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14. ABSTRACT

The over-proliferation of prostate epithelial cell is the first critical step that leads to prostate tumorigenesis. We demonstrated that p44 cytoplasm localization associates with this growth process and is essential for growth of prostate epithelial and prostate cancer cells. The proposed research tested the feasibility to target this growth process for prostate cancer treatment. We report that inhibition of p44 cytoplasm translocation by identified compounds suppressed growth and progression of prostate tumors in both tumor xenografts and Pten gen knockout mouse model. These results provided a new paradigm and a new strategy for treatment of prostate cancer. In addition, we also found that the posttranslational modifications of p44 regulated its subcellular localization during rpostate tumorigenesis.

15. SUBJECT TERMS
androgen receptor cofactor p44, cyroplasm transport, prostate cancer

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1. **INTRODUCTION:** We identified a 44-kDa protein (p44) as a novel androgen receptor (AR)-interacting protein. P44 localizes in the cytoplasm of epithelial cells at the early stage of prostate development and is essential for growth of prostate epithelial cells. In contrast, p44 in the nucleus in adult prostate inhibits proliferation and promotes differentiation of epithelial cells. In addition, p44 translocation from the nucleus into the cytoplasm is an essential step for initiating proliferation of epithelial cell in the aged prostate gland and in prostate cancer. Therefore, p44 nuclear export is a novel target site for the age-related growth of prostate epithelial cells and prostate cancer. By screening a small chemical compound library, we identified 6 compounds that specifically inhibit p44 nuclear export. The objective of the proposed research proposed research is test whether the identified compounds have the potential to serve as agents for treatment of prostate cancer.
2. **KEYWORDS:** *androgen receptor cofactor p44, cytoplasm transport, prostate cancer*.
3. **ACCOMPLISHMENTS:**
    - **What were the major goals of the project?**

        - ***Task 1: To investigate whether inhibition of p44 cytoplasm translocation by identified compounds suppresses growth and progression of prostate tumors. (months 1-36).***
          Task 1.1: To evaluate the toxicity of the identified compounds (months 1-6).
          Task 1.2: To investigate whether inhibition of p44 cytoplasm localization inhibits growth of LNCaP tumor xenografts (months 6-14).
          Task 1.3: To investigate whether inhibition of p44 cytoplasm localization suppresses the hormone-refractory progression and growth of prostate tumors (months 15-24).
          Task 1.4: To investigate whether inhibition of p44 cytoplasm localization suppresses growth of the androgen-refractory prostate tumors (months 25-36).
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        - ***Task 3: To determines the signals that control p44 subcellular localization (months 1-36).***
          Task2.1: To investigate nuclear transport signals in the control of p44 subcellular localization (months 1-12).
          Task 2.2: To investigate how the p44-associated proteins affect p44 subcellular localization (months 12-36).
          Task 2.2: To investigate how identified compounds affect the p44 nuclear transport signals (months 24-36).

    - **What was accomplished under these goals?**

        - ***Task 1: To investigate whether inhibition of p44 cytoplasm translocation by identified compounds suppresses growth and progression of prostate tumors. (months 1-36).***
          The task 1.1 was finished in the first year (09/01/2012 - 8/31/2013). See the annual report (09/10/2013).
          The task 1.2 was finished in the first year (09/01/2012 - 8/31/2013). See the annual report (09/10/2013).
          The task 1.3 was finished in the second year (09/1/01/2013 - 8/31/2014). See the annual report (11/24/2014).

The Task 1.4 was finished in the sencod year (09/01/2013 - 8/31/2014) but has not been reported due to the institution transfer of the PI. The results were reported here. LNCaP cells (1 x 10^6) are injected into the back of male nude mice (n=30). When the tumors reach the size of 6 mm in diameter (30 days after tumor cell injection), the mice are castrated. When tumor sizes reach 9 mm, the mice are randomized for the treatment with 2 chemical compounds (04G11 and 41G8) 30 days after castration. The control mice (n=10) were treated by administration of the diluent by I.P. twice weekly and treated mice (n=20, 10 for each chemical compound) were treated by administration of 04G11 and 41G8 compounds in the diluent by I.P. twice weekly. The treatment will last for 1 month (total 8 injections of each compound). The tumor sizes were measured with a caliper. The treatment with both compounds significantly decreased the tumor sizes (by 2-fold after treated for 30 days) in the castrated mice (Figure 1). After treatment, mice are sacrificed and tumors are excised and weighed. The tumor weigts from the treated mice are about 2.5-fold lower than that from the control mice (Figure 2). These results suggested that chemical compounds inhibited growth of androgen-refractory prostate tumor.

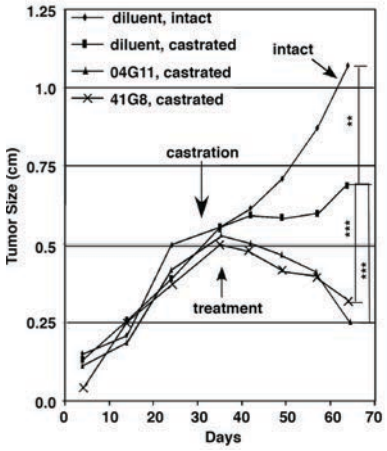


Fig. 1. The tumor sizes in intact mice, castrated mice, or castrated mice treated with the diluent or compounds (04G11 or 41G8).

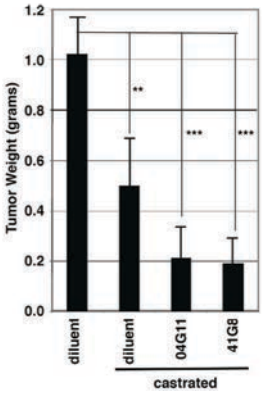


Fig. 2. The tumor weights in intact mice, castrated mice, or castrated mice treated with diluent or compounds (04G11 or 41G8).

- ***Task 2: To investigate whether identified compounds inhibit prostate tumors initiated by the Pten gene deletion (months 13-36).***

The task 2.1 was finished in the second year (09/1/01/2013 - 8/31/2014). See the annual report (11/24/2014).

The task 2.2 was finished in the second year (09/1/01/2013 - 8/31/2014). See the annual report (11/24/2014).

The task 2.3 was finished in the current year (09/01/2018 - 08/31/2019). We performed to evaluate several biomarkers to characterize the efficacy of two compounds for prostate cancer treatment. The Ki67 immunostaining was used to determine cellular proliferation (Figure 3A, top two panels). The compound 04G11 or 41G8 treatment significantly decreased cellular proliferation (the Ki67-positive cells) (Figur 3B), indicating in the decreased proliferating rate of hyperplasia and tumor proliferation associated with p44 nuclear localization. In contrast, cell apoptosis was not affected by the treatment with these two compounds (Figure 3A, bottom panel; Figure 3B). Expression of p21 and p27 was increased but expression of CDK2 and cyclin A was decreased in the treated mouse prostate (Figure 3C). These observations are consistent with the published results that p44 nuclear location up-regulated p21 expression and down-regulated CDK2 and cyclin A expression to inhibit cell proliferation (38, 53). The proliferating epithelial cells in hyperplasia lesion are not quiescent and

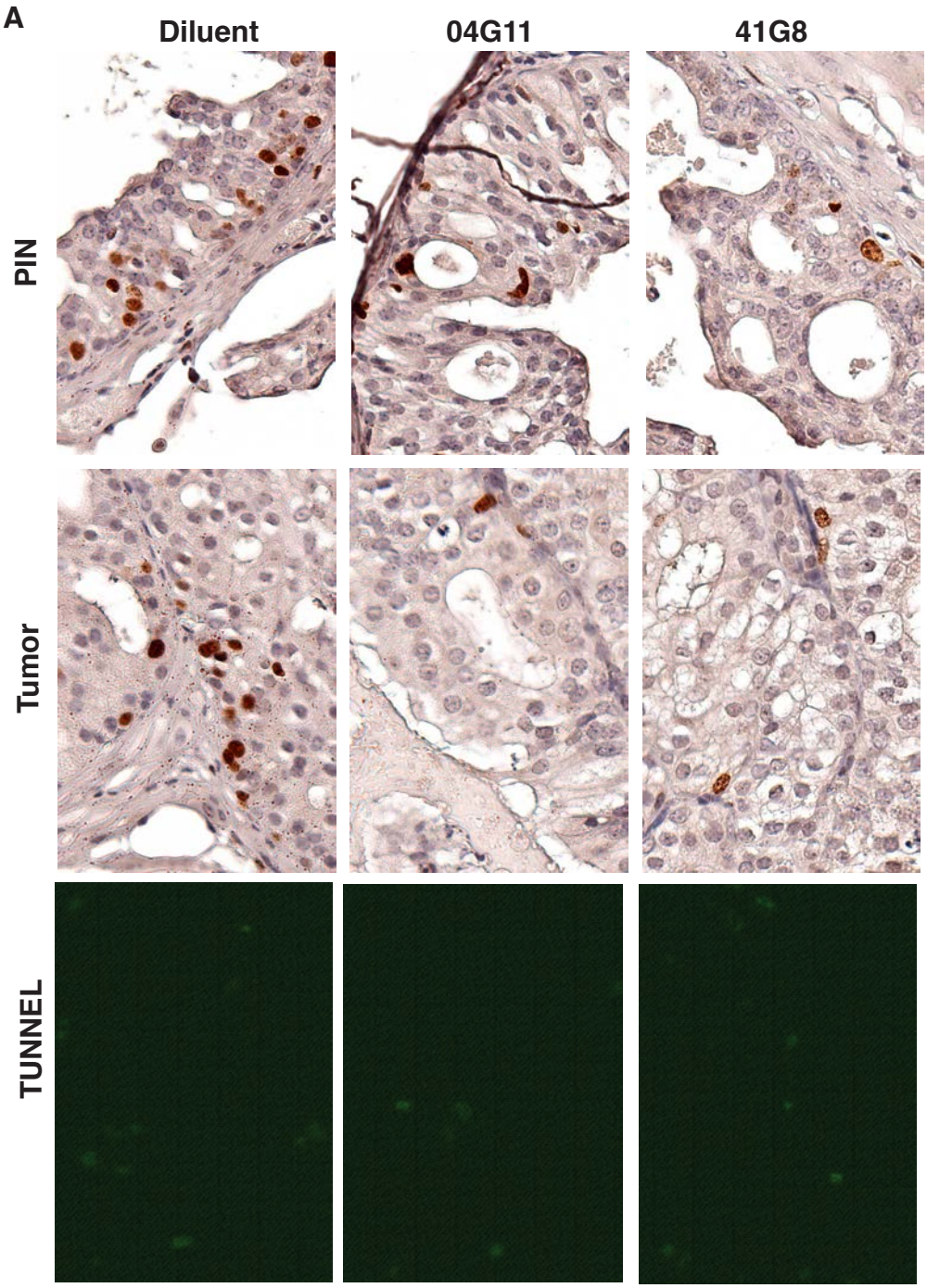
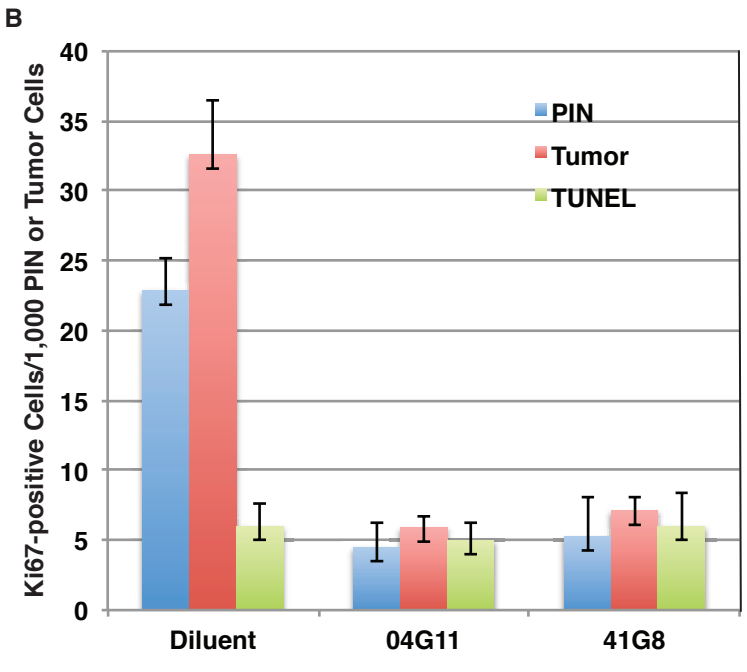
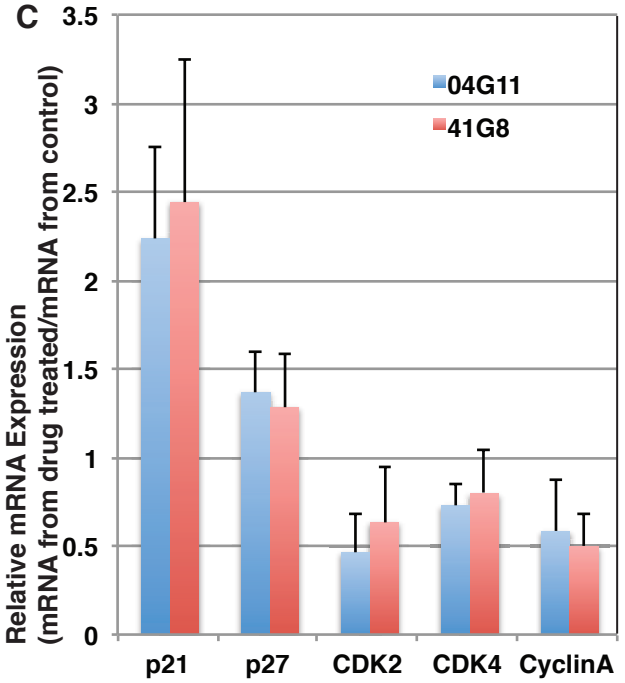


Fig. 3. A, Ki67 immunostating (top two panels) of PIN and tumor regions of prostate tissues derived from Pten KO mice treated with the diluent or compound (04G11 or 41G8). The Ki67-positive (proliferating) cells were stained in brown. TUNEL assay (bottom panel). The apoptotic cells were stained in green color.
B, The diagram shows the KI67- or TUNEL-positive cells per 1,000 epithelial cells in the samples of top two panels.
C, RT-PCR analysis of expression of genes as indicated. Relative expression = mRNA levels in drug treated sample/ mRNA levels in diluent treated samples.





posses a phenotype of intermediate differentiation (7, 10, 35, 48, 49). The treatment also enhanced differentiation (CK18 expression) of epithelial cells in the hyperplasia lesion. This observation is consistent with the fact that the nuclear p44 promotes differentiation of prostate epithelial cells (19).

- ***Task 3: To determines the signals that control p44 subcellular localization (months 1-36).***
  The task3.1 was finished in the first year (09/021/2012 - 8/31/2013). See the annual report (09/10/2013). The alternative approach was reported in the annual report (09/24/2018).
  The task 3.2 was finished in the second year (09/1/021/2013 - 8/31/2014).
  The task 3.3 was finished in the third year (09/1/021/2017 - 8/31/2018). See the annual report (09/24/2018).

- *1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

- **What opportunities for training and professional development has the project provided?**
  - *Nothing to Report.*
- **How were the results disseminated to communities of interest?**
  - Nothing to Report.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *Nothing to Report.*

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
   - **What was the impact on the development of the principal discipline(s) of the project?**
     - The age-related growth of prostate is the first critical step that leads to the abnormal proliferation and prostate cancer. However, very little is known about what regulates this age-related growth process and how this growth process is related to prostate cancer. Our studies demonstrated that p44 in the nucleus inhibits proliferation and promotes differentiation of prostate epithelial cells and that p44 translocation from the nucleus into the cytoplasm is an essential step for initiating proliferation of epithelial cell and prostate cancer cells. Thus, the proposed research has direct impact on prostate tumor biology.
     - The p44 nuclear export is a novel target site for growth of prostate cancer. By screening a small chemical compound library, we identified 6 compounds that specifically inhibit the p44 nuclear export. The proposed research demonstrated that two identified compounds inhibited prostate tumor growth in xenograft and Pten gene knock out mouse models. Thus, they have the potential to serve as agents for treatment of prostate cancer. Therefore, the proposed research has direct impact on therapy of prostate cancer.
     - Prostate cancer is dependent on androgens for growth and survival and therefore, androgen ablation has been the standard therapy for metastatic prostate cancer. This treatment rarely cures cancer because the initial response is almost always followed by a relapse to an un-responsive, hormone-refractory stage. Thus, there is a great need to identify novel targets and develop compounds to inhibit these targets for treatment of advanced prostate cancer. The proposed research showed that two identified compounds also inhibited

growth of castration resistant prostate cancer. Thus, this study has great impact on developing effective treatments for advanced prostate cancer.

- **What was the impact on other disciplines?**
  - *Nothing to Report.*
- **What was the impact on technology transfer?**
  - The patern will be applied for the identified compounds as potential therapeutic agents for prostate cancer.
- **What was the impact on society beyond science and technology?**
  - *Nothing to Report.*

5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
   - Nothing to Report.
6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*
   - **Publications, conference papers, and presentations**
     Report only the major publication(s) resulting from the work under this award.
     - **Journal publications.** Wang Z. The essential role of WD repeat domain 77 in prostate tumor initiation induced by Pten loss. *Oncogene*. 2018 Jul;37(30):4151-4163. doi: 10.1038/s41388-018-0254-8. Epub 2018 Apr 30. PMID: 29706654.
   - **Inventions, patent applications, and/or licenses**
     In the preparation for a patent application.
7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**
     - *Zhengxin Wang, Guangchang Zhou, Deon O'Bryant, Vanessa Adams*
     - *no change.*
   - **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
     - *Nothing to Report.*
   - **What other organizations were involved as partners?**
     - *Nothing to Report.*
8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** *Nothing to Report*
   - **QUAD CHARTS:** *Nothing to Report*
9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

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

Deon O'Bryant and Zhengxin Wang*

The Center for Cancer Research and Therapeutic Development, Department of Biological Sciences, Clark Atlanta University, Atlanta, GA 30314, USA.

Running Title: The essential role of Wdr77 in prostate cancer initiation

Keywords: WD repeat domain 77, Pten, E2F transcriptional factors, TGFβ, prostate cancer, cancer initiation

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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 TGFβ signaling, leading to attenuated cellular proliferation induced by loss of *Pten*. These findings establish a critical role of Wdr77 for prostate tumor initiation.

Keywords: Wdr77; Pten; E2F; TGFβ; prostate cancer; cancer 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 methylosome 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 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 TGFβ 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 TGFβ signaling *in vivo*, suggesting that *Wdr77* plays a role in relieving growth suppression of the TGFβ 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 Figure 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 Figure 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 Figure 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 Figure 2a, panels B-E versus panel A; Supplementary Figure 2A, lanes 2 and 4 versus lanes 1 and 3), indicating that *Wdr77* gene deletion did not affact AKT activation induced by *Pten* loss. Loss of the *Wdr77* gene did not affect expression of the *Pten*

gene or reverse versa (Supplementary Figure 2b, lanes 2 and 4 versus 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 (Figure 1a). The tumor incidence was increased as mice aged (Figure 2b). Interestingly, examination of $Pten^{pc-/-};Wdr77^{pc-/-}$ mouse prostate revealed a few PIN lesions (PIN, circled) and small tumors (T, circled) (Figure 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 (Figure 2). Gland sizes are significant increased (Figure 2c) and epithelial enfoldings are lacking (Figure 2d) in the DLP prostate of the $Pten^{pc-/-}$ mouse due to tumor growth. Loss of the *Wdr77* gene decreased gland sizes (Figure 2c) as well as epithelial enfolgings (Figure 2d), consistent with the critical role of Wdr77 in growth and differentiation of prostate epithelial cells. But *Pten* gene deletion could not restore grand size and enfolding numbers decreased by *Wdr77* deletion (Figure 2c, 2d).

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 (Figure 3a). In contrast, Wdr77 was absent in the benign epithelial cells (Figure 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 (Figure 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 (Figure 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 locolized in the nucleus, it inhibited cell growth and promoted cellular differention (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*, $Wdr77^{f/f}$ $Wdr77^{pc-/-}$, $Pten^{pc-/-}$, and $Pten^{pc-/-};Wdr77^{pc-/-}$ prostate sections (Figure 4a). In *WT* prostate, about 4% of epithelial cells were Ki-67-positive (Figure 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 (Figure 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 (Figure 4b, left panel). However, the proliferation rate of 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 (Figure 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* (Figure 4c, lane 2) and *Pten* plus *Wdr77* genes (lanes 4 and 6). Of note, the efficiency of Ad-mediated gene deletion is between 95-100% (24). *Pten* deletion led to AKT phosphorylation (Supplementary Figure 3a, lane 2 versus lane 1) and enhanced growth of prostate epithelial cells (Figure 4d, left), consistent with previous reports (18, 25). However, deletion of both *Pten* and *Wdr77* genes also led to AKT phosphorylation (Supplementary Figure 3a, lane 4 versus lane 3) but inhibited growth of prostate epithelial cells (Figure 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 (Figure 4e). On the converse, *Pten*- and *Wdr77*-null cells exhibited a significant decrease in cell proliferation relative to that of control cells (Figure 4e). Thus, Wdr77 mediates cellular proliferation induced by the *Pten* gene deletion. The isolated epithelial cells lost AR expression (Figure 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 investiageted 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 GSK3β (Supplementary Figure 3a, lane 2) but loss of *Wdr77* did not afftect these phosphorylation events (lane 4), indicating that loss of *Wdr77* had no affect on Pten-AKT-GSK3β (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 Figure 3b) and Western blot analysis of PARP protein (Supplementary Figure 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 pRB-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 Figure 5, *WT* mice presented very low expression of E2F3 (Figure 5a, panel A; Figure 5b). However, *Pten* deletion resulted in strong nuclear expression of E2F3 (Figure 5a, panel B; Figure 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 (Figure 5a, panel C; Figure 5b). Of note, high levels of E2F3 are observed in the tumor region (Figure 5a, panel C, circled by red line; Figure 5b), where *Wdr77* is not deleted (Figure 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 (Figure 5a, panel D; Figure 5c), as opposed to the strong pRb signal exhibited in the *Pten*-null mouse prostate (Figure 5a, panel D; Figure 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 (Figure 5a, panel F; Figure 5c). Thus, loss of *Wdr77* did not affect Rb phosphorylation induced by *Pten* deletion.

TGFβ 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, TGFβ-mediated transcription, and TGFβ2 and TGFβ receptor type II (TβRII) expression were dramatically induced when Wdr77 expression was silenced (14). We further demonstrated that Wdr77 expression caused the non-sensitivity of proliferating cells to TGFβ 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 TGFβ were over-represented on the gene list, whose expression is altered in prostate cancer (Supplementary Figure 4a). The gene heatmap reports show the altered expression of TGFβ target genes in majority of prostate cancer patients (Supplementary Figure 4b). Thus, the TGFβ 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 TGFβ were over-represented on the gene list, whose expression is altered in *Pten* knockout prostate (Figure 6a). The gene heatmap

reports changes of TGFβ target genes in *Pten* knockout prostate versus WT prostate (Figure 6b). Among these genes, 23 genes (up-regulated by TGFβ in *WT* prostate) are down regulated and 5 genes (repressed by TGFβ in *WT* prostate) are up regulated in the *Pten-null* prostate. This analysis suggests that the TGFβ 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 (Figure 6c, Some are indicated by black arrows.), indicating that the TGFβ signaling is active. SMAD3 protein was diffusing into the cytoplasm in hyperplasia and tumor cells (Figure 6c, panels B and C), suggesting loss of the TGFβ signaling. Consistent with this observation, *Pten* loss led to significant down regulation of TGFβ2, TGFBRII and TGFBRIII expression in prostate epithelial cells (Supplementary Figure 5). Loss of *Wdr77* reactivated the TGFβ signaling (p-SMAD3 expression) inactivated by *Pten* loss *in vivo* (Figure 6c, panel D, circled) and restored TGFβ2 and TGFBRIII expression in *Pten*-null epithelial cells (Supplementary Figure 5). Our findings suggest that Wdr77 may also play an important role to block the anti-proliferative effects of the TGFβ signaling during prostate tumorigenesis.

DISCUSSION

Wdr77 regulates proliferation and differentiation of prostate epithelial cells during the development through its subcellular 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 (Figure 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-GSK3β 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 promotes 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), GSK3β and BAD (data not shown) proteins (Supplementary Figure 2A, lane 2 versus 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 Figure 3a, lane 4 versus lane 3) and induce cell apoptosis (Supplementary Figure 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 results 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 Wdr77requirment for cellular proliferation of mutiple 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. TGFβ 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 TGFβ 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 TGFβ signaling was observed in prostate cancer patients when compared to the normal prostate tissues. Similarly, the TGFβ signaling is also down-regulated in the *Pten* knockout prostate. In this study, we observed inactivation of the TGFβ 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 TGFβ 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 TGFβ 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 Figure 6, panels B-D versus 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 Figure 7). We previously reported that *Wdr77* loss selectively affected expression of a set of AR-target genes in the mouse prostate (42) and in prostate cancer cells (9). We previously observed that Wdr77 regulates cellular proliferation is independent on the AR signaling (12, 43, 44). 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 TGFβ 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 (42). 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 (45) 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 (18, 42). Prostate glands derived from five to seven aminals 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 and 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 ^{o}C. The fixed prostate gland was embedded in paraffin and sectioned (5μm). 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 minute treatment with 3% H_20_2 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 (5x 10^5 cells per well) were plated in a 24 well culture plate in triplicate and counted every day for four days. For BrdU incorporation assay, cells were plated in a chamber slide (8-well, $1.0x10^4$ cells/per well) and grown overnight. BrdU was added to the medium at the final concentration of 10 μM. Cells were incubated for 2 hrs 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 6V overnight. NC membrane was blocked for 30 minutes 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 hours. NC membranes were then washed 3x with TBST in five minute intervals, followed by incubation in secondary antibody labeled with horseradish peroxidase (HRP) in 2% BSA-TBST, for 1.5 hours. NC membranes were then washed 4x5 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 ^oC. Cells were pelleted by centrifugation at 2,000 rpm 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 ± 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure legends

Figure 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.

Figure 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.

Figure 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).

Figure 4. Loss of *Wdr77* inhibited cellular proliferation induced by *Pten* gene deletion. (**a**) The prostate tissue were derived from the *WT*, $Wdr77^{f/f}$, $Wdr77^{pc-/-}$, $Pten^{pc-/-}$, 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), $Wdr77^{f/f}$ (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 $Pten^{loxP/loP}$ and $Pten^{loxP/loxP};Wdr77^{loxP/loxP}$ mice and infected with adenovirus harboring GFP ($Pten^{f/f}$, 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: $Pten^{f/f}$; Right: $Pten^{f/f};Wdr77^{f/f}$) 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 hr and submitted for immunostaining for BrdU. (**f**) Western blot of whole cell lystates made from LNCaP, $Pten^{f/f}$, and $Pten^{f/f};Wdr77^{f/f}$ cells with anti-AR antibody and anti-actin antibodies.

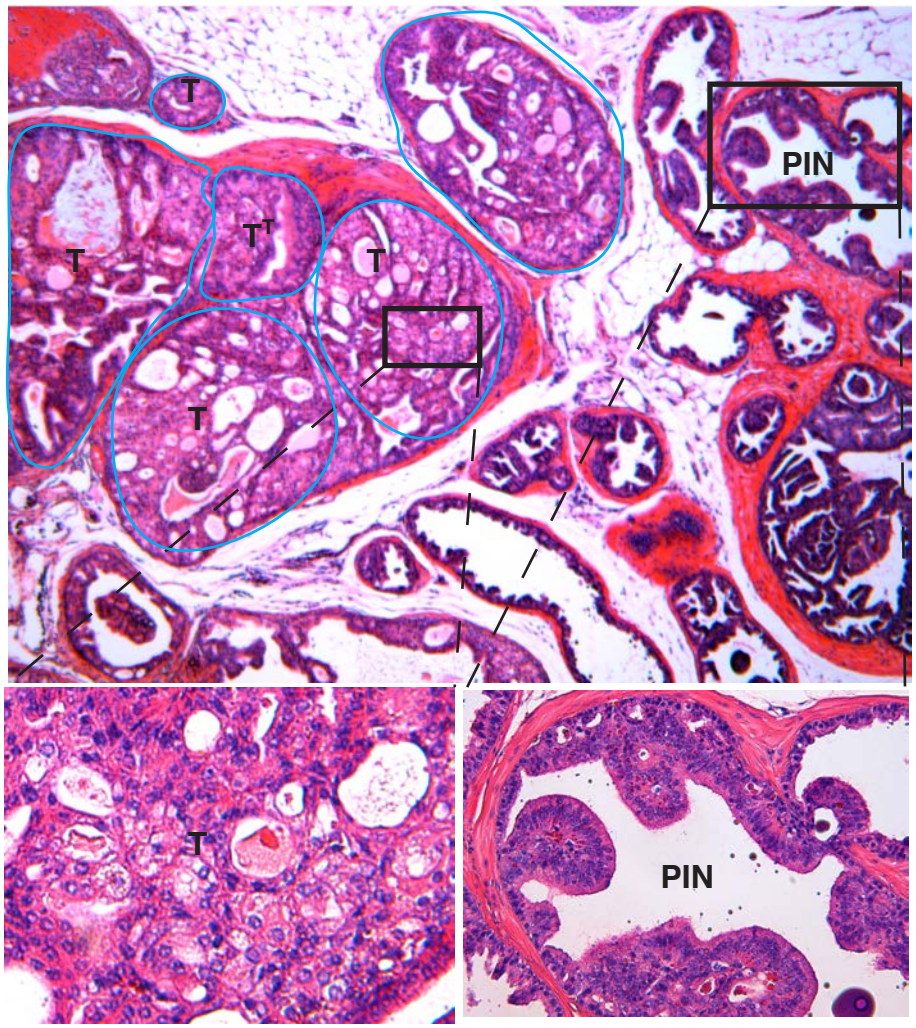
Figure 5. Loss of *Wdr77* blocked E2F3 expression induced by *Pten* gene deletion. (a) The prostate tissues were derived from the *WT*, $Pten^{pc-/-}$, and $Pten^{pc-/-};Wdr77^{pc-/-}$ mice at the age of 2 months and immunostained for E2F3 and phosporylated Rb (pRb). Quantalization of nuclear E2F3 (b)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

Figure 6. Loss of Wdr77 prevented inactivation of the TGFβ signaling. (**a**) GSEA enrichment plot indicates that genes regulated by TGFβ were over-represented on the gene list, whose

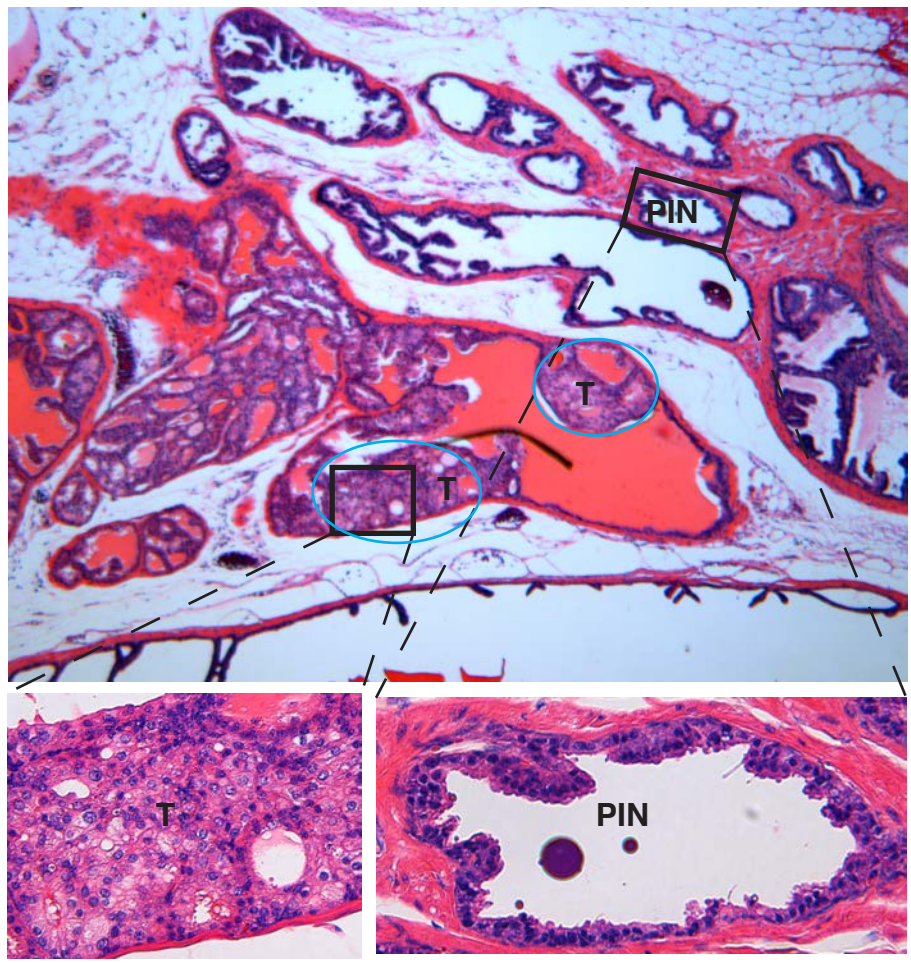
expression is regulated by *Pten* loss. (**b**) Genes targeted by TGFβ are visualized by heatmap. The red and blue colors represent higher than average and lower than average expression of particular gene in the *Pten*-null prostate, respectively. (**c**) Immunostaining of phosphorylated SMAD3 (pSMAD3) 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.

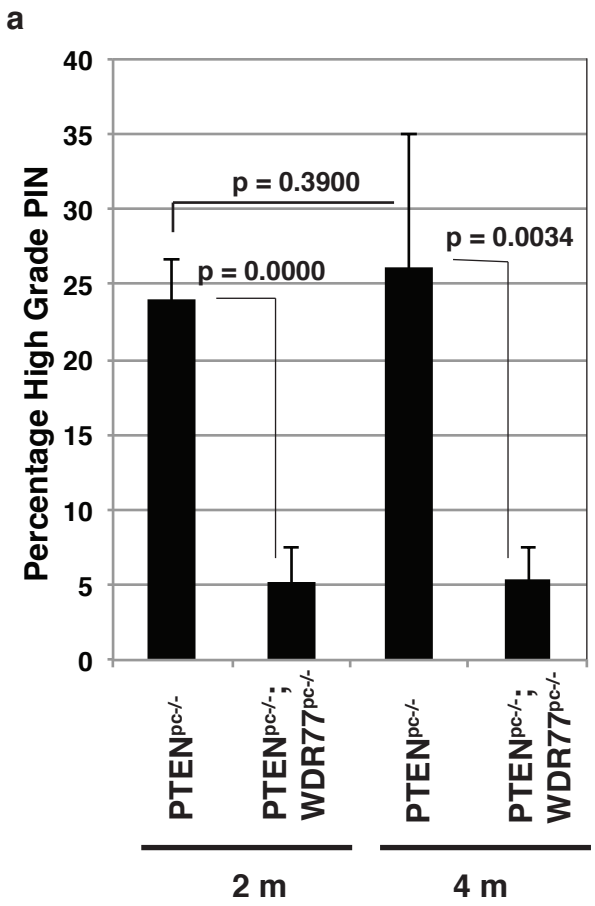
Table 1. Ages, genomic types and numbers of mice analyzed.

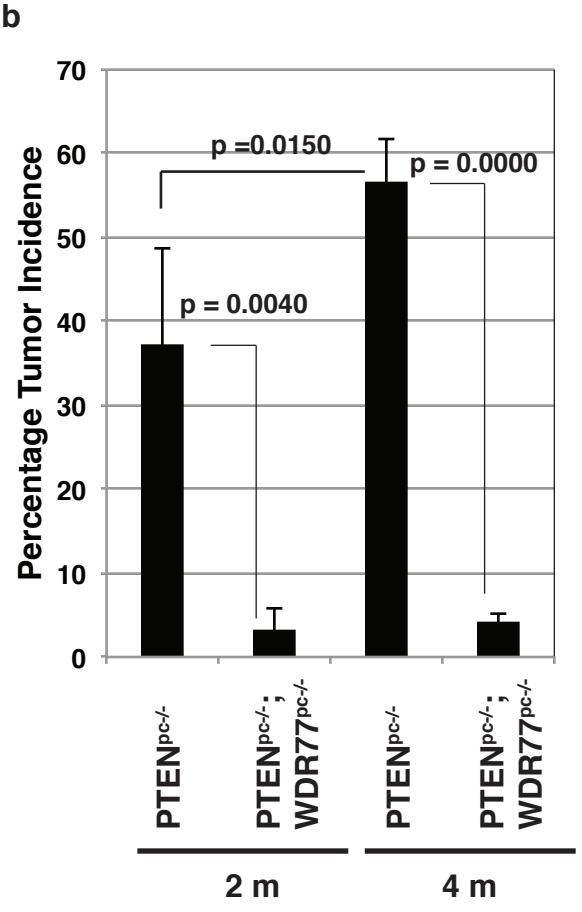
a

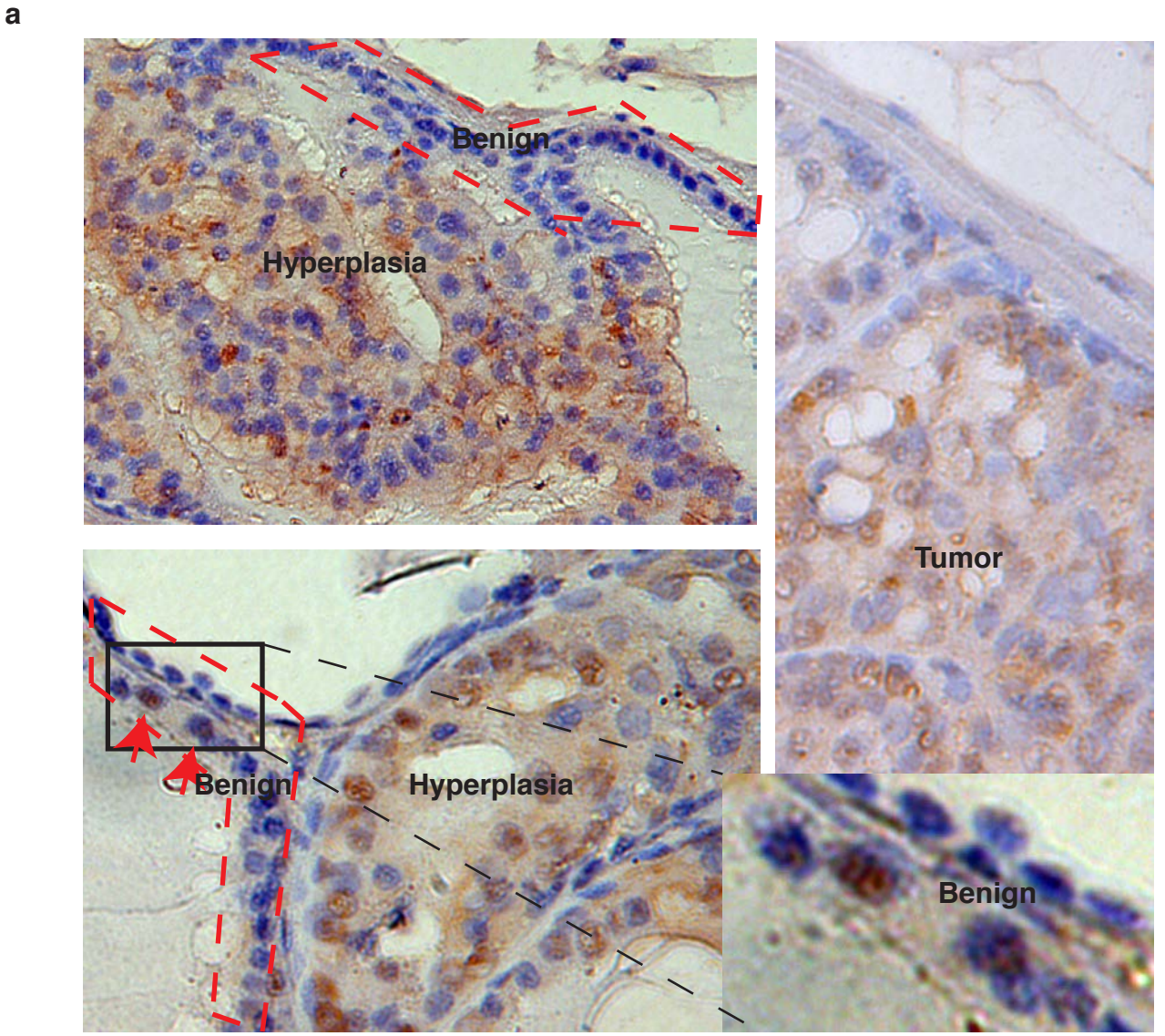


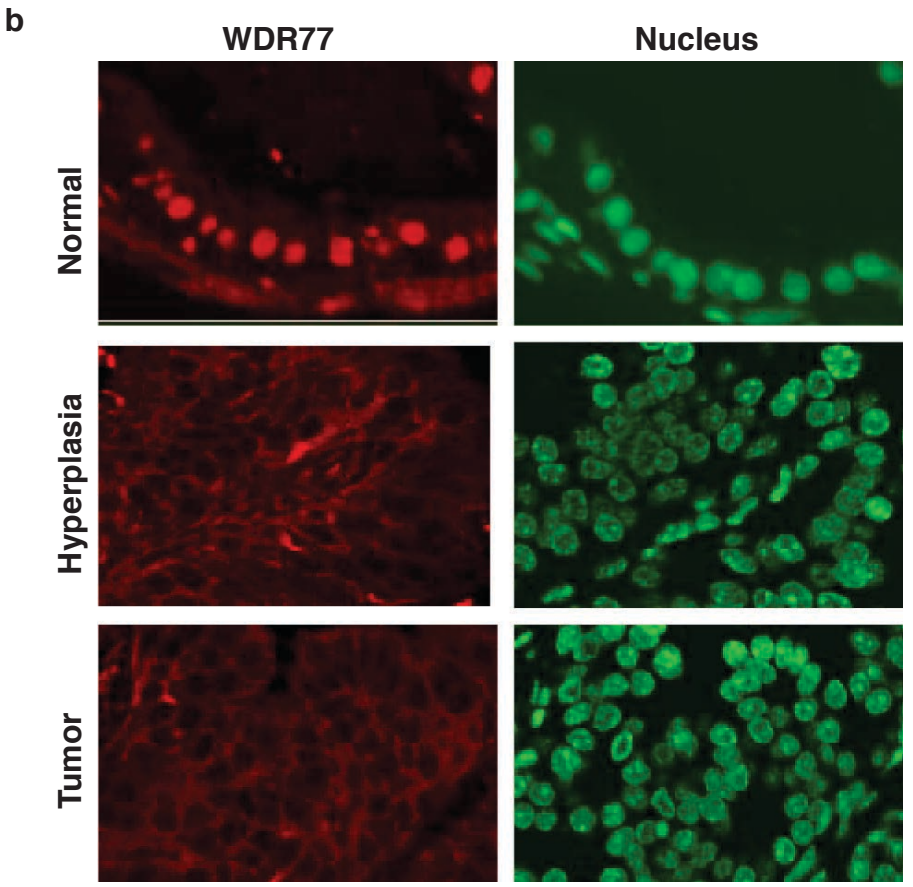
b

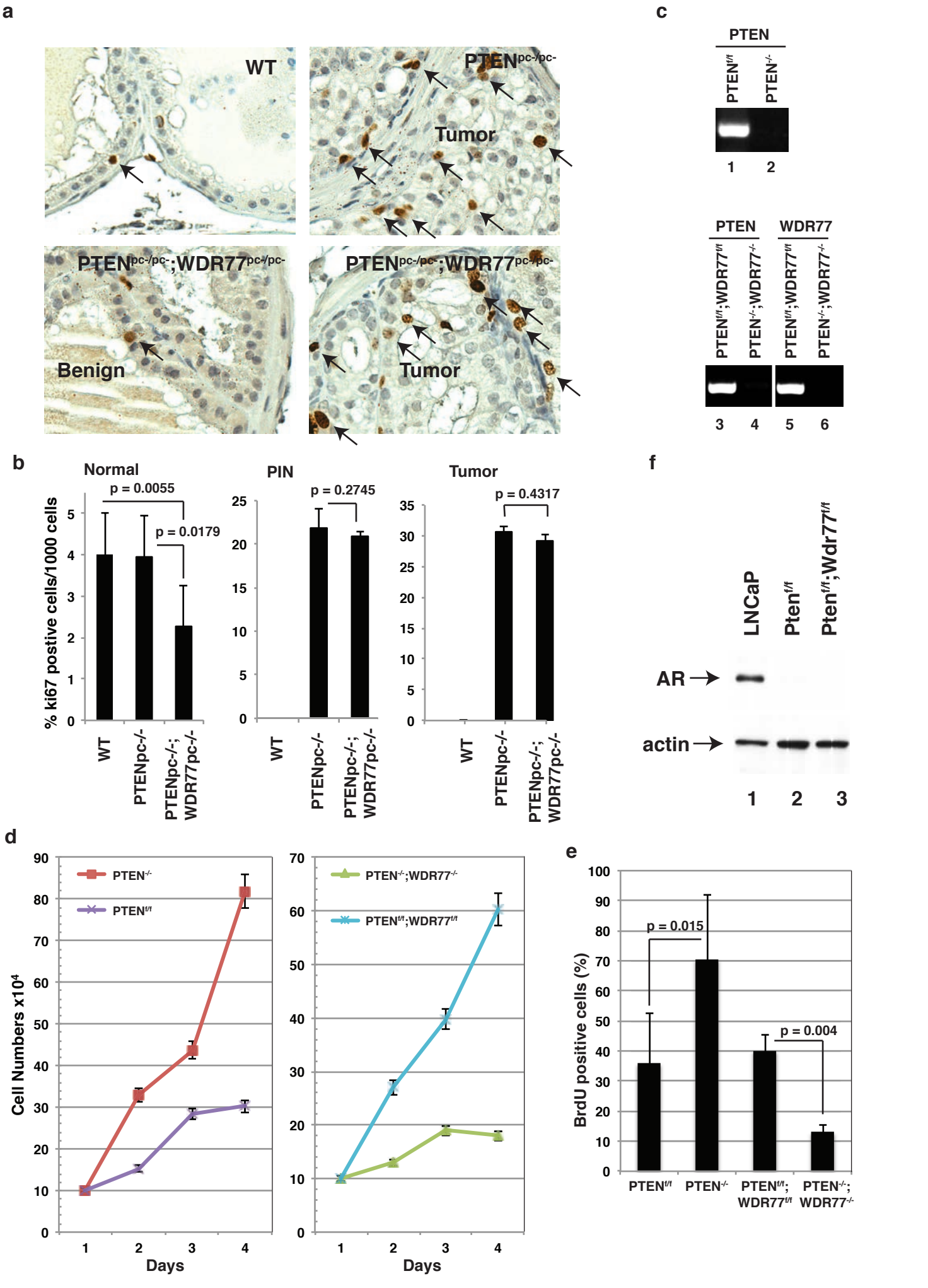


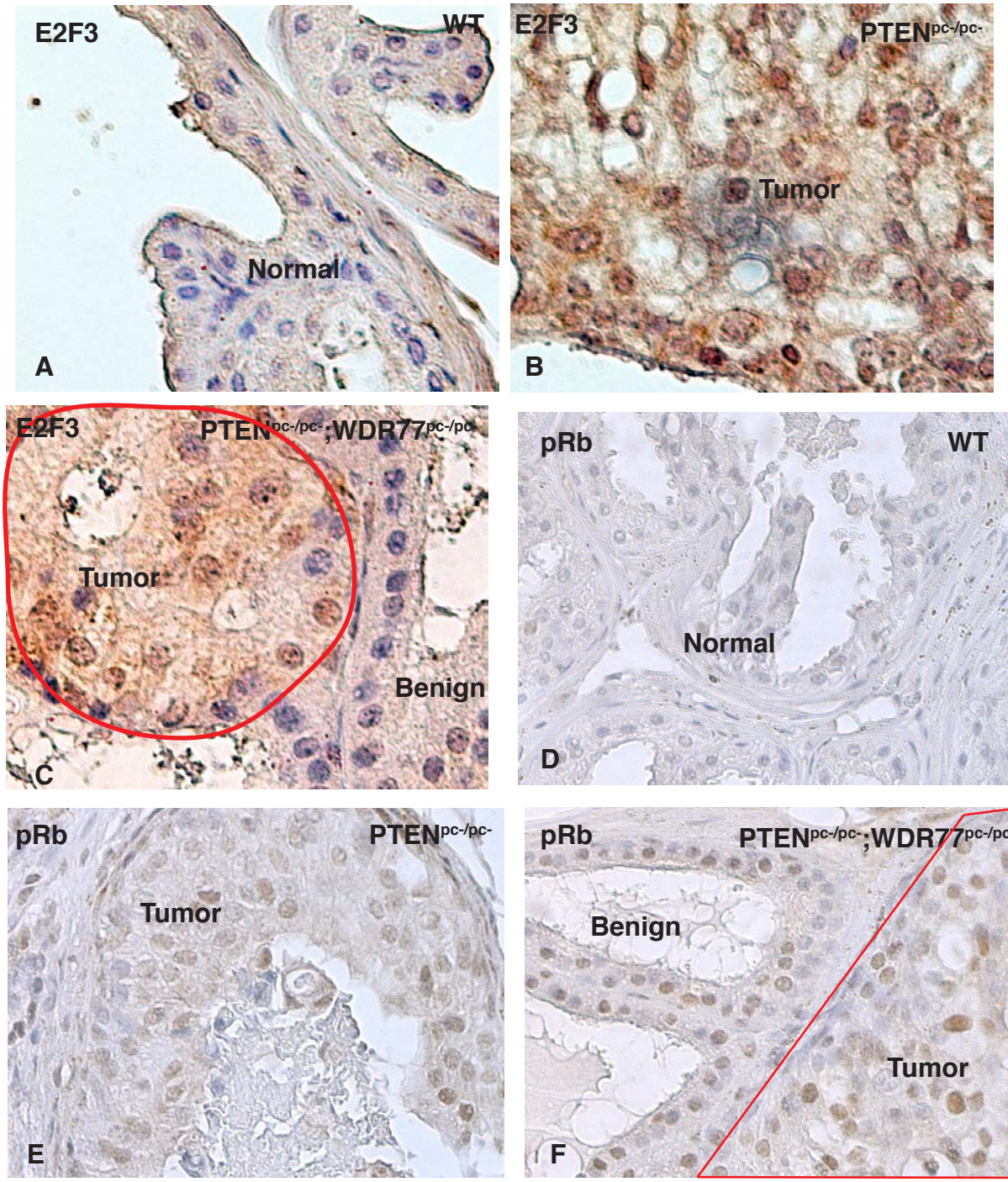

a
Percentage High Grade PIN
40
35
30
25
20
15
10
5
0
p = 0.3900
p = 0.0000
p = 0.0034
PTEN pc-/-
PTEN pc-/-; WDR77 pc-/-
PTEN pc-/-
PTEN pc-/-; WDR77 pc-/-
2 m
4 m

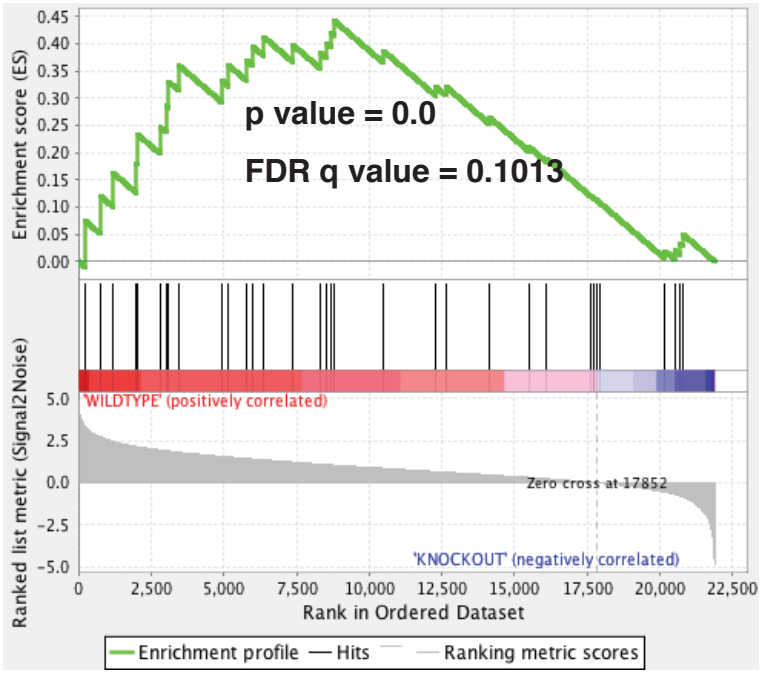

b
Percentage Tumor Incidence
70
60
50
40
30
20
10
0
p =0.0150
p = 0.0000
p = 0.0040
PTEN pc-/-
PTEN pc-/-; WDR77 pc-/-
PTEN pc-/-
PTEN pc-/-; WDR77 pc-/-
2 m
4 m

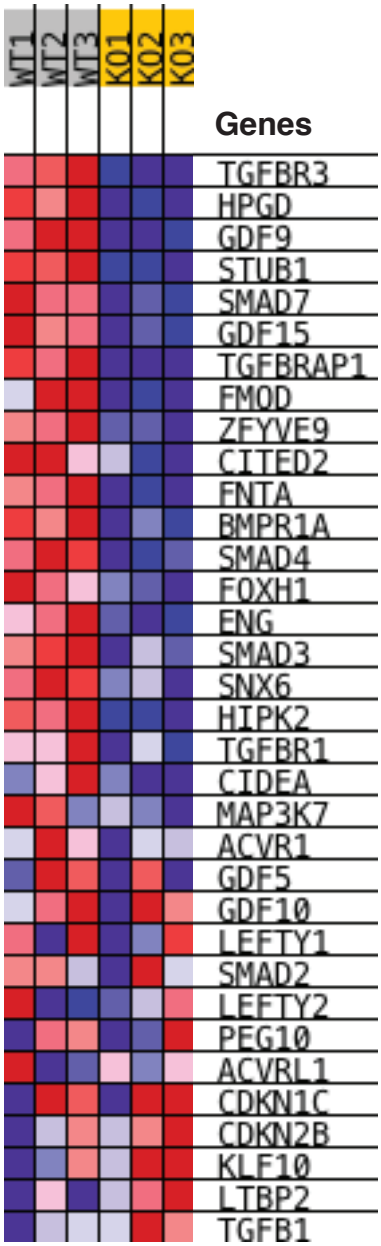

a
Benign
Hyperplasia
Tumor
Benign
Hyperplasia
Benign


b
WDR77
Nucleus
Normal
Hyperplasia
Tumor


a
WT
PTENpc-/pc-
Tumor
PTENpc-/pc-;WDR77pc-/pc-
Benign
PTENpc-/pc-;WDR77pc-/pc-
Tumor
c
PTEN
PTENf/f
PTEN-/-
1 2
PTEN
WDR77
PTENf/f;WDR77f/f
PTEN-/-;WDR77-/-
PTENf/f;WDR77f/f
PTEN-/-;WDR77-/-
3 4 5 6
b
Normal
PIN
Tumor
% ki67 postive cells/1000 cells
p = 0.0055
p = 0.0179
p = 0.2745
p = 0.4317
WT
PTENpc-/-
PTENpc-/-; WDR77pc-/-
f
LNCaP
Ptenf/f
Ptenf/f;Wdr77f/f
AR
actin
1 2 3
d
PTEN-/-
PTENf/f
PTEN-/-;WDR77-/-
PTENf/f;WDR77f/f
Cell Numbers x10^4
Days
e
BrdU positive cells (%)
p = 0.015
p = 0.004
PTENf/f
PTEN-/-
PTENf/f; WDR77f/f
PTEN-/-; WDR77-/-


E2F3
WT
Normal
A
E2F3
PTEN pc-/pc-
Tumor
B
E2F3
PTEN pc-/pc-;WDR77 pc-/pc-
Tumor
Benign
C
pRb
WT
Normal
D
pRb
PTEN pc-/pc-
Tumor
E
pRb
PTEN pc-/pc-;WDR77 pc-/pc-
Benign
Tumor
F


a
p value = 0.0
FDR q value = 0.1013
Enrichment score (ES)
'WILDTYPE' (positively correlated)
Zero cross at 17852
'KNOCKOUT' (negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


b
WT1
WT2
WT3
K01
K02
K03
Genes
TGFBR3
HPGD
GDF9
STUB1
SMAD7
GDF15
TGFBRAP1
FMOD
ZFYVE9
CITED2
FNTA
BMPR1A
SMAD4
FOXH1
ENG
SMAD3
SNX6
HIPK2
TGFBR1
CIDEA
MAP3K7
ACVR1
GDF5
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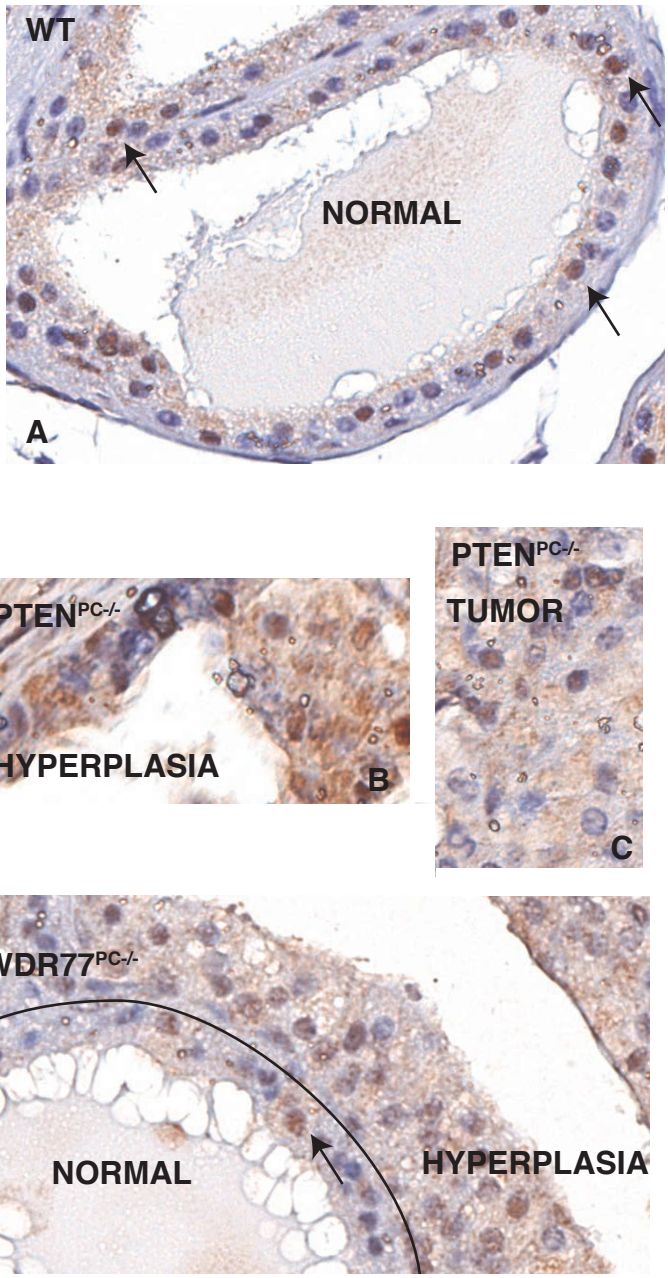
CDKN1C
CDKN2B
KLF10
LTBP2
TGFB1


c
WT
NORMAL
A
PTEN PC-/-
HYPERPLASIA
B
PTEN PC-/-
TUMOR
C
PTEN PC-/-;WDR77 PC-/-
NORMAL
HYPERPLASIA
D

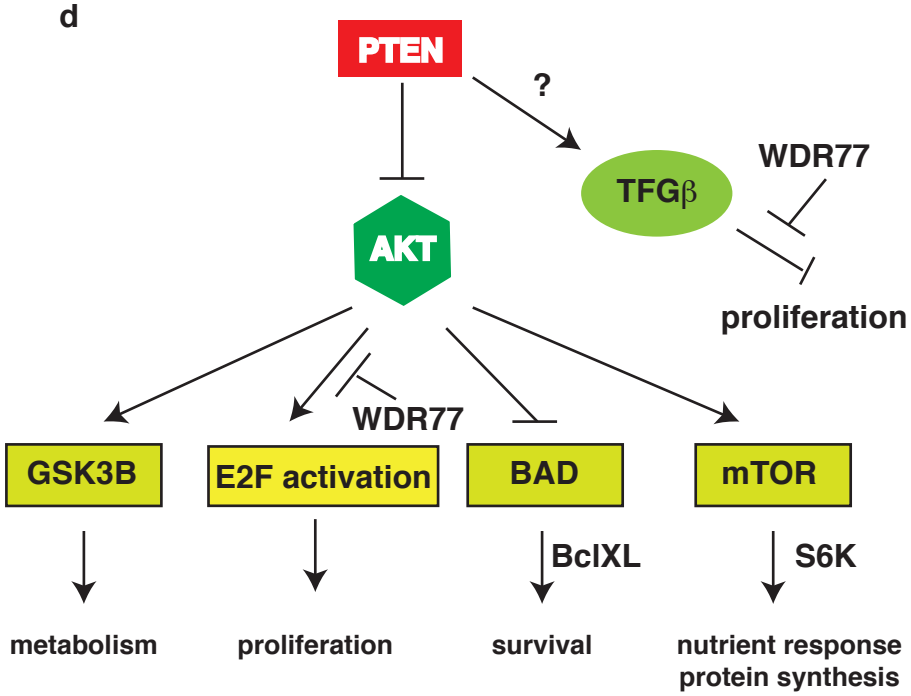
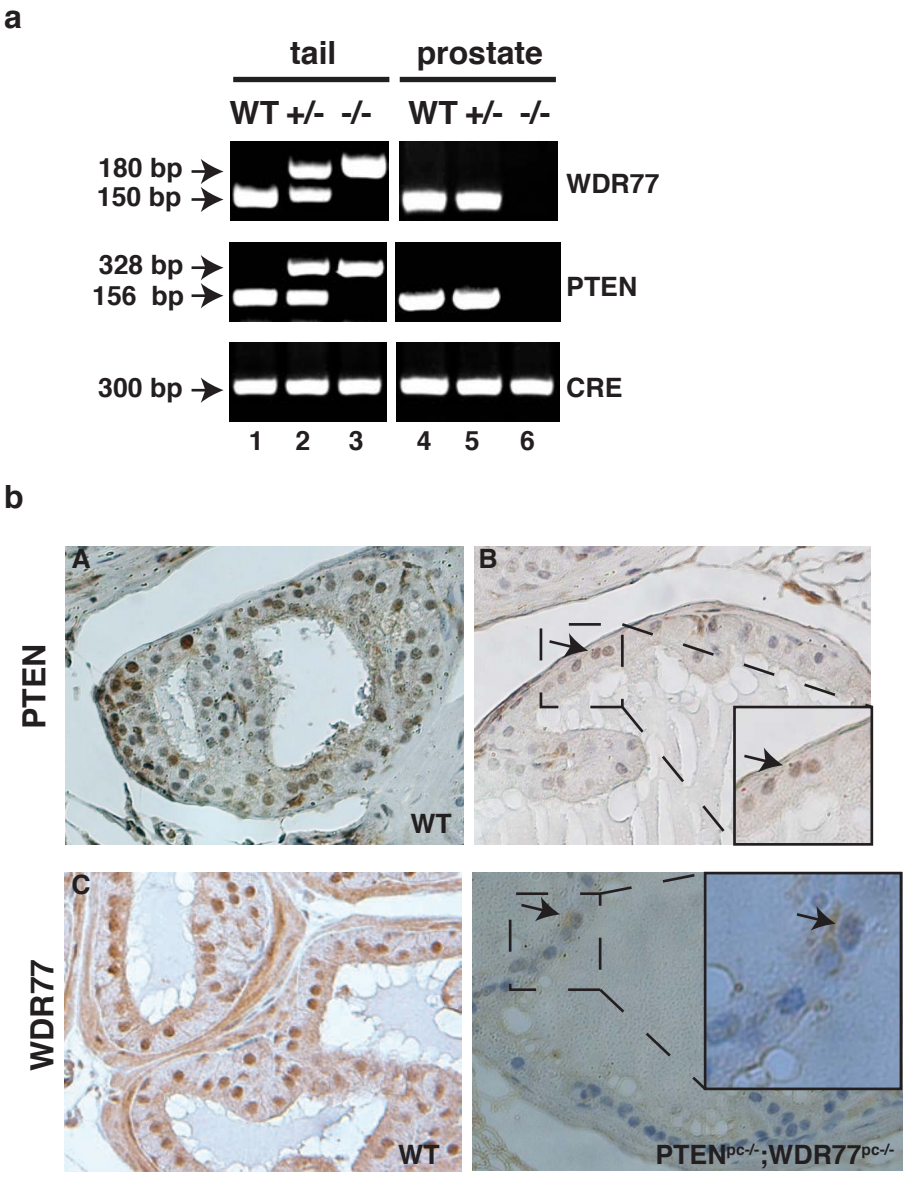
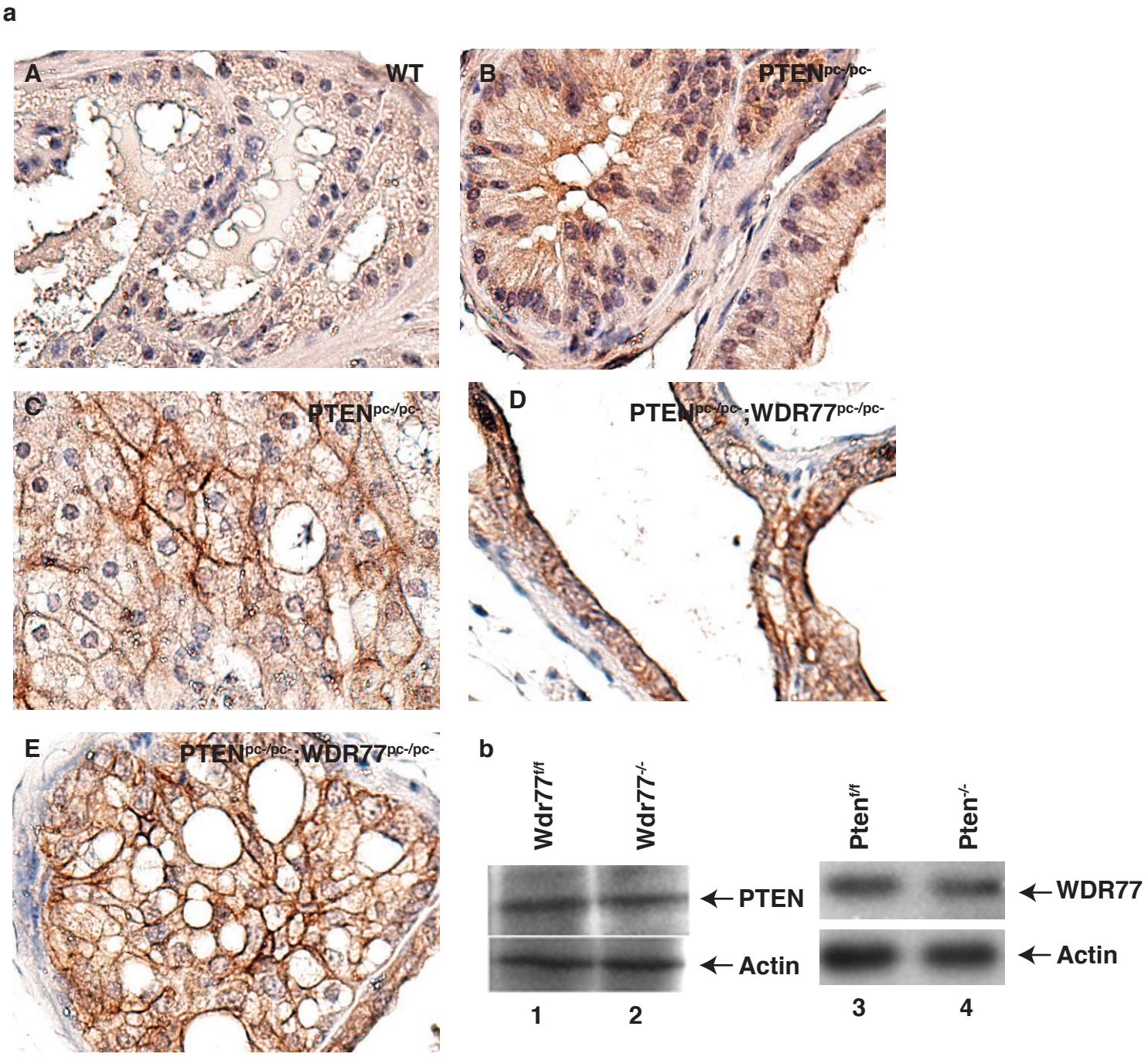

d
PTEN
?
AKT
TFGβ
WDR77
proliferation
WDR77
GSK3B
E2F activation
BAD
mTOR
BcIXL
S6K
metabolism
proliferation
survival
nutrient response
protein synthesis

