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Loss of *ATF3* Promotes Hormone-induced Prostate Carcinogenesis and the Emergence of CK5^{+}CK8^{+} Epithelial Cells

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Abstract

Steroid sex hormones can induce prostate carcinogenesis, and are thought to contribute to the development of prostate cancer during aging. However, the mechanism for hormone-induced prostate carcinogenesis remains elusive. Here we report that activating transcription factor 3 (ATF3) – a broad stress sensor – suppressed hormone-induced prostate carcinogenesis in mice. While implantation of testosterone and estradiol ($T+E_2$) pellets for 2 months in wild-type mice rarely induced prostatic intraepithelial neoplasia (PIN) in dorsal prostates (1 out of 8 mice), loss of *ATF3* led to the appearance of not only PIN but also invasive lesions in almost all examined animals. The enhanced carcinogenic effects of hormones on *ATF3*-deficient prostates did not appear to be caused by a change in estrogen signaling, but were more likely a consequence of elevated androgen signaling that stimulated differentiation of prostatic basal cells into transformation-preferable luminal cells. Indeed, we found that hormone-induced lesions in *ATF3*-knockout mice often contained cells with both basal and luminal characteristics, such as p63^{+} cells

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Author Contributions

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

Conflict of Interest

The authors declare that they have no conflict of interest.

(a basal cell marker) showing luminal-like morphology, or cells double-stained with basal ($CK5^+$) and luminal ($CK8^+$) markers. Consistent with these findings, low *ATF3* expression was found to be a poor prognostic marker for prostate cancer in a cohort of 245 patients. Our results thus support that ATF3 is a tumor suppressor in prostate cancer.

Keywords

ATF3; estrogen; androgen; prostate carcinogenesis; differentiation

Introduction

Characterized by its strong association with aging, prostate cancer remains as one of the major health threats to men worldwide. While genetic alterations that can cause transformation of prostatic epithelia are inevitably accumulated during aging, aging men also produce fewer androgens. As a result, the ratio of plasma estrogens (e.g., estradiol, E_2) to androgens (e.g., testosterone) is increased with age[1]. Estrogens produced by adipose tissue, adrenal glands and testicles, or converted from testosterone in the prostate locally, can bind estrogen receptor α (ERα) or β (ERβ) to regulate prostatic branching, and appears to be required for normal prostate development[2]. The increase in the relative level of prostatic estrogens in aging men is therefore thought to be one of the major contributors to the occurrence and development of prostate cancer[1,2]. Indeed, not only epidemiological studies have demonstrated that an elevated plasma estrogen level correlates with a high risk of prostate cancer[3], but estradiol in combination with testosterone mimicking the hormone imbalance in aging men has been shown to induce prostate carcinogenesis in rodents[4,5]. Although the underlying mechanism remains elusive, hormone-induced carcinogenesis in mouse prostates appears to require both estrogen and androgen signaling, as mice null for ERα are refractory to the carcinogenic effect[5] while a treatment regimen devoid of testosterone rather induces squamous metaplasia and keratinization of prostate epithelia without neoplastic transformation[6]. Squamous metaplasia is thought to be caused by extensive proliferation of basal epithelial cells that is stimulated by paracrine signaling mediated by ERα predominantly expressed in adult prostatic stroma[7]. On the other hand, androgen signaling triggered by binding of testosterone to the androgen receptor (AR) in the prostatic luminal epithelium or stroma is indispensable for sustaining the prostate epithelium in a differentiated and relatively growth-quiescent state[8]. Elevated androgen signaling, however, can also drive prostatic proliferation and is responsible for the outgrowth and survival of prostate cancer cells[9,10]. Indeed, targeting androgen signaling or AR is one of the major strategies for treating prostate cancer. Not surprisingly, the AR activity, presented as transactivation of androgen-responsive genes, is regulated by a complex network comprised of transcription co-factors that are often aberrantly expressed in prostate cancer[11,12].

Previously, we identified ATF3 (activating transcription factor 3), a member of the ATF/CREB family member, as a major AR repressor in the prostatic epithelium and prostate cancer cells[13]. ATF3 binds AR at its DNA-binding domain, thereby preventing AR from binding to androgen-responsive genes[13]. ATF3 can also bind the AR C-terminal ligand-binding domain, disrupting the AR intramolecular interaction required for its transcriptional

activity[13]. Best known for its rapid induction by broad cellular stresses including DNA damage, oxidative stress, and oncogenic stimuli[14], ATF3 engages in a number of cellular signal pathways, such as those mediated by p53, TGFβ, and Toll-like receptor 4, through interacting with other proteins or binding to the consensus ATF/CREB *cis*-regulatory element[15–17]. While it is generally believed that ATF3 is important for triggering appropriate cellular responses to immune and oncogenic stimulation[18,19], aberrant ATF3 expression is frequently associated with human diseases such as prostate cancer[20]. Indeed, unbiased cancer profiling analyses have revealed that ATF3 expression is often down-regulated in prostate cancer, particularly in advanced diseases[21]. Using a genetically-engineered mouse model, we recently demonstrated that ATF3 suppresses the development of prostate cancer caused by Pten loss[21] – one of the most frequent genetic alterations occurring in human prostate cancer. We also showed that *ATF3* deficiency leads to increased Akt signaling in both transformed mouse prostatic epithelia and human prostate cancer cells[21]. These results in combination with the earlier findings that ATF3 is an AR repressor and can activate the tumor suppressor p53[13,15] strongly argue for a notion that ATF3 plays an important role in the suppression of prostate cancer[22]. However, ATF3 has also been shown to be oncogenic in other cellular contexts, such as in breast cancer[23].

Given that hormone signaling may function as an oncogenic stimulus to promote prostate cancer development, we sought to test whether *ATF3* deficiency in mice also contributes to prostate carcinogenesis induced by steroid sex hormones. Our results indicate that loss of *ATF3* in mice accelerated hormone-induced prostate carcinogenesis, an effect which was likely achieved through promoting differentiation of basal epithelial cells into luminal cells. The latter cell type appears to be favored as the cell of origin for prostate cancer[24]. We therefore provide an additional line of genetic evidence supporting that ATF3 is a tumor suppressor for prostate cancer.

Results

Low ATF3 expression is a poor prognosis marker for prostate cancer

Previous studies found that *ATF3* expression is frequently down-regulated in prostate cancer[21,25,26]. To further explore the role of ATF3 in prostate cancer, we examined *ATF3* expression in 419 prostate cancer samples and 52 normal tissues using the RNA-seq data deposited in the Cancer Genome Atlas (TCGA) database. Consistent with previous reports, we found that the *ATF3* expression level was significantly lower in prostate tumors than that in normal tissues ($p = 0.0004$) (Fig 1A). Further comparison of *ATF3* expression between prostate tumors and their corresponding adjacent normal tissues also showed decreased *ATF3* expression in tumors ($p = 0.005$, $n = 52$) (Fig 1B). We also carried out immunohistochemical (IHC) staining on 14 prostate cancer samples and their corresponding normal prostate tissues. We found that the ATF3 staining intensity was significantly lower in 9 out of 14 prostate tumor samples (64.2%) as compared to their normal prostatic epithelia (Fig 1C). In contrast, elevated ATF3 staining was found in only one of these tumors. Intriguingly, when the survival data for prostate cancer patients registered in the TCGA database were analyzed, we found that low *ATF3* expression was significantly associated

with a poor relapse-free survival in patients (p=0.006) (Fig 1D). Our results thus support the role of ATF3 that plays in the suppression of prostate cancer.

ATF3 is hormone inducible and expressed in both basal and luminal cells

As hormone signaling can promote prostate carcinogenesis[1,2], we asked whether ATF3 also suppresses prostate carcinogenesis induced by steroid sex hormones. To explore this possibility, we first tested whether *ATF3* expression is induced by hormone stimulation. We respectively treated PC3 cells that carry functional ERα and LNCaP cells known to express AR[27] with estradiol (E_2) and a synthetic androgen R1881 for Western blotting. While these hormones induced expression of ER/AR target genes progesterone receptor (PR) and NKX3.1 as expected, we found that E_2 and R1881 rapidly induced an increase in the ATF3 protein level (Fig 2A and 2B). The hormones also rapidly increased the *ATF3* mRNA levels (Fig 2C), suggesting that they likely induced *ATF3* expression at the transcription level. As ER and AR regulate prostatic basal and luminal epithelial cells respectively, we examined *ATF3* expression in these two distinct cell types by IHC. In support of the possibility that ATF3 may regulate hormone-induced events, *ATF3* was expressed in both basal (red arrows) and luminal cells (black arrows) of human (Fig 2D) and mouse prostates (Fig 2E). Of note, p63 staining was used to label basal cells in the mouse tissue (Fig 2E).

ATF3 deficiency does not affect hormone-induced squamous metaplasia in anterior prostates

We next determined the contribution of ATF3 to hormone-induced prostate carcinogenesis by subcutaneously implanting pellets embedded with 25 mg of testosterone (T) and 2.5 mg of E_2, or placebo, into *ATF3* wild-type (WT) or knockout (KO) mice (C57BL/6). These pellets allow for continuous release of the hormones for 2 months until we subjected mouse prostates to histopathological examinations. Treating mice with T+E_2 at these doses were expected to reproduce circulating plasma hormone levels similar to those found in aging men[5]. While we did not notice apparent alteration in the growth and behavior of mice, the hormone treatments induced squamous metaplasia in mouse anterior prostates (APs) evidenced by epithelial keratinization and dramatic expansion of enlarged, flat epithelial cells in these glands (Fig 3A). These highly proliferative (PCNA positive) cells were often positive for p63 staining ($p63^+$) (Fig 3A), indicating their basal-cell origin. These results were different from those in an early report that T+E_2 embedded in Silastic tubing induced prostatic intraepithelial neoplasia (PIN) in mouse APs[5]. This discrepancy might be due to different hormone release kinetics. The pellets used in our study might release E_2 more efficiently and thus predominantly stimulate estrogen signaling in APs - the glands most sensitive to estrogens. Indeed, squamous metaplasia, known as a major estrogenic effect in rodents[6], was only seen in APs in our experiments. Moreover, expression of PR, a well-characterized ER target gene, was strongly induced by the hormone treatments (Fig 3A). Importantly, loss of *ATF3* did not appear to alter the estrogenic effects, as the hormones induced squamous metaplasia in *ATF3*-deficient APs to the same extent as the wild-type glands (Fig 3A). Consistent with these results, *ATF3* deficiency did not change the number of p63-positive or PCNA-positive cells (Fig 3B and 3C). These results indicate that ATF3 had a negligible effect on estrogen signaling. Indeed, knockout of *ATF3* expression by a

single-guided RNA in PC3 cells[21] only marginally altered PR expression induced by E_2 (Fig 3D).

Loss of ATF3 promotes hormone-induced carcinogenesis in dorsal prostates

As ATF3 can repress androgen signaling in the prostatic epithelium[13], we next asked a question as to whether loss of *ATF3* leads to different hormone responses in less-estrogenic glands like dorsal prostates (DPs). While we did not find obvious abnormality in ventral and lateral prostates (Table 1), the hormone treatments appeared to induce PIN lesions in DPs of one WT mice, but hyperplasia, in other WT mice (Fig 4A and 4B), suggesting a minor carcinogenic effect of the hormones on DPs. Intriguingly, *ATF3* deficiency appeared to promote this carcinogenic effect, as 11 out of 12 KO mice developed PIN lesions in their DPs (Fig 4A and 4B). Moreover, staining for α-smooth muscle actin (α-SMA) expression in these ATF3-deficient DPs revealed that a number of lesions (6–30%) were surrounded by disintegrated smooth muscle layers that were invaded by transformed epithelial cells (Fig 4A and 4C), indicating their invasiveness. In contrast, only one of the WT mice appeared to have invasive lesions (Fig 4B). These results thus demonstrate that *ATF3* deficiency promoted prostate carcinogenesis induced by $T+E_2$. Of note, DPs of placebo-treated KO mice were hyperplastic (Fig 4A and 4B), a finding reminiscent of our previous results[13].

ATF3 deficiency promotes epithelial cell proliferation in dorsal prostates

To explore the underlying mechanism for carcinogenesis promoted by loss of ATF3, we determined the proliferation rate of prostatic epithelial cells by PCNA staining. Without the hormone treatments, *ATF3*-deficient DPs had a higher proliferation rate than WT glands (Fig 5A and 5B), consistent with the results that the KO glands were hyperplastic (Fig 4B). As expected, while the hormone treatments significantly increased the proliferation rate, loss of *ATF3* further enhanced prostatic epithelial proliferation induced by the hormones (Fig 5A and 5B). As hormone-induced PR expression (as a marker for ER signaling) was not significantly different between WT and KO glands (Fig 5A and 5C), the increased proliferation in KO cells was more likely caused by increased sensitivity to androgen stimulation. Indeed, we have previously demonstrated that loss of ATF3 can promote androgen signaling in the prostatic epithelium[13].

Hormone-induced KO tumors contain cells with a basal-cell origin

Prostate cancer can be originated from either basal cells or luminal cells[28]. While luminal cells are more susceptible to transformation, prostate tumorigenesis originated from basal cells has a long latency and appears to require basal-to-luminal differentiation[29,30]. To better understand the mechanism underlying the promotion of hormonal carcinogenesis by ATF3, we stained DPs with antibodies to AR and p63 to label luminal and basal cells, respectively. Whereas AR positivity was mainly found in columinal luminal cells in the placebo group as expected, most cells in the hormone-induced prostate lesions were also AR-positive but negative for p63 staining (Fig 6A). On the contrary, p63 staining was mainly found in basal cells (Fig 6A). As a consequence of growth stimulation by E_2, the number of p63-positive ($p63^+$) basal cells in both WT and KO glands was increased by the hormone treatments (Fig 6B). Although there was no difference in the total number of $p63^+$ cells, we found, to our surprise, that the prostate lesions in KO mice were comprised of many $p63^+$ cells with a

luminal-like morphology, *i.e.*, large oval-shaped nuclei aligned vertically to the basement membrane (Fig 6A, red arrows). These luminal-like $p63^+$ cells were often dislodged and moved away from the basement membrane (Fig 6A). Given that basal cells can be differentiated into luminal cells under certain conditions such as AR expression[31,32], these luminal-like $p63^+$ cells were likely derived from basal-to-luminal differentiation. Although we occasionally found these intermediate cells in the hormone-treated WT glands, the number of glands containing such cells was significantly smaller than that of the KO glands (Fig 6C). As luminal cells appear to be the favorable cell of origin for prostate cancer[24], these results suggest that basal-to-luminal differentiation promoted by *ATF3* deficiency would generate luminal cells that are more transformation-competent[29] thereby facilitating prostate carcinogenesis induced by hormones.

