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Loss of FOXO1 cooperates with TMPRSS2-ERG overexpression to promote prostate tumorigenesis and cell invasion

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Abstract

E26 transformation-specific (ETS) transcription factor ERG is aberrantly overexpressed in approximately 50% of all human PCa due to *TMPