}* ($n = 5$), *Wdr77^{pc/-/-}* ($n = 5$), *Pten^{pc/-/-}* ($n = 5$), and *Pten^{pc/-/-};Wdr77^{pc/-/-}* ($n = 7$) prostate. **c** Gene typing of prostate epithelial cells derived from the *Pten^{loxP/loxP}* and *Pten^{loxP/loxP};Wdr77^{loxP/loxP}* mice and infected with adenovirus harboring GFP (*Pten^{fl/fl}*, lanes 1, 3, 5) or Cre recombinase (*Pten^{-/-}*, lanes 2, 4, 6). **d** The growth curves of prostate epithelial cells derived from the *Pten^{loxP/loxP}* (left), and *Pten^{loxP/loxP};Wdr77^{loxP/loxP}* (right) mice and infected with adenovirus harboring GFP (left: *Pten^{fl/fl}*; Right: *Pten^{fl/fl};Wdr77^{fl/fl}*) or Cre recombinase (Left: *Pten^{-/-}* and Right: *Pten^{-/-};Wdr77^{-/-}*). **e** Percentage of BrdU-positive epithelial cells infected with adenovirus harboring GFP or Cre recombinase. Cell were grown in the presence of BrdU for 2 h and submitted for immunostaining for BrdU. **f** Western blot of whole cell lysates made from LNCaP, *Pten^{fl/fl}*, and *Pten^{fl/fl};Wdr77^{fl/fl}* cells with anti-AR antibody and anti-actin antibodies



note, the efficiency of Ad-mediated gene deletion is between 95–100% [24]. *Pten* deletion led to AKT phosphorylation (Supplementary Fig. 3a, lane 2 vs. lane 1) and enhanced growth of prostate epithelial cells (Fig. 4d, left), consistent with previous reports [18, 25]. However, deletion of both *Pten* and *Wdr77* genes also led to AKT phosphorylation (Supplementary Fig. 3a, lane 4 vs. lane 3) but

inhibited growth of prostate epithelial cells (Fig. 4d, right). To assess proliferation, we used a BrdU incorporation assay. As reported [25], the percentage of BrdU-positive *Pten*-null epithelial cells was significantly higher than that of control epithelial cells (Fig. 4e). On the converse, *Pten*-null and *Wdr77*-null cells exhibited a significant decrease in cell proliferation relative to that of control cells (Fig. 4e).

Fig. 5 Loss of *Wdr77* blocked E2F3 expression induced by *Pten* gene deletion. **a** The prostate tissues were derived from the WT, *Pten^{pc-/-}*, and *Pten^{pc-/-};Wdr77^{pc-/-}* mice at the age of 2 months and immunostained for E2F3 and phosphorylated Rb (pRb). Quantalization of nuclear E2F3 (b) and pRb (c) immunostaining signals using ImageJ32 software (NIH)