Loss of ATF3 promotes the emergence of $CK5^+CK8^+$ epithelial cells

To confirm the existence of intermediate cells in the lesions of KO mice, we double-stained DPs with antibodies to cytokeratin 5 (CK5) and cytokeratin 8 (CK8) to visualize basal and luminal cells simultaneously. As expected, CK8-positive ($CK8^+$) luminal cells rested on a layer of CK5-positive ($CK5^+$) basal cells and extruded towards lumens in DPs of the placebo-treated mice (Fig 7A). Whereas the hormones increased the numbers of $CK5^+$ and $CK8^+$ cells as expected, we saw a few CK5 and CK8 double positive ($CK5^+CK8^+$) cells scattered in WT DPs. In striking contrast, we found a significantly-larger number of $CK5^+CK8^+$ cells in DPs of hormone-treated KO mice (Fig 7A). Localized on the top of a layer of $CK5^+$ basal cells, these $CK5^+CK8^+$ cells had cuboidal shapes, aligned vertically to the basement membrane, and often mingled with $CK8^+$ luminal cells (Fig 7A). Overall, more than 20% of epithelial cells in the ATF3-deficient DPs were $CK5^+CK8^+$, comparing to 2% of $CK5^+CK8^+$ cells in the WT glands (Fig 7B). Moreover, a significantly higher number of ATF3-deficient glands contained $CK5^+CK8^+$ cells as compared to their WT counterparts (Fig 7C). When CK5 staining was examined alone, $CK5^+$ cells appeared to frequently form two or more layers in the KO glands (Fig 7A). Indeed, we found that the number of glands with multiple layers of $CK5^+$ cells was significantly increased in the ATF3-deficient DPs (Fig 7D). These results thus suggest that loss of *ATF3* could promote basal-to-luminal transformation in mouse prostates. As a simple test of the possible effect of ATF3 on prostate epithelial differentiation, we knocked down *ATF3* expression in RWPE-1 cells using an ATF3-specific siRNA (siATF3, Fig 7F)[13] and stained the cells for differentiation markers. As RWPE-1 is a normal human prostate epithelial cell line mainly harboring a basal-cell phenotype, few than 1% of cells were positive for CK8 staining (Fig 7E and 7F). In line with the notion that *ATF3* deficiency could promote basal-to-luminal differentiation, knockdown of *ATF3* expression significantly increased the number of $CK8^+$ cells by 5 folds (Fig 7E and 7F). Of note, most of these $CK8^+$ cells remained positive for CK5 staining. Therefore, ATF3 appears to induce human prostate basal cells to differentiate into luminal cells.

Discussion

Decrease in androgen production and accumulation of genetic alterations during aging are two major risk factors for prostate cancer. While it has been established that ATF3 plays important roles in provoking cellular responses to oxidative stresses that can accumulate

naturally and cause genetic alterations during aging[33,34], we demonstrated in this report that loss of ATF3 promoted prostate carcinogenesis in mice under a condition mimicking the estrogen/androgen imbalance in aging men[5]. Our results thus argue for a notion that ATF3 dysfunction contributes to the genesis and the development of prostate cancer. Indeed, while it was frequently found that *ATF3* expression is down-regulated in human prostate cancer[21], decreased *ATF3* expression appeared to be associated with poor survival of prostate cancer patients (Fig 1D). This notion is in line with previous findings that ATF3 is proapoptotic[35,36] and can repress androgen signaling required for the outgrowth and survival of prostate cancer cells[13]. Moreover, we recently demonstrated that *ATF3* deficiency promotes prostate tumorigenesis induced by genetic ablation of *Pten* in mice[21]. Together, our studies provide direct genetic evidence supporting the role of ATF3 that plays in the suppression of prostate cancer.

Hormone-induced prostate carcinogenesis in mice requires both estrogen and androgen signaling[5]. While estrogens stimulate proliferation of basal cells and are likely the driving force for cellular transformation, the role of androgen signaling in prostate carcinogenesis is unclear and thought to outcompete with estrogens to preserve a glandular phenotype and prevent prostatic atrophy[5]. Different from the previous report[5], however, the T+E_2 combination only induced squamous metaplasia in the most estrogen-sensitive anterior prostates in our animal model (Fig 3). These results indicate that the estrogenic effects appear to be dominant in APs in our model. Although *ATF3* expression could be transiently induced by E_2 in prostatic epithelial cells (Fig 2A), ATF3 did not appear to affect estrogen signaling, as loss of *ATF3* neither caused phenotypic changes in APs, nor altered PR expression induced by the hormones (Fig 3). Accordingly, it was more likely that loss of *ATF3* promoted hormonal carcinogenesis through increasing androgen signaling. Although our efforts in determining effects of ATF3 on androgen signaling in our mouse model were hindered by the lack of commercial antibodies suitable for detecting androgen-responsive genes by IHC, we have previously demonstrated that loss of *ATF3* is sufficient to enhance androgen signaling in mouse prostatic epithelia[13]. Interestingly, *ATF3* expression could be induced by both androgen stimulation (Fig 2B) and androgen deprivation[13]. While these observations are consistent with the notion that ATF3 is a broad stress sensor, it would be interesting to further elucidate the mechanism(s) underlying ATF3 induction by androgen alterations for a better understanding of the regulation of androgen signaling by ATF3.

An important finding from this study is that loss of *ATF3* induced the emergence of prostate epithelial cells expressing both basal-cell and luminal-cell markers, suggesting that *ATF3* deficiency might promote prostatic basal epithelial cells to differentiate into luminal cells. Not only luminal-like $p63^+$ cells were wide-spread in ATF3-knockout prostate lesions, but we found a significant increase in the number of $CK5^+CK8^+$ cells that were likely intermediate between basal and luminal cells. Although both basal cells and luminal cells can be self-sustained and serve as the cell of origin for prostate cancer[29], basal cells are often considered stem-like cells[31] and can be converted into luminal cells likely through asymmetrical division[37]. Indeed, the intermediate cells found in the ATF3-deficient lesions were often localized vertically to the basement membrane (Fig 6A and 7A), suggesting that they might be generated from asymmetrical division of stem-like basal cells. While the definite origin of these intermediate cells would need to be determined through cell lineage