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

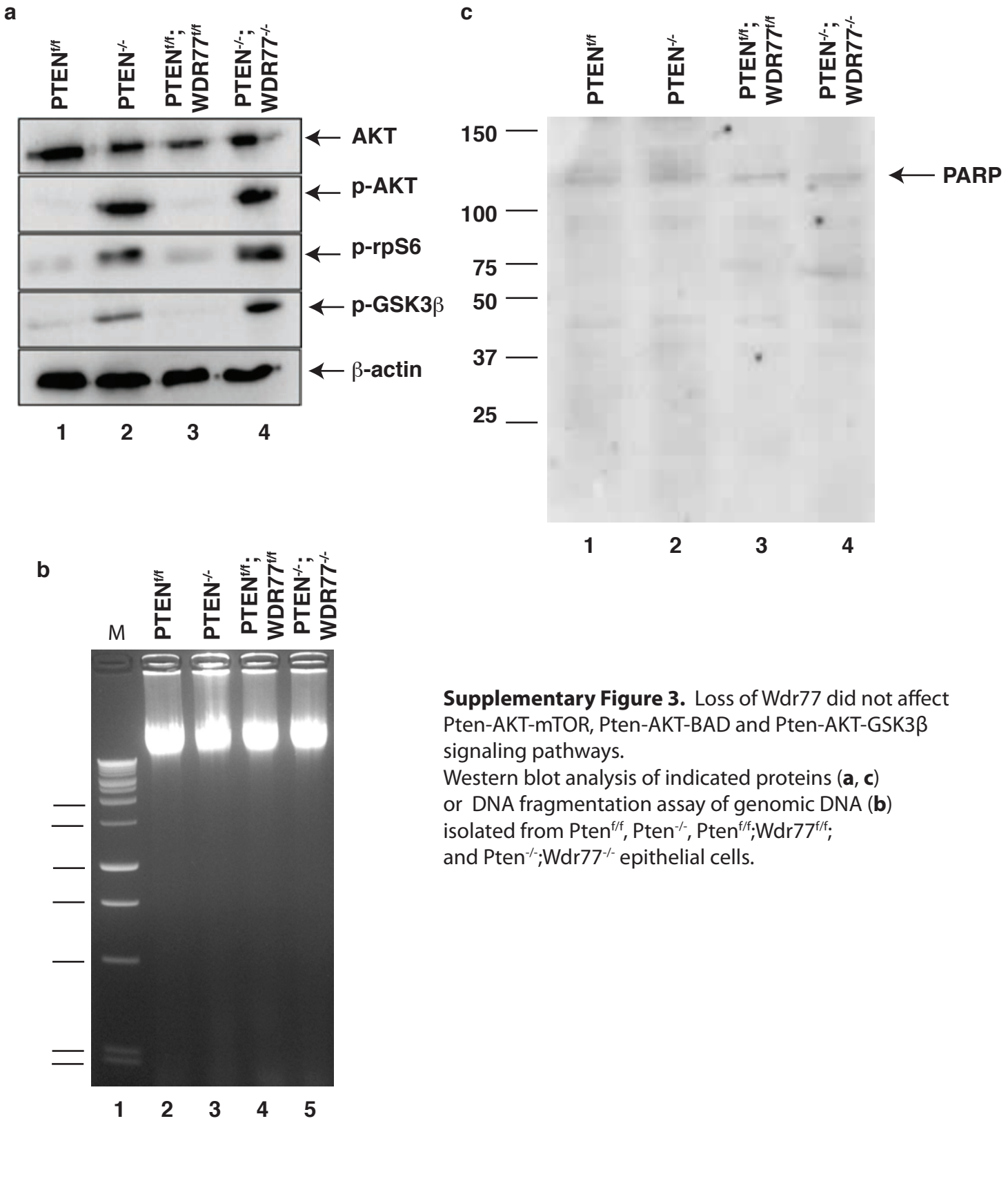
Age (month)	Genomic Type			
	$PTEN^{loxP/loxP}$	$PTEN^{pc-/-}$	$PTEN^{loxP/loxP}$; $WDR77^{loxP/loxP}$	$PTEN^{pc-/-}$; $WDR77^{pc-/-}$
1	2	5	2	5
2	2	5	2	5
4	2	5	2	7



Supplementary Figure 1. The prostate-specific deletion of Pten and Wdr77 genes in the mouse. (a) Gene typing of genomic DNA isolated from tails (lanes 1-3) and prostate glands. (b) Immunostaining of Pten and Wdr77 proteins in prostate glands of WT (a, c) and PtenloxP/loxP;WdrloxP/loxP;Cre (b, d) mice.

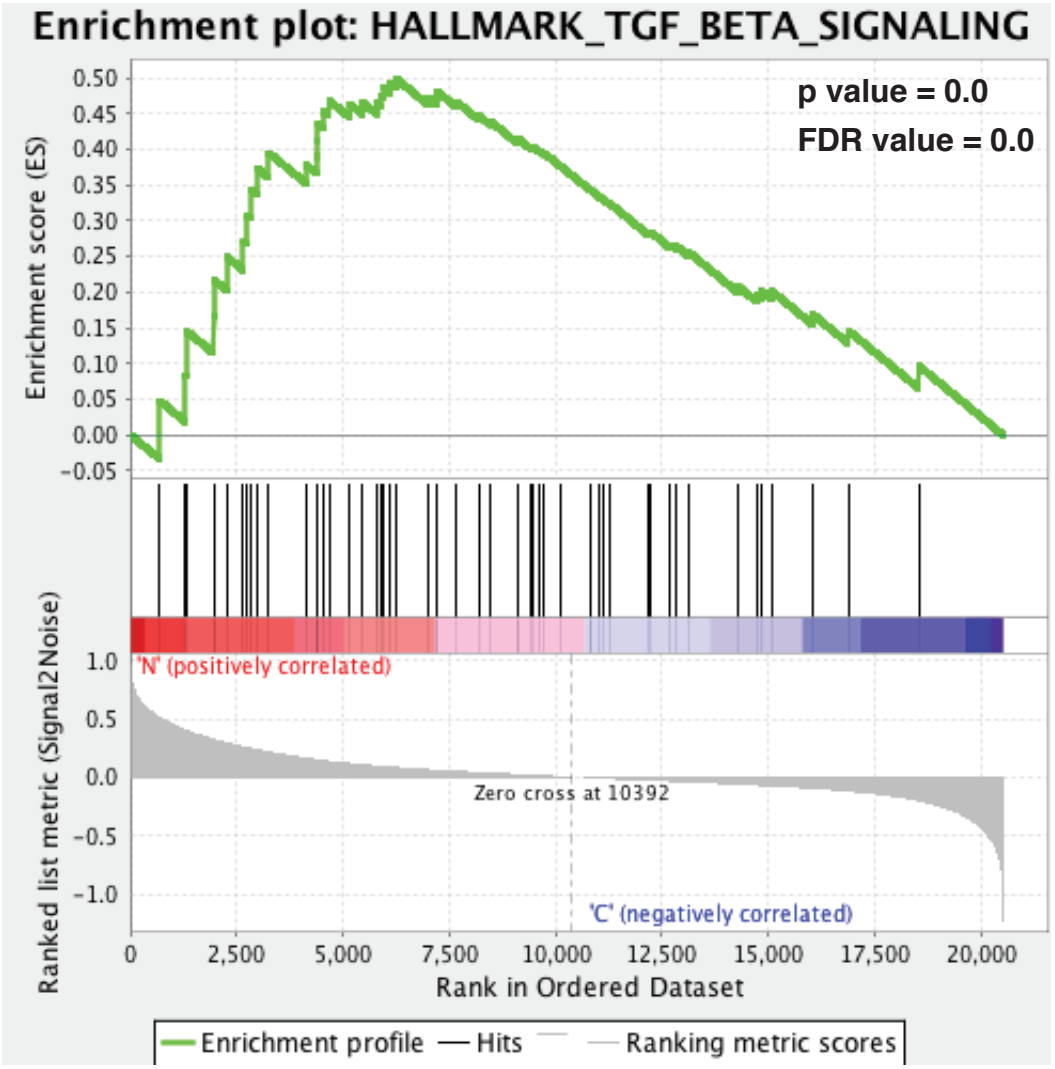


Supplementary Figure 2. Immunostaining and Western blot analysis. (**a**) AKT phosphorylation induced by Pten gene deletion. Immunostaining of phosphorylated AKT (pAKT) in prostate tissues derived from WT, Ptenpc-/-, and Ptenpc-/-;Wdr77pc-/- mice. (**b**) Western blot analysis of Pten and Wdr77 expression in WT and Pten- or Wdr77-deleted prostate cells.

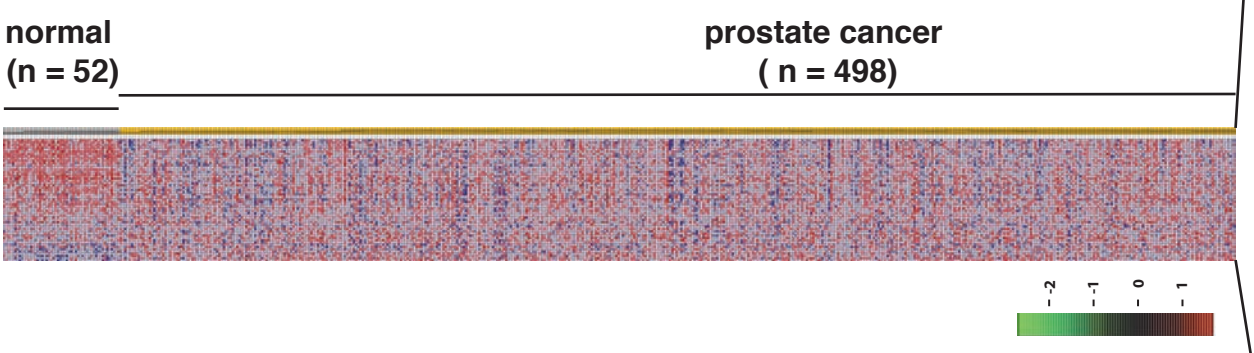


Supplementary Figure 3. Loss of Wdr77 did not affect Pten-AKT-mTOR, Pten-AKT-BAD and Pten-AKT-GSK3β signaling pathways.
Western blot analysis of indicated proteins (**a**, **c**) or DNA fragmentation assay of genomic DNA (**b**) isolated from $Pten^{f/f}$, $Pten^{-/-}$, $Pten^{f/f}$;$Wdr77^{f/f}$; and $Pten^{-/-}$;$Wdr77^{-/-}$ epithelial cells.

a



b



PIK3C2G
CA14
GCNT4
ASPA
PGM5P2
NKAPL
KY
IGSF1
SCN5A
CXCR2
HRASLS5
KCNJ15
PAK7
C2orf88
RPE65
DHDPSL
PAK3
C10orf82
C14orf64
GPX2
RAB9B
PCP4L1
DAB1
PDE1C
AQP5
FAM83B
ARSF
CCBE1
LGR6
ZNF157
MGAT4C
SLC2A9
SOSTDC1
CPNE6
EPHB1
TMIE
LY6G6D
STOX2
NPY6R
NRG2
OPRT
GPR156
HPD
MCF2
GLRA4
TMLHE
CLCA2
RHBDL3
DCC
CSRNP3
NKX2-3
LOC100128675
DLX1
HOXC6
SLC45A2
DLX2
SNHG3
HOXC4
ZIC2
HOXC5
PRR7
HJURP
CCDC108
UCN
LMX1B
EPHA10
ONECUT2
CASKIN1
C2CD4C
SNHG4
CLEC18B
SLIT1
GAL
GSC
ZIC5
MMP26
LASS1
MYBL2
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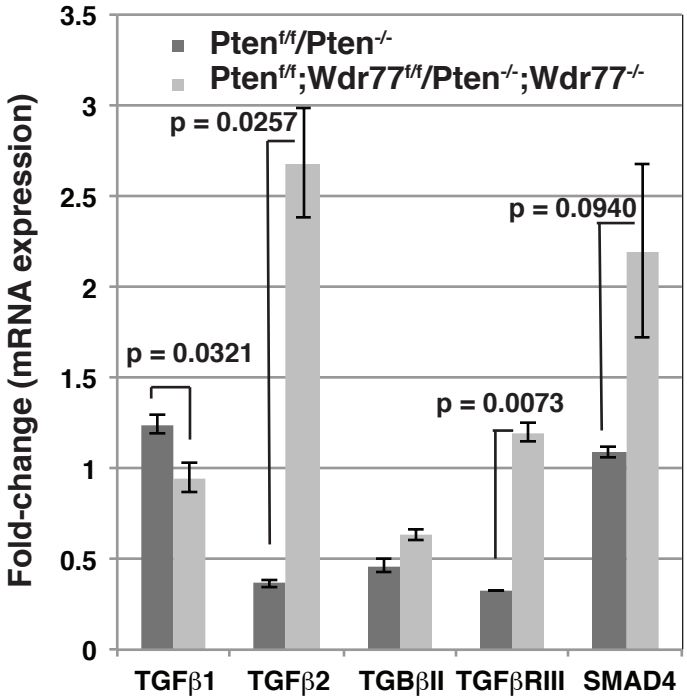
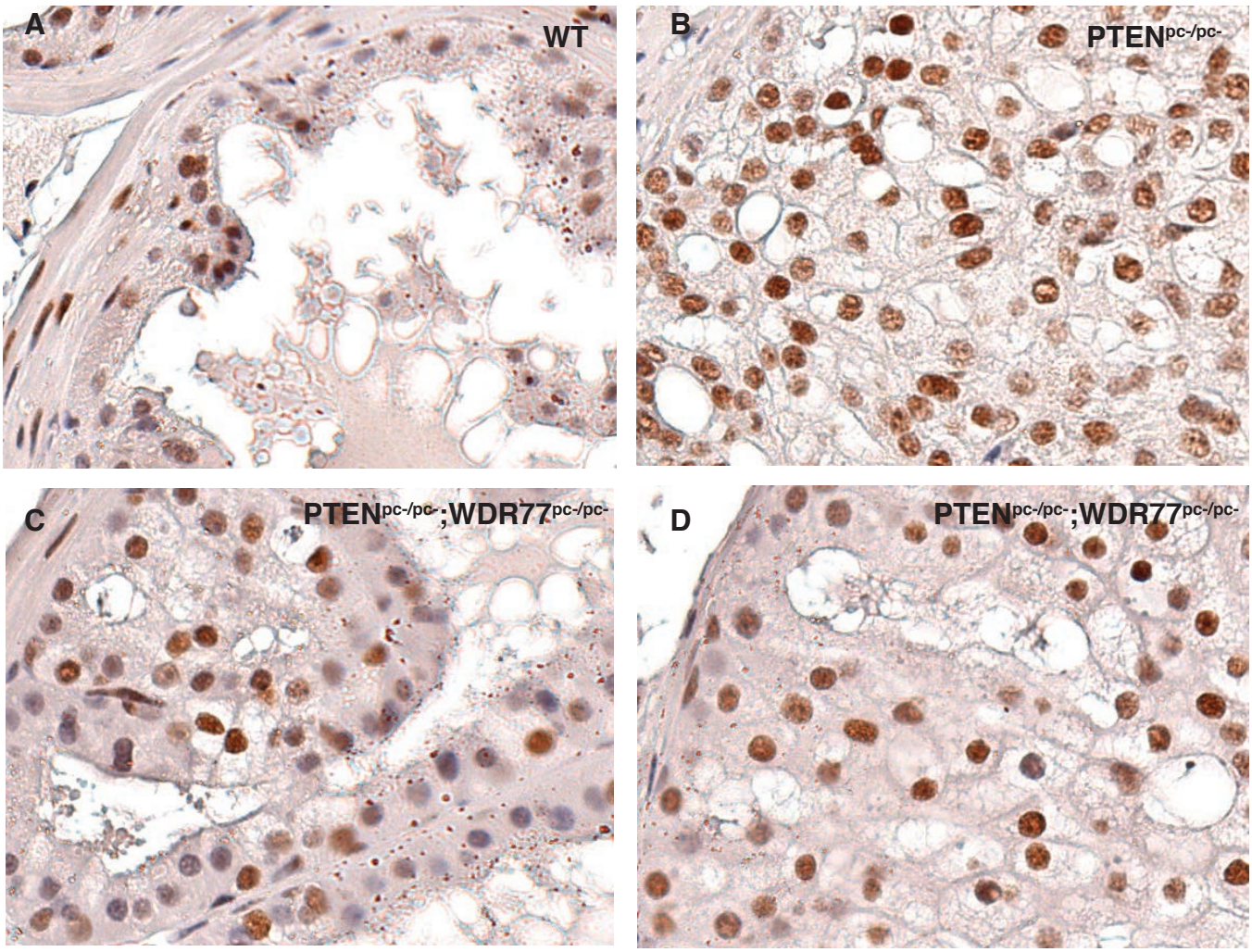
PAQR6
CENPA
NET02
LOC100130872
PCA3
C9orf163
C10orf95
MATK
LOC100287718
FOXD1
IQGAP3
NLRP12
CCDC78
CDC25C
TDRD1
SIM2
LOC100133612

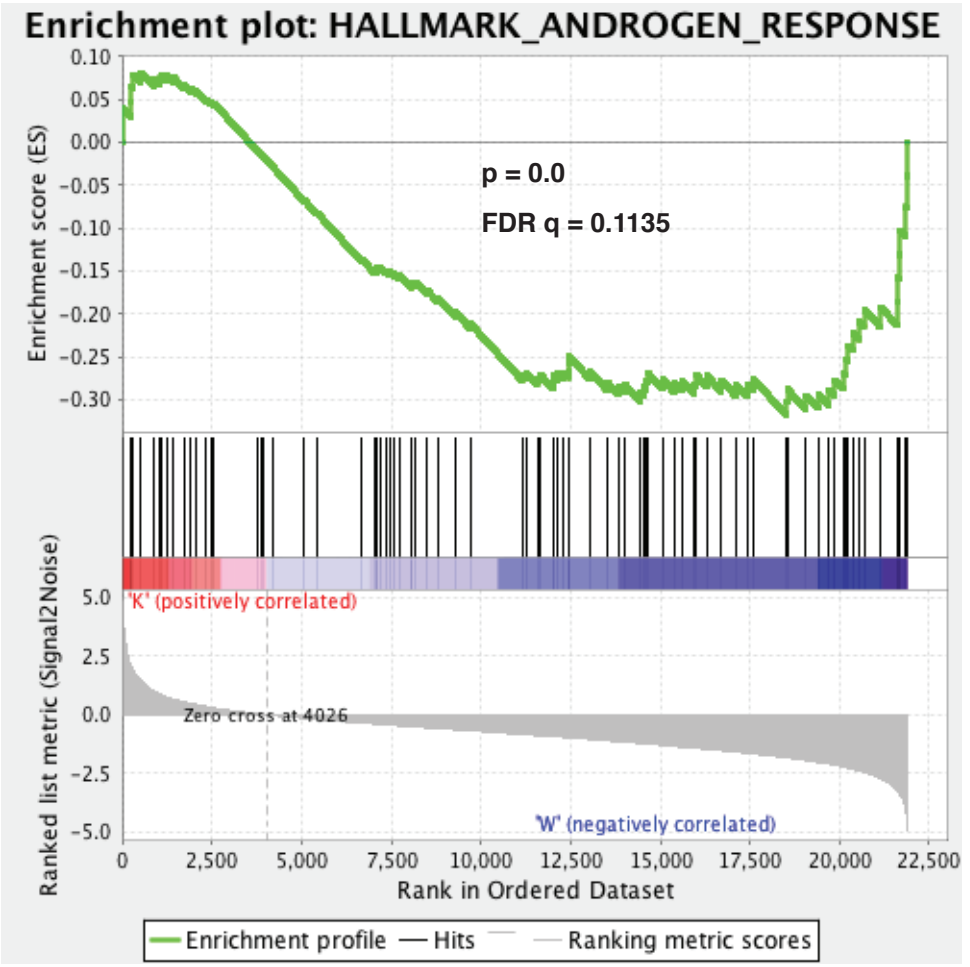
Supplementary Figure 4. Loss of TGFβ signaling associated with prostate cancer.
(**a**) GSEA enrichment plot indicates that genes regulated by TGFβ were over-represented on the gene list, whose expression is altered in prostate cancer.
(**b**) Genes targeted by TGFβ are visualized by heatmap.

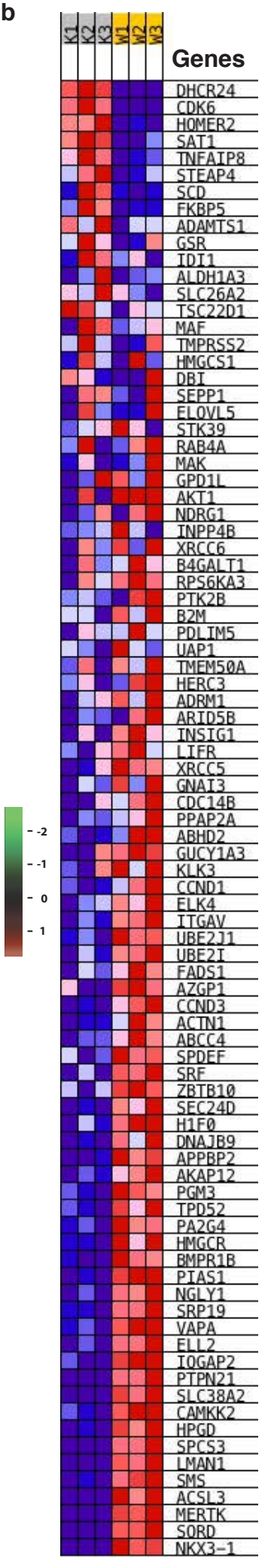


Supplementary Figure 5. RT-PCR analysis of expression of genes involved in TGFβ signaling RNAs were isolated from $Pten^{f/f}$, $Pten^{-/-}$, $Pten^{f/f}$;$Wdr77^{f/f}$; and $Pten^{-/-}$;$Wdr77^{-/-}$ epithelial cells and aubmitted for real-time PCR analysis. Fold-change = relative expression in loxP-floxed cells/ relative expression in cells with deletion of Pten or Pten plus Wdr77 genes.



Supplementary Figure 6. AR expression is induced by Pten loss. Immunostaining of AR in prostate tissues derived from WT, Pten$^{pc-/-}$, and Pten$^{pc-/-}$;Wdr77$^{pc-/-}$ mice.





Supplementary Figure 7. Pten loss decreased the AR signaling in the prostate. (**a**) GSEA enrichment plot indicates that genes regulated by AR were over-represented on the gene list, whose expression is altered by Pten loss. (**b**) Genes targeted by AR are visualized by heatmap.

sTable 1: Mouse Primers for Real Time PCR	
Target Genes	Sequence 5'-3'
TGFβ1	F: AGCTGCGCTTGCAGAGATTA
	R: AGCCCTGTATTCCGTCTCCT
TGFβ2	F: TCCCCTCCGAAAATGCCATC
	R: TGCTATCGATGTAGCGCTGG
TGFBRII	F: CCAAGTCGGATGTGGAAATGG
	R: TGTCGCAAGTGGACAGTCTC
TGFBRIII	F: CTGCCAAGGGAGGTTCACAT
	R: AACCCTCCGAAACCAGGAAG
Smad4	F: GAGAGAGCGAGGTTGCACAT
	R: ACCTTTATATACGCGCTTGGGT