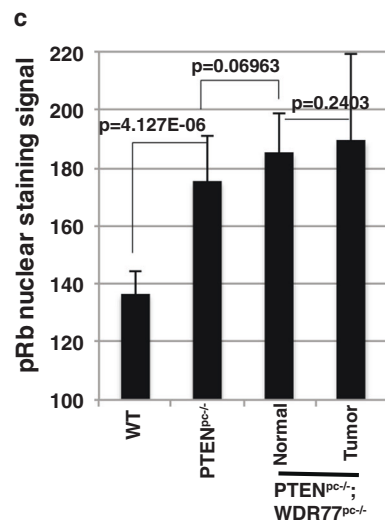
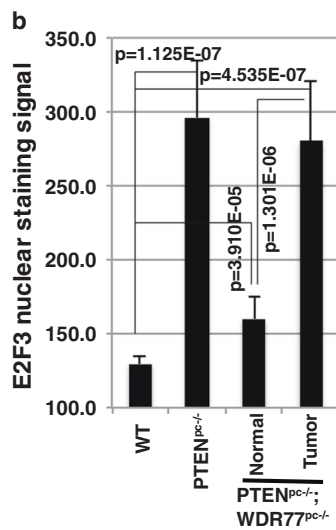
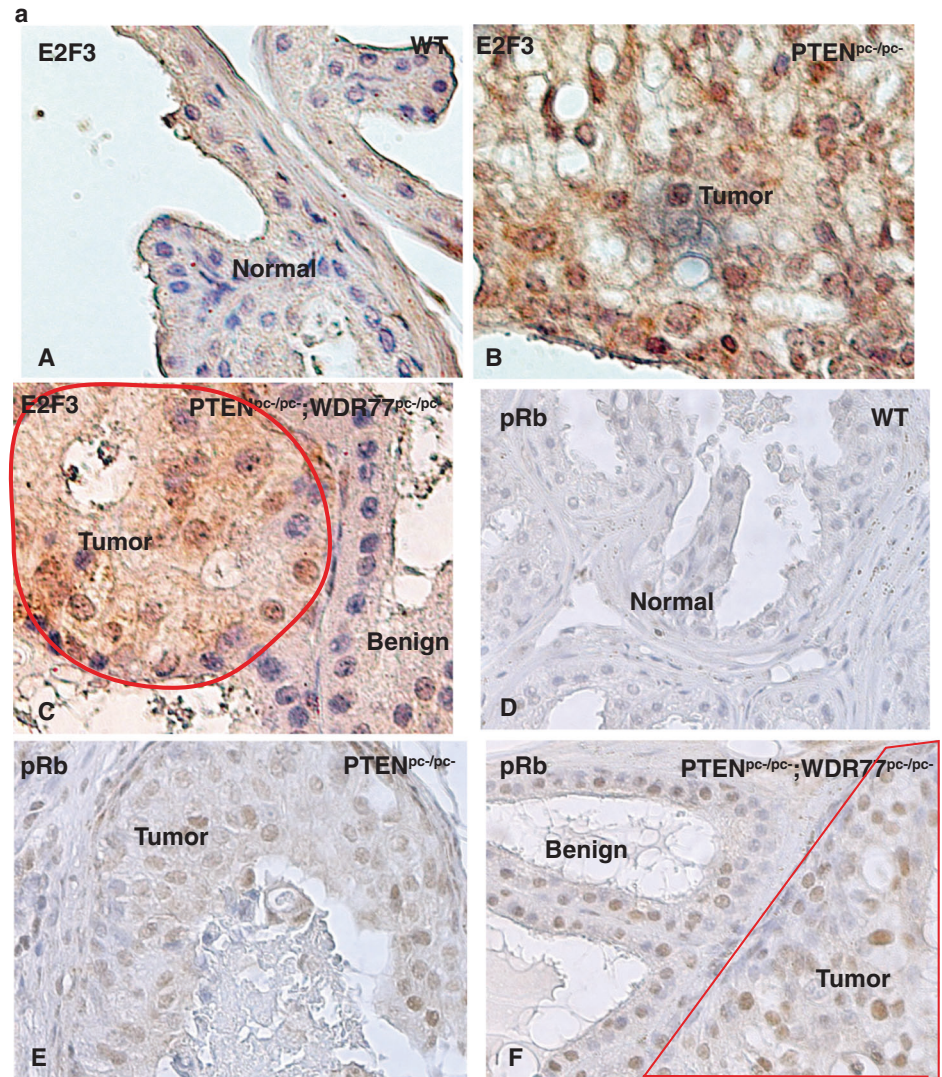
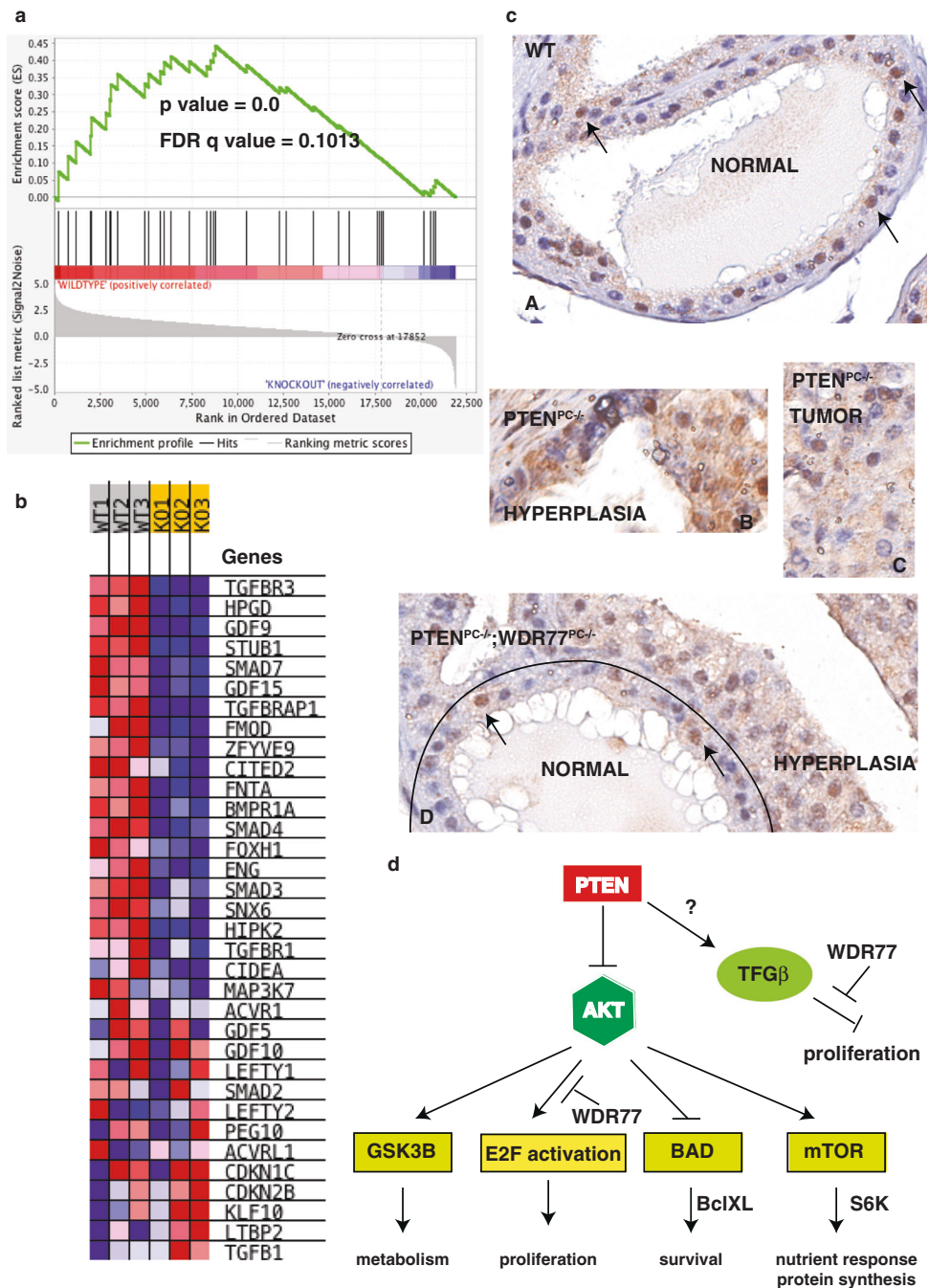


Fig. 6 Loss of *Wdr77* prevented inactivation of the TGF β signaling. **a** GSEA enrichment plot indicates that genes regulated by TGF β were over-represented on the gene list, whose expression is regulated by *Pten* loss. **b** Genes targeted by TGF β are visualized by heatmap. The red and blue colors represent higher than average and lower than average expression of particular gene in the *Pten*-null prostate, respectively. **c** Immunostaining of phosphorylated SMAD3 (pSMAD3) in prostate tissues derived from WT, *Pten*^{pc-/-}, and *Pten*^{pc-/-};*Wdr77*^{pc-/-} mice at the age of 2 months. **d** Diagram of the *Pten*-AKT signaling and its downstream targets



Thus, *Wdr77* mediates cellular proliferation induced by the *Pten* gene deletion. The isolated epithelial cells lost AR expression (Fig. 4f, lanes 2 and 3), suggesting that the regulation of cell growth by *Pten* and *Wdr77* is AR-independent.

Pten loss promotes phosphorylation of AKT, which in turn phosphorylates multiple targets resulting in an increase in cell proliferation, cell survival, metabolism, and protein synthesis [16]. We next investigated which signaling(s) activated by *Pten* loss was affected by *Wdr77* gene deletion. As reported [25], *Pten* loss resulted in phosphorylation of

AKT, S6 ribosomal protein (rpS6), and GSK3 β (Supplementary Fig. 3a, lane 2) but loss of *Wdr77* did not affect these phosphorylation events (lane 4), indicating that loss of *Wdr77* had no effect on *Pten*-AKT-GSK3 β (metabolism) and *Pten*-AKT-mTOR-S6K (protein synthesis) signalings induced by *Pten* loss. Loss of *Pten* gene or *Pten* and *Wdr77* genes did not induce cell apoptosis indicated by DNA fragmentation assay (Supplementary Fig. 3b) and Western blot analysis of PARP protein (Supplementary Fig. 3c). We also observed that loss of *Wdr77* gene did not inhibit phosphorylation of the BAD protein induced by *Pten*

deletion (data not shown). So, *Wdr77* gene deletion also did not affect cell survival induced by *Pten* loss. It was reported previously that *Pten* loss enhanced cellular proliferation by activating the RB-E2F pathway [26, 27]. The pRB-E2F pathway plays a critical role in regulating cellular proliferation because it regulates expression of many genes that are required for cell cycle progression [28]. To determine whether *Wdr77* exerts influence on this process, we performed immunostaining of E2F3, a member of the E2F family of transcription factors [29]. As shown in Fig. 5, *WT* mice presented very low expression of E2F3 (Fig. 5a, panel A; Fig. 5b). However, *Pten* deletion resulted in strong nuclear expression of E2F3 (Fig. 5a, panel B; Fig. 5b), consistent with previous reports [27]. Interestingly, upon simultaneous deletion of *Wdr77* and *Pten* genes, E2F3 expression in the benign region resembled that of the *WT* mouse with little to no expression (Fig. 5a, panel C; Fig. 5b). Of note, high levels of E2F3 are observed in the tumor region (Fig. 5a, panel C, circled by red line; Fig. 5b), where *Wdr77* is not deleted (Fig. 3a). This finding indicates that *Wdr77* is required for expression of E2F3 induced by *Pten* deletion.

Rb interacts with E2F proteins to block its function in the activation of S-phase genes [28]. The phosphorylation of Rb abolishes its interaction with E2F proteins, thereby relieving its inhibitory effect on cell-cycle progression. In *WT* mice, Rb phosphorylation was minimal (Fig. 5a, panel D; Fig. 5c), as opposed to the strong pRb signal exhibited in the *Pten*-null mouse prostate (Fig. 5a, panel D; Fig. 5c). This result indicates that *Pten* loss leads to Rb phosphorylation to relieve its inhibitory effect on E2F transcriptional factors. In the double knockout mutant, pRb presented in epithelial cells of both normal (*Wdr77*-null) and tumor (circled by red lines, *Wdr77*-positive) regions (Fig. 5a, panel F; Fig. 5c). Thus, loss of *Wdr77* did not affect Rb phosphorylation induced by *Pten* deletion.