tracing using fluorescent reporters, our results suggest that hormone-induced prostate lesions had a basal-cell origin. This notion appears to contradict a recent report, which concluded that luminal cells are favored as the cell of origin for hormone-induced prostate cancer[24]. However, the lineage tracing approach used in this recent study has a limitation in that only a portion of basal cells are fluorescently labeled[29]. Thus, the possibility that hormone-induced prostate lesions were partially derived from a small portion of unlabeled basal cells cannot be excluded. It is important to note that prostate tumors derived from basal cells often have a long latency[29], but appear to be more invasive likely due to alterations in the *Spp1* and *Smad4*-mediated pathways that have been shown to promote invasion and metastasis of prostate cancer in a genetically-engineered mouse model[38,39]. It is thus likely that differentiation of basal cells into luminal cells (which are more competent to transformation) is a contributing factor for prostate tumorigenesis with a basal-cell origin[29,30]. Interestingly, while $T+E_2$ can induce PINs in the wild-type prostate glands (albeit at a low frequency in our experiments, also see reference[5]), we found that prostate lesions generated in the ATF3-deficient mice were invasive in almost all animals examined (Fig 4A and 4B). This observation provides an additional support to the notion that loss of ATF3 promoted basal-to-luminal differentiation leading to prostate lesions that were originated, at least in part, from basal cells. As AR has been shown to promote basal-to-luminal differentiation[32], it is highly likely that the enhanced androgen signaling led to the differentiation in the ATF3-deficient prostates. Interestingly, when $CK5^+CK8^+$ cells were examined in the prostate lesions generated by genetic ablation of the tumor suppressor Pten, we only saw very few double-stained cells and *ATF3* deficiency did not appear to increase the number of intermediate cells in this mouse model (data not shown). These observations were not unexpected, given that it is known that AR signaling is impaired in *Pten*-deficient prostates[40, 41]. These results thus provide an additional support to the notion that enhanced androgen signaling due to loss of *ATF3* likely lead to the basal-to-luminal differentiation found in the hormone-induced prostate lesions. Accordingly, given that the role of androgen signaling in hormone-induced prostate carcinogenesis is unclear, it would be interesting to test whether testosterone serves to induce basal-to-luminal differentiation thereby facilitating cellular transformation stimulated by estradiol.

Materials and Methods

Animals and hormone treatments

Animal experiments were carried out according to protocols approved by the Institutional Committee of Animal Care and Use (ICACU) of the Albany Medical College and the ICACU of Georgia Regents University. *ATF3* knockout (*ATF3*$^{-/-}$) mice were described previously[13,42]. For prostate carcinogenesis, 8 to 12 mice (C57BL/6) were subcutaneously implanted with pellets embedded with 25 mg of testosterone propionate (SA-211) and 2.5 mg of β-estradiol 17-acetate (SE-271) (Innovative Research of America, Sarasota, FL, USA), or placebo (SC-111), at the age of 8 weeks. Mice were sacrificed 2 months later, and subjected to histopathological examinations as described previously[13].

Immunohistochemistry and immunofluorescence staining

These were carried out essential as described previously[13]. In brief, prostate sections were treated with a hot citrate buffer, and subjected to IHC staining using the ABC Elite Kit and the DAB Kit (Vector laboratories, Burlingame, CA, USA) according to the manufacturers' protocols. PCNA (sc-56, 1:1000), ATF3 (sc-188, 1:200), AR (sc-816, 1:200), and p63 (sc-8430, 1:200) antibodies were purchased from Santa Cruz (Dallas, TX, USA). α-smooth muscle actin (α-SMA) staining were performed using an alkaline phosphatase (AP)-conjugated anti-α-smooth muscle actin antibody (Sigma, 1:600) and SIGMAFAST Fast Red TR/Naphthol AS-MX tablets (F4523, Sigma-Aldrich, St Louis, MO, USA) according to the supplier's protocol. For CK5/CK8 double staining, prostate sections were incubated with CK5 (PRB-160P, 1:500) and CK8 (MMS-162P, 1:500) antibodies (Biolegend, San Diego, CA, USA), followed by incubation with Alexa Fluor 594-conjugated anti-rabbit IgG (A-24923) and Alexa Fluor 488-conjugated anti-mouse IgG (A-21131, Life Technologies).

Cell culture and generation of ATF3-knockout PC3 cells

LNCaP and PC3 cells, originally from ATCC, were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. For hormone treatments, cells were first cultured in charcoal-stripped medium, followed by supplementing with 1 nM of R1881 (Perkin Elmer) or 10 nM of E2 (Sigma). To generate ATF3-knockout PC3 cells using the CRISPR-Cas9 technology, oligonucleotides containing a sequence for an ATF3-specific guided RNA (5'-AAAATGATGCTTCAACACCC-3') was inserted into pSpCas9(BB)-2A-Puro[43]. PC3 cells were transfected with the resulted construct for 2 days, selected with puromycin for 2 days, and then plated at a low density in 100-mm dishes. Individual clones were then expanded, and subjected to Western blotting for screening for clones lacking ATF3 expression. RWPE-1 cells were purchased from ATCC, and cultured in keratinocyte serum free medium (Invitrogen). Cells (the 3rd passage) in 24-well plates were transfected with 50 pmol of siATF3 or siLuc[13] for 3 days and then stained for CK5/CK8 expression.

Western blotting assays

Western blotting assays were performed as described previously[13]. In brief, cells were lysed in RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche), and then resolved in SDS-polyacrylamide electrophoresis for immunoblotting.

Data and statistical analysis

ATF3 expression data and clinical features of prostate cancer patients were retrieved from the Cancer Genome Atlas (TCGA) database. To test for the difference in ATF3 expression between normal and tumor samples, Student's t-test or paired t-test was used. In the survival analysis, all percentiles between the lower and upper quartiles of ATF3 expression were computed and the best performing threshold was used as a cut-off point for high and low ATF3 expression. The Kaplan-Meier method and the log-rank test were used to compare recurrence-free survival curves between high and low ATF3 expression groups.

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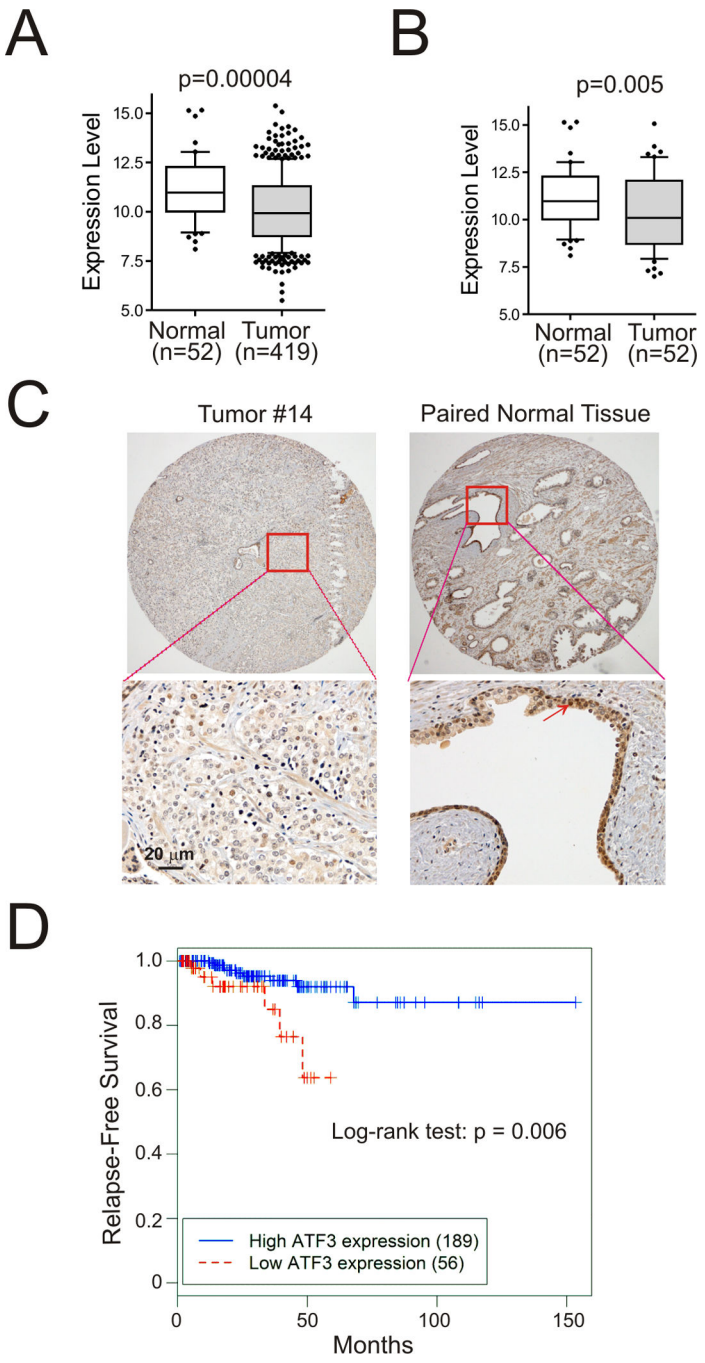

Figure 1. ATF3 expression is down-regulated in human prostate cancer

(**A**) ATF3 expression data measured by RNA-seq were retrieved from TCGA, and used for comparison between prostate cancer samples and normal tissues. The data are presented as box and whiskers (10–90 percentile). The p value was calculated by Student's t-test. (**B**) ATF3 expression was compared between prostate cancer samples and their paired normal tissues. The p value was calculated by paired Student's t-test. (**C**) Representative IHC results of ATF3 expression in human prostate tumors and their paired normal tissue. Tissue array slides from Super Bio Chips and US Biomax were stained for ATF3 expression by IHC. The

arrow indicates normal prostate epithelial cells with higher nuclear staining. (**D**) The Kaplan-Meier survival curves for patients with high or low ATF3 expression shows low ATF3 expression is a poor prognosis marker for prostate cancer.

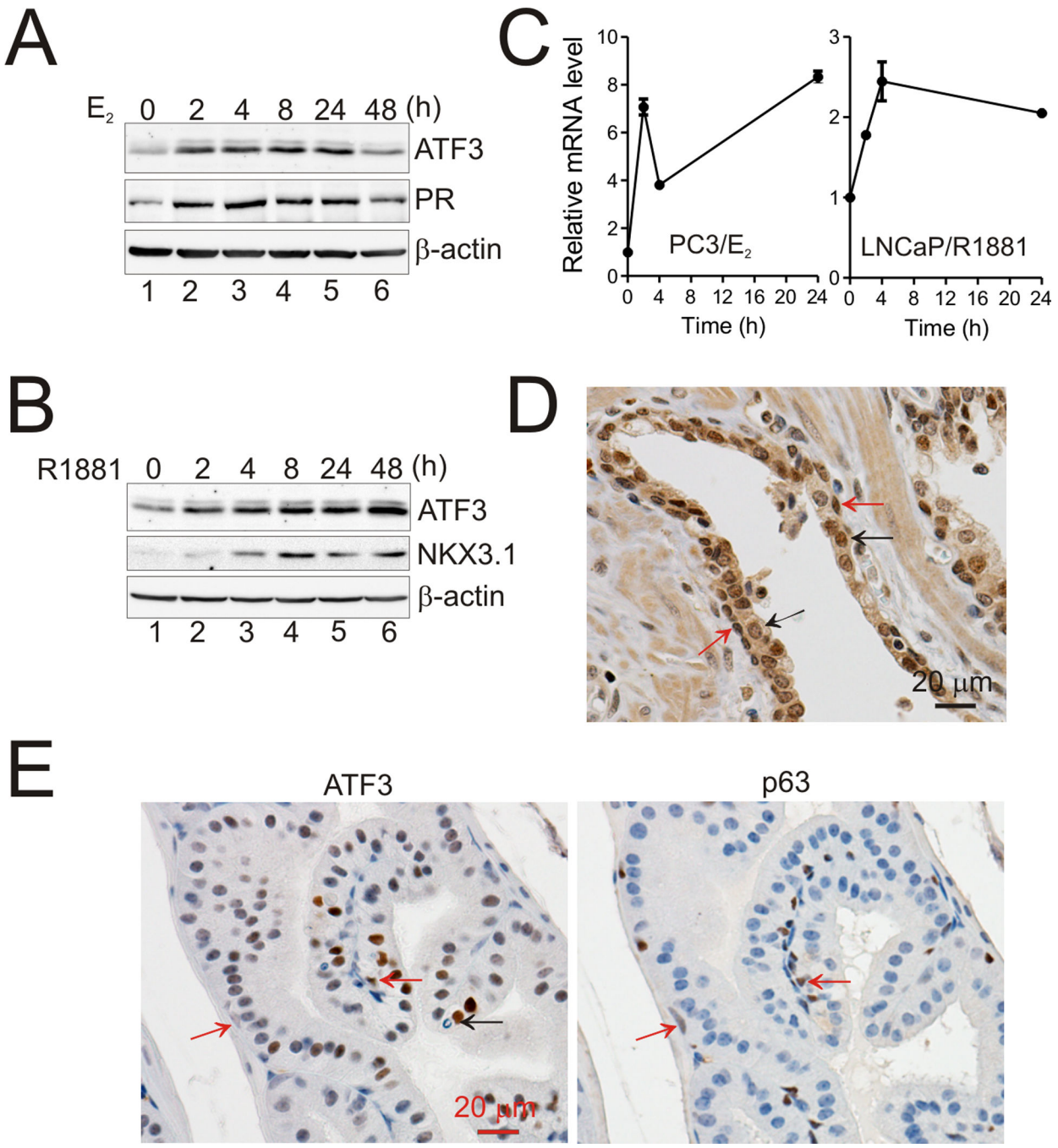

Figure 2. ATF3 is hormone-inducible and expressed in both prostate basal and luminal cells

(**A**) PC3 cells were treated with 10 nM of E_2 for Western blotting assays. (**B**) LNCaP cells were treated with 1 nM of R1881 for Western blotting analysis. (**C**) PC3 and LNCaP cells were treated with E2 and R1881, respectively, and then subjected to qRT-PCR to measure the ATF3 mRNA level. (D) Normal human prostate samples were stained for ATF3 expression by IHC. Red and black arrows indicate basal and luminal cells, respectively. (**E**) Adjacent serial sections of mouse anterior prostates were stained for ATF3 and p63

expression by IHC. Red arrows indicate two representative basal cells positive for both ATF3 and p63 staining.

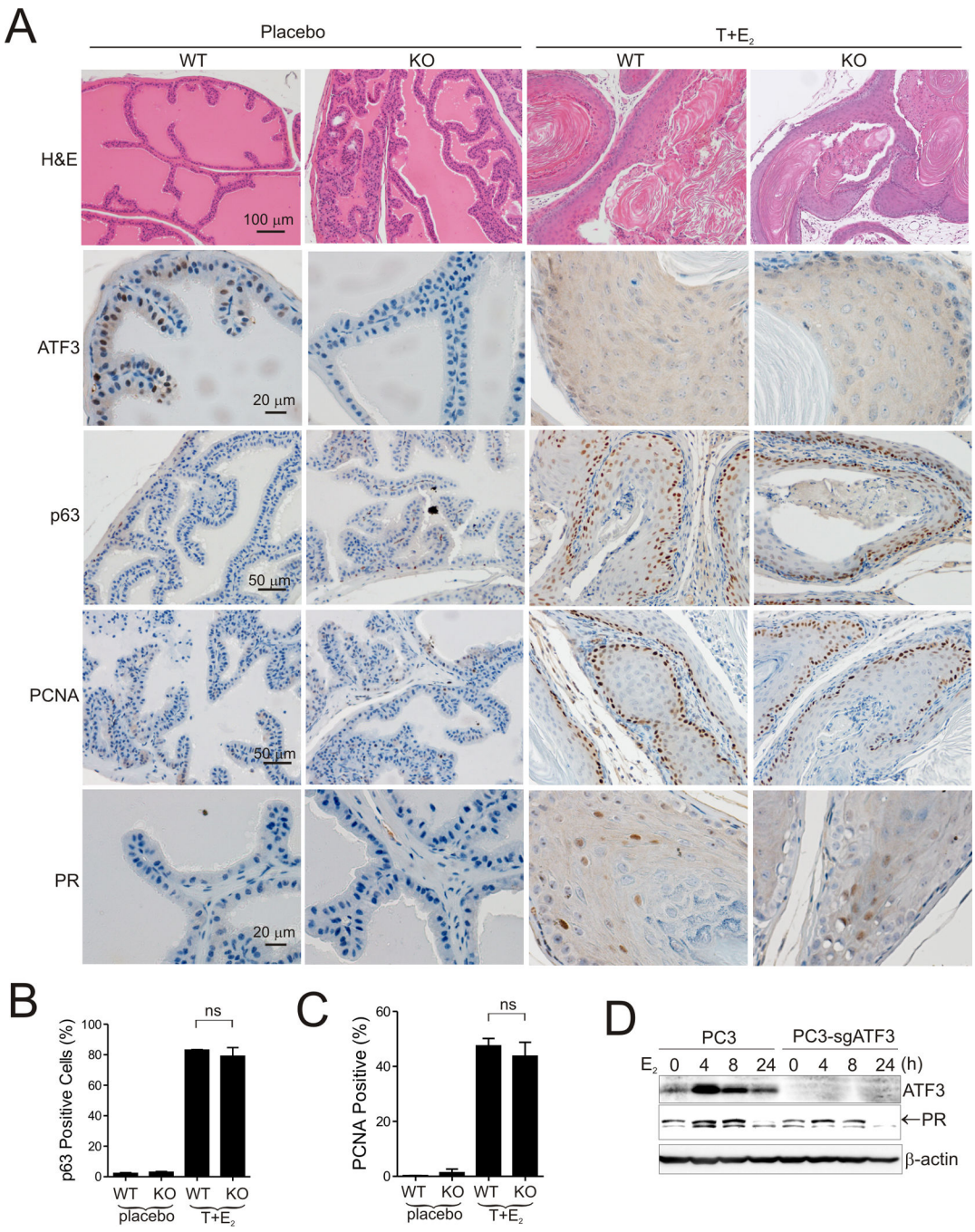

Figure 3. *ATF3* deficiency does not affect hormone-induced squamous metaplasia in anterior prostates

(**A**) Sections of anterior prostates from placebo and hormone-treated mice (T+E_2) were stained with H&E, or subjected to IHC staining, as indicated. (**B, C**) p63-positive (B) or PCNA-positive (C) cells were counted from random microscopic fields. Percentages of positive cells were depicted as mean ± SD and showed in the graphs. ns, no significant difference, Student's t-test. (**D**) PC3 cells and a clone lacking ATF3 expression (generated

by a single-guided RNA, i.e., sgATF3) were treated with 10 nM of E_2 for different time, and subjected to Western blotting for PR expression.

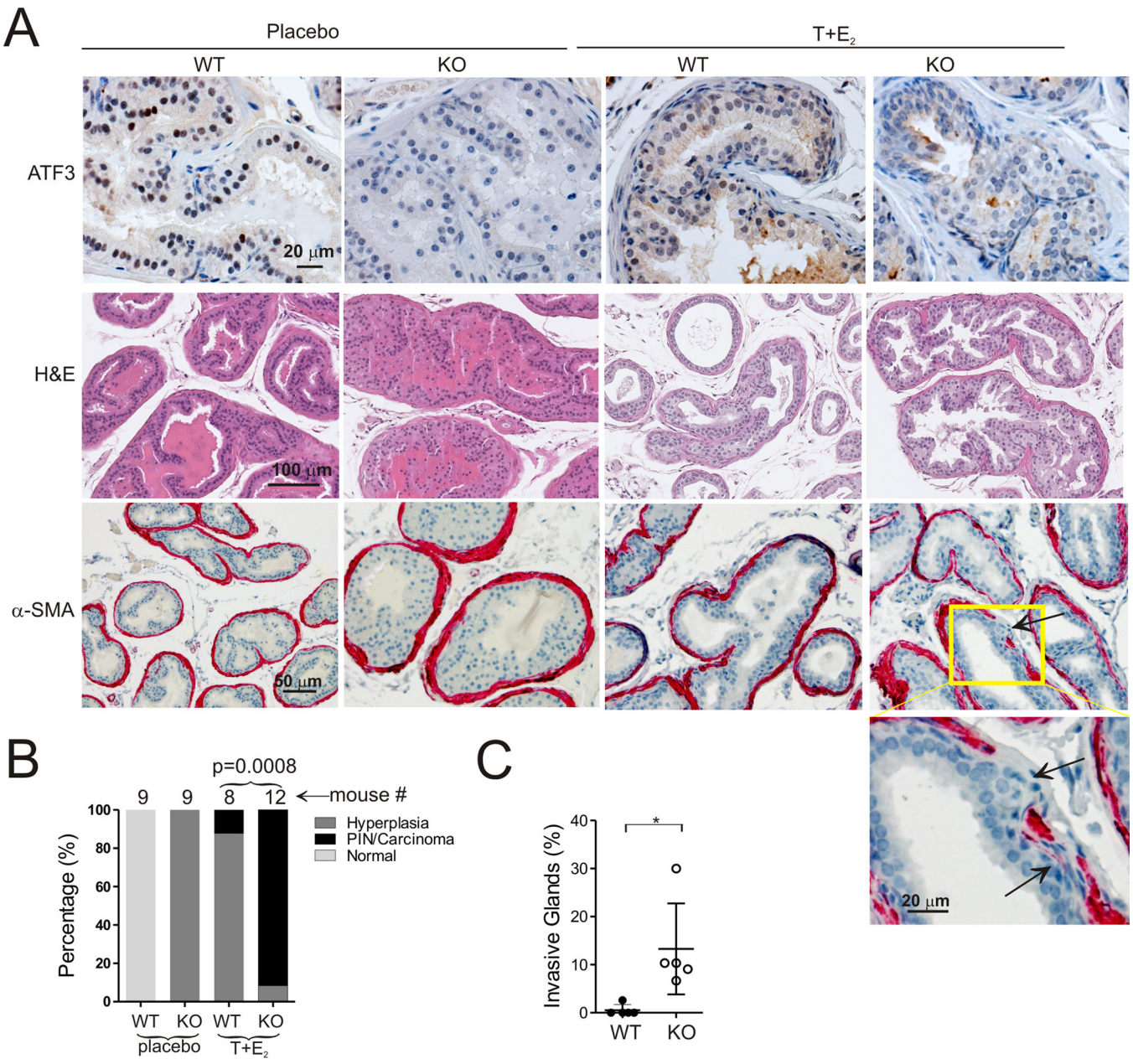

Figure 4. Loss of ATF3 promotes hormone-induced prostate carcinogenesis in dorsal prostates

(**A**) DP sections were subjected to H&E staining, or IHC staining for ATF3 and α-SMA expression as indicated. Arrows indicate invasion sites. (**B**) Percentages of mice with hyperplasia or PIN/carcinoma in their dorsal prostates. Fisher's Exact test. (**C**) Percentages of invasive glands in WT and KO mice (5 mice for each genotype) as determined by α-SMA staining. *, $p < 0.05$, Mann-Whitney test.