TGF β signaling is activated in *Pten*^{pc-/-};*Wdr77*^{pc-/-} mice

The anti-proliferative effects of TGF- β signaling are lost during tumorigenesis, leading to hyper cellular proliferation [30–32]. More recently, we found that Smad2/3 phosphorylation, TGF β -mediated transcription, and TGF β 2 and TGF β receptor type II (T β RII) expression were dramatically induced when *Wdr77* expression was silenced [14]. We further demonstrated that *Wdr77* expression caused the non-sensitivity of proliferating cells to TGF β signaling, thereby contributing to cellular proliferation during lung tumorigenesis [14]. We analyzed the TCGA prostate cancer data set (<https://cancergenome.nih.gov/>). Gene set enrichment analysis (GSEA) indicates that genes regulated by TGF β were over-represented on the gene list, whose expression is

altered in prostate cancer (Supplementary Fig. 4a). The gene heatmap reports show the altered expression of TGF β target genes in majority of prostate cancer patients (Supplementary Fig. 4b). Thus, the TGF β signaling is lost in prostate cancer. We also analyzed the DNA microarray data set (GSE25140) generated from the mouse with the prostate-specific *Pten* deletion [33]. GSEA indicates that genes regulated by TGF β were over-represented on the gene list, whose expression is altered in *Pten* knockout prostate (Fig. 6a). The gene heatmap reports changes of TGF β target genes in *Pten* knockout prostate vs. *WT* prostate (Fig. 6b). Among these genes, 23 genes (up-regulated by TGF β in *WT* prostate) are down regulated and 5 genes (repressed by TGF β in *WT* prostate) are up regulated in the *Pten*-null prostate. This analysis suggests that the TGF β signaling is down-regulated in the *Pten* gene knockout prostate. We performed immunostaining of SMAD3 in *WT*, *Pten*^{pc-/-}, and *Pten*^{pc-/-};*Wdr77*^{pc-/-} prostate sections and detected nuclear SMAD3 staining in normal prostate epithelial cells (Fig. 6c, Some are indicated by black arrows.), indicating that the TGF β signaling is active. SMAD3 protein was diffusing into the cytoplasm in hyperplasia and tumor cells (Fig. 6c, panels B and C), suggesting loss of the TGF β signaling. Consistent with this observation, *Pten* loss led to significant down regulation of TGF β 2, TGFBR2, and TGFBR3 expression in prostate epithelial cells (Supplementary Fig. 5). Loss of *Wdr77* reactivated the TGF β signaling (p-SMAD3 expression) inactivated by *Pten* loss in vivo (Fig. 6c, panel D, circled) and restored TGF β 2 and TGFBR3 expression in *Pten*-null epithelial cells (Supplementary Fig. 5). Our findings suggest that *Wdr77* may also play an important role to block the anti-proliferative effects of the TGF β signaling during prostate tumorigenesis.

Discussion

Wdr77 regulates proliferation and differentiation of prostate epithelial cells during the development through its sub-cellular localization. This developmental process is re-activated during prostate tumorigenesis. The data from this study indicate that *Wdr77* expression, as well as its cytoplasmic localization is required for prostate tumor initiation induced by *Pten* loss.

Pten is frequently altered in cancers, suggesting it plays a fundamental role in many malignancies. *Pten* loss promotes phosphorylation of AKT, which in turn phosphorylates multiple targets resulting in an increase in cell proliferation, cell survival, and protein synthesis (Fig. 6d). An important question is which signaling activated by *Pten* loss plays an essential role in prostate tumorigenesis. Sox4 expression was up-regulated as a result of activation of *Pten*-AKT-mTOR signaling induced by *Pten* loss and loss of the *Sox4*

gene attenuated invasive phenotype of prostate tumors [34]. β -Catenin, a downstream target of Pten-AKT-GSK3 β signaling, is essential for many developmental processes and has been implicated in tumorigenesis in many tissues, including prostate cancer [35, 36]. It has been shown that β -catenin is required for prostate development and its over-expression can promote invasive prostate cancer in the *Pten* deletion model [37]. These findings suggest that the activation of Pten-AKT-mTOR or/and Pten-AKT-GSK3B signaling is critical for prostate cancer progression driven by *Pten* loss. *Pten* loss resulted in phosphorylation of AKT, S6 ribosomal protein (rpS6), GSK3 β , and BAD (data not shown) proteins (Supplementary Fig. 2a, lane 2 vs. lane 1), indicating the activation of Pten-AKT-mTOR, Pten-AKT-BAD, and Pten-AKT-GSK3B signaling pathways. But, deletion of *Wdr77* gene did not affect the activation of these pathways (Supplementary Fig. 3a, lane 4 vs. lane 3) and induce cell apoptosis (Supplementary Fig. 3b and 3c), suggesting that *Wdr77* did not affect Pten-AKT-mTOR, Pten-AKT-BAD, and Pten-AKT-GSK3B signaling pathways. However, we found that *Pten* loss induced E2F3 expression and Rb phosphorylation in prostate epithelial cells. As the consequence, cellular proliferation was significantly enhanced. Deletion of *Wdr77* abolished prostate tumor initiation induced by *Pten* loss, correlated with decreased E2F3 expression but not Rb phosphorylation. This finding suggests that Pten-AKT-E2F signaling axis may be required for prostate tumor initiation induced by *Pten* loss.

We observed that *Pten* loss significantly enhanced BrdU-positive (proliferative) epithelial cell populations and induced E2F3 expression and Rb inactivation (phosphorylation), consistent with the fact that *Pten* gene loss induces cellular proliferation [17, 22, 25]. Loss of *Wdr77* significantly reduced cellular proliferation induced by *Pten* loss. This result is consistent with our previous observations that *Wdr77* plays an essential role in proliferation of prostate epithelial [23] and cancer cells [21]. *Wdr77* deletion reduced E2F3 expression induced by *Pten* loss, suggesting that *Wdr77* is necessary for the maintenance of E2F3 protein levels, which is required for cell cycle progression from G1 to S phase. The mechanism of how *Wdr77* regulates E2F3 expression is currently under investigation. *Wdr77* also targets several growth factors, as well as growth inhibitors [13]. Whether expression of these factors by *Wdr77* contributes to prostate tumorigenesis induced by *Pten* loss in vivo is also under investigation. It is most likely that *Wdr77* is not only required for the tumor onset induced by *Pten* loss, but also required for tumor initiation by other oncogenic factors because of the critical role of *Wdr77* in cellular proliferation. Results supporting this statement include i) a general *Wdr77* requirement for

cellular proliferation of multiple tested cancer cell lines (PC3, DU145, LNCaP, A549, PC14, PANC1, ASPC1, mDA MD231, HCT116, and WM2664) independent on the *Pten* status; ii) an essential role of *Wdr77* for growth (proliferation) of mouse tumor xenografts of PC3, LNCaP, A549, and PC13; iii) a requirement of *Wdr77* for tumor formation of prostate epithelial cells transformed by large T antigen and Ras (G12V) in the nude mouse.

Most normal adult cells are fully differentiated and generally quiescent. TGF β acts as a key physiological factor that ensures the maintenance of cell quiescence [38, 39]. Tumorigenesis is involved in the loss of cellular sensitivity to TGF β signaling [40]. Our analysis of the TCGA prostate cancer dataset across 498 prostate cancer patients and 52 normal prostate tissues supports this conclusion. Down-regulation of the TGF β signaling was observed in prostate cancer patients when compared to the normal prostate tissues. Similarly, the TGF β signaling is also down-regulated in the *Pten* knockout prostate. In this study, we observed inactivation of the TGF β signaling (loss of p-SMAD3 immunostaining) in prostate epithelial cells following loss of *Pten*, which was blocked by loss of *Wdr77*. In the previous study, we described that *Wdr77* abrogated TGF β growth suppression in proliferating cells [14]. These results suggest that *Wdr77* cytoplasmic translocation induced by loss of *Pten* is not only required for cellular proliferation but may also play an important role to block the anti-proliferative effects of the TGF β signaling, which is essential for prostate tumorigenesis.