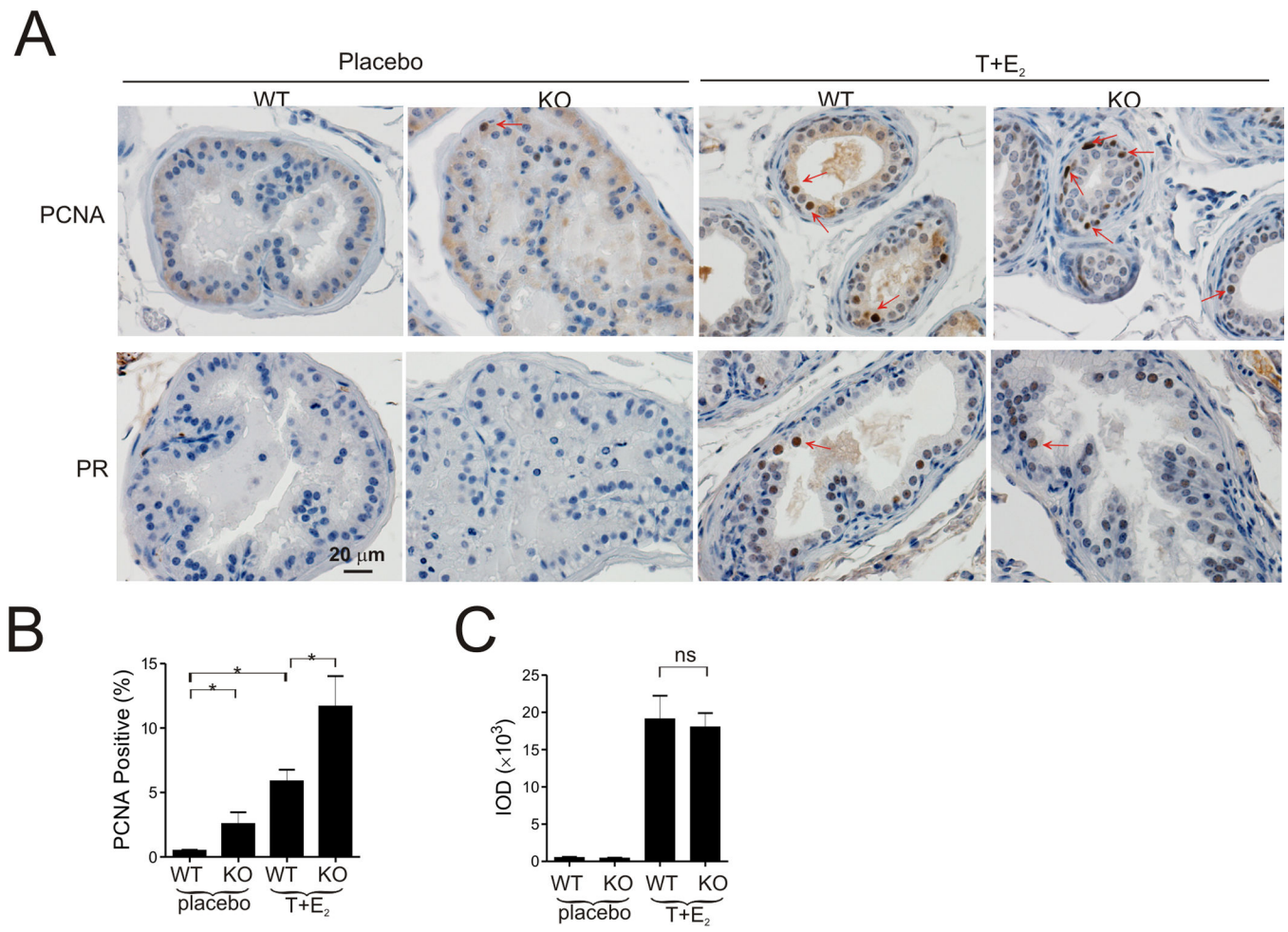

Figure 5. *ATF3* deficiency increases proliferation of prostatic epithelial cells in dorsal prostate

(**A**) DP sections were stained for PCNA and PR expression. Arrows indicate positive cells. (**B**) PCNA-positive cells were counted from random microscopic fields. The data were presented as mean ± SD. *, $p < 0.05$, Student t-test. (**C**) PR staining was quantitated using the Image-Pro Plus software and presented as integrated optical density (IOD). ns, not significant, Student's t-test.

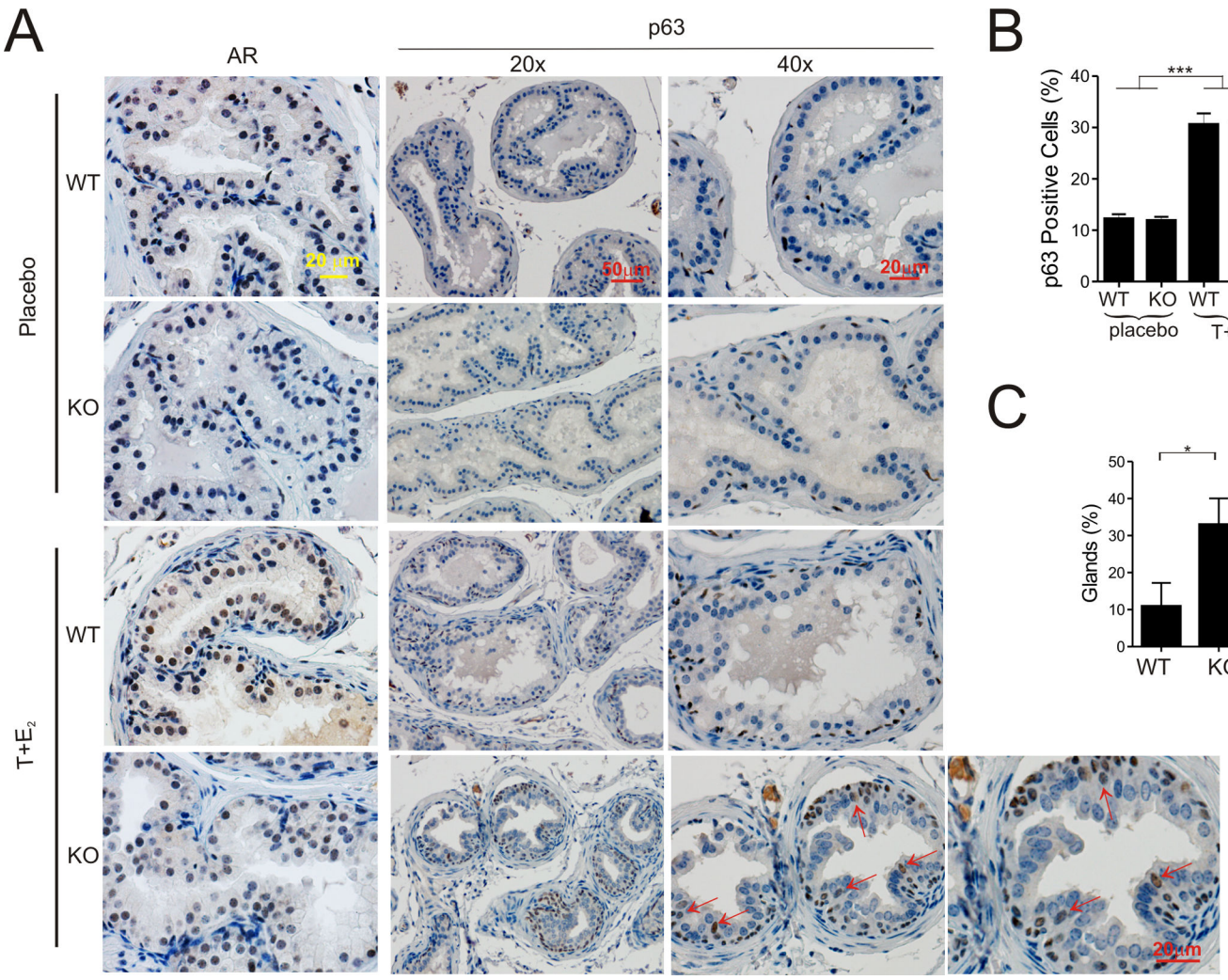

Figure 6. *ATF3*-deficient prostate lesions contain luminal-like, p63$^+$ cells

(**A**) DP sections were subjected to IHC staining for AR and p63 expression. Arrows indicate liminal-like, p63$^+$ cells. (**B**) Percentages of p63$^+$ cells. Cells in random fields (×20) (sections from 3 mice for each group) were counted. (**C**) DP glands containing luminal-like, p63+ cells were counted as (B) and presented as percentages in the graph. *, $p < 0.05$; ***, $p < 0.001$; Student's t-test.

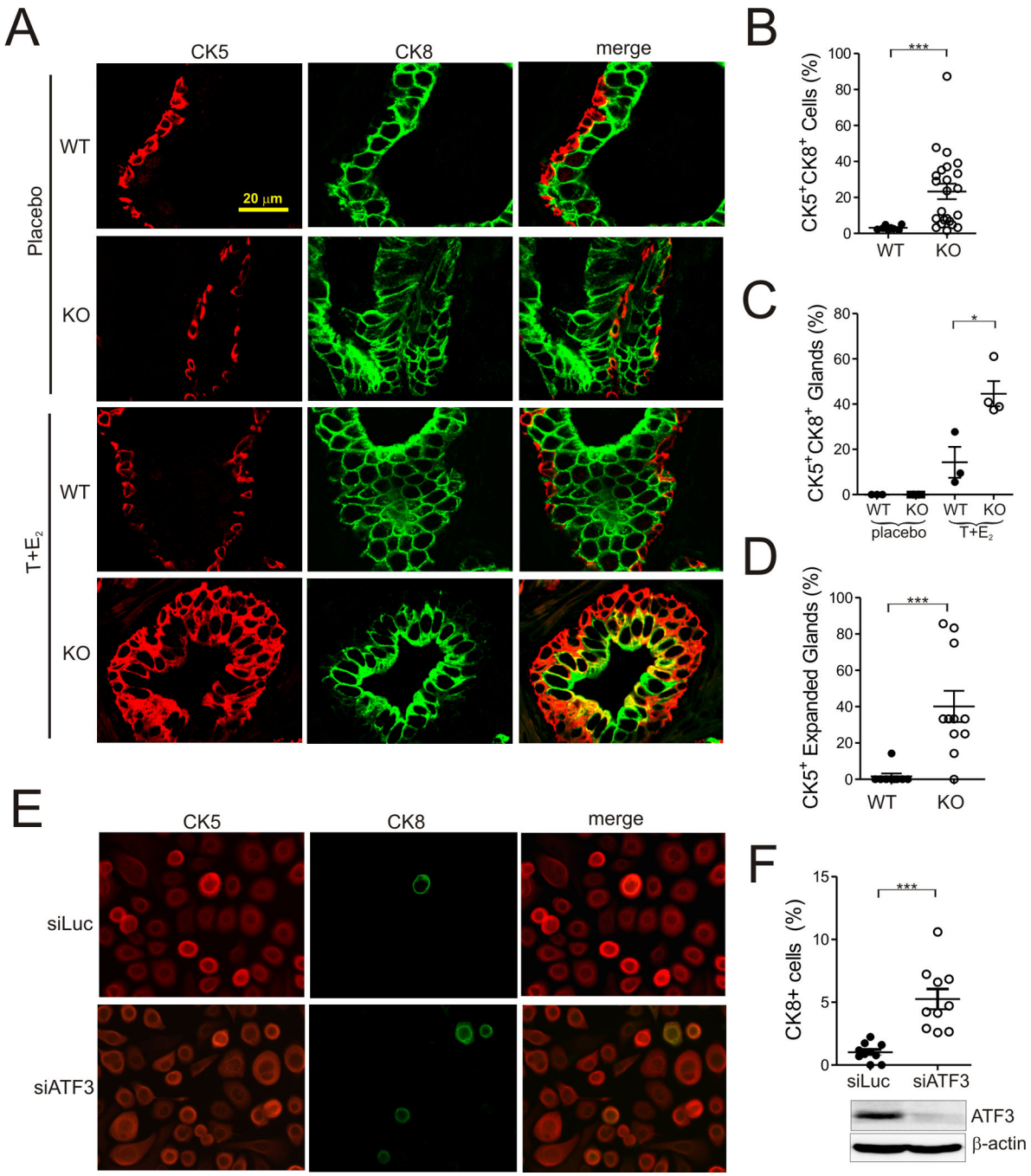

Figure 7. Loss of ATF3 increased the CK5$^+$CK8$^+$ cell number in T+E$_2$-treated dorsal prostates

(**A**) DP sections were double-stained with CK5(red) and CK8(green) antibodies, and observed under a confocal microscope. (**B**) CK5$^+$CK8$^+$ cells in each DP (from 3 to 4 mice each group) were counted, and presented as percentages of total epithelial cells. (**C**) Percentage of glands containing CK5$^+$CK8$^+$ cells for each group (3 to 4 mice for each group) is shown. (**D**) Glands with multiple CK5+ layers in random 20× fields were counted and presented as percentages of total glands. Only hormone-treated samples were scored in panel B and D. (E, F) RWPE-1 cells were transfected with siATF3 or a control siRNA

(siLuc) for 3 days, and then stained for CK5 and CK8 expression. CK8+ cells in ten random microscopic fields (×20) were counted and the numbers were depicted in (F). The Western blots under the graph show decreased ATF3 expression in siATF3-transfected cells. *, $p < 0.05$; ***, $p < 0.001$; Student's t-test.

Table 1

Mouse Phenotypes Induced by the Hormones

	ATF3 WT	ATF3 KO
AP	Squamous metaplasia	Squamous metaplasia
DP	1/8 PIN/invasive carcinoma 7/8 hyperplasia	11/12 PIN/invasive carcinoma 1/12 hyperplasia
VP/LP	Normal	Normal