It was reported that the loss of the *Pten* gene resulted in up-regulation of AR expression [41]. We observed the same result in *Pten*^{pc-/-} and *Pten*^{pc-/-};*Wdr77*^{pc-/-} mice (Supplementary Fig. 6, panels B–D vs. panel A). Although AR protein levels were slightly increased, the androgen signaling (reflected by expression of androgen hallmark genes) was down-regulated in the *Pten*-null mouse prostate (Supplementary Fig. 7). We previously reported that *Wdr77* loss selectively affected expression of a set of AR-target genes in the mouse prostate [10] and in prostate cancer cells [9]. We previously observed that *Wdr77* regulates cellular proliferation is independent on the AR signaling [11, 12, 42]. We obtained the same result that *Wdr77* regulated proliferation of mouse epithelial cells in which AR protein is not expressed. How the AR signaling altered by *Pten* loss or/and *Wdr77* subcellular transportation may contribute to prostate tumorigenesis requires additional studies.

This study suggests that *Wdr77* expression and its cytoplasmic translocation are required for cellular proliferation by maintaining E2F3 expression and inactivating the TGF β signaling, which represents a necessary step in prostate tumor initiation.

Materials and methods

Prostate-specific deletion of *Pten* and *Wdr77* genes in the mouse

Pten^{loxP/loxP} mice (C;129S4-*Pten*^{tm1Hwu}; Stock Number 006440) were purchased from Jackson Laboratory and *Wdr77*^{loxP/loxP} mice were generated as previously described by us [10]. The *Pten*^{loxP/loxP} mouse was crossed with the *Wdr77*^{loxP/loxP} mouse to generate *Pten*^{loxP/loxP};*Wdr77*^{loxP/loxP} mouse. *Pten*^{loxP/loxP} or *Pten*^{loxP/loxP};*Wdr77*^{loxP/loxP} mouse was then crossed with *PRR2Bi-Cre* mouse [43] to generate mice that were prostate-specific deletion of *Pten* gene (*Pten*^{loxP/loxP};*Cre* or *Pten*^{pc-/-}) or both *Pten* and *Wdr77* genes (*Pten*^{loxP/loxP};*Wdr77*^{loxP/loxP};*Cre* or *Pten*^{pc-/-};*Wdr77*^{pc-/-}). For gene typing, the genomic DNA was isolated from tail or prostate gland of mouse at the age of 21 day old and subjected to PCR analysis with primers as described [10, 18]. Prostate glands derived from five to seven animals per genotype were used for analysis. No randomization and blinding were used. Mice were handled in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Morehouse College School of Medicine's Institutional Animal Care and Use Committee approved all the experimental procedures used for mice in this study.

Antibodies

Antibodies against Pten (D4.3), Ki-67 (D3B5), Akt (pan C67E7), p-AKT (Ser473), and p-S6 ribosomal protein (Ser235/236) were obtained from Cell Signaling Technology. Antibodies against AR (N-20), E2F3 (N-20), and pSMAD3 (Ser208) were obtained from Santa Cruz Biotechnology. The BrdU antibody was obtained from BD Biosciences. The antigen purified anti-*Wdr77* antibody was described previously [8].

Immunohistochemistry

The prostate gland was dissected from *WT*, *Pten*^{pc-/-} or *Pten*^{pc-/-};*Wdr77*^{pc-/-} mouse at the age of 21, 60, or 120 days and fixed with 10% formalin overnight at 4 °C. The fixed prostate gland was embedded in paraffin and sectioned (5 μm). Prostate sections were subjected to washes in xylene, dehydration in graded ethanol (70–100%), and then washed in phosphate buffered saline (PBS). Following dehydration, the sections were subjected to the citrate antigen retrieval followed by a 12 min treatment with 3% H₂O₂ in PBS for endogenous peroxidase blocking. To reduce nonspecific binding of the primary antibody, the sections were blocked with 4% fish gelatin for

30 min. The prostate sections were incubated with the primary antibody overnight at 4 °C and then with a secondary peroxidase-labeled antibody (Biocare Medical) for 30 min, washed with PBS and incubated with 4+ Streptavidin-HRP (Biocare Medical) for 30 min. The sections were then washed with PBS and the signal was visualized by application of 3,3'-Diaminobenzidine (DAB) (Vector Laboratories).

Cell culture and cell growth assay

Mouse prostate epithelial cells were isolated from prostate glands of *Pten*^{loxP/loxP} and *Pten*^{loxP/loxP};*Wdr77*^{loxP/loxP} mice as described previously by us [23] and cultured in Keratinocyte Serum Free Medium (KSFM) (Gibco) supplemented with 2% Fetal Bovine Serum plus 1% Pen-Strep, epidermal growth factor (0.1 ng/mL), and bovine pituitary extract (23 μg/mL). The *Pten* gene or both *Pten* and *Wdr77* genes were deleted by infection of epithelial cells with Ad5-CMV-Cre-GFP (15 particles per cell; Vector Development Laboratory, Baylor College of Medicine) as described [23]. The control cells were infected with Ad5-CMV-GFP at the same time with the same viral particles per cell. Cells were harvested at 7 days post adenovirus infection for analysis. For cell growth assay, cells (5 × 10⁵ cells per well) were plated in a 24 well culture plate in triplicate and counted every day for 4 days. For BrdU incorporation assay, cells were plated in a chamber slide (8-well, 1.0 × 10⁴ cells/per well) and grown overnight. BrdU was added to the medium at the final concentration of 10 μM. Cells were incubated for 2 h and immunostained with anti-BrdU antibody (BD Biosciences) according to a previously published protocol [23].

Western blot analysis

Whole cell lysates were obtained using the Passive Lysis Buffer (Promega) supplemented with Protease and Phosphatase inhibitors (Fischer Scientific). Protein concentration was determined using the Bradford Assay (Bio-Rad) with BSA as the standard. Following SDS-PAGE, protein was transferred to a nitrocellulose membrane (NC) at 6 V overnight. NC membrane was blocked for 30 min in 3% milk in TBST (Tris buffered saline with 0.05% Tween-20). Thereafter, the membranes were incubated in primary antibody in 2% BSA-TBST, for 2 h. NC membranes were then washed 3 × with TBST in 5 min intervals, followed by incubation in secondary antibody labeled with horseradish peroxidase (HRP) in 2% BSA-TBST, for 1.5 h. NC membranes were then washed 4 × 5 min with TBST. Protein was then detected using an enhanced chemiluminescent substrate (Western Lightning-Plus ECL, Perkin Elmer).

Cell cycle analysis

Cells were trypsinized, washed with PBS and fixed with cold ethanol (70%) for overnight at 4 °C. Cells were pelleted by centrifugation at 2000 r.p.m. for 5 min and washed with PBS. Cells were stained with propidium iodide (PI) and submitted to cell cycle analysis on the Accuri C6 Flow Cytometer (BD Biosciences). Data were analyzed using FloJo software (FloJo).

Real-time PCR

Total RNA was isolated from cells using the TRIzol reagent (ThermoFisher). cDNA was generated using the Superscript III First-Strand Synthesis System (ThermoFisher). Real-time PCR was performed with Go-Taq qPCR master mix (Promega) and specific primers (Supplementary Table 1) (40 cycles of 15 s at 95 °C and 20 s at 60 °C).

Statistical analysis

Data are presented as the means of three or more independent experiments \pm the standard deviation. A 2-tailed unpaired student *t*-test was used to determine whether differences between control and experiment samples were statistically significant. *P* values less than 0.05 were considered statistically significant.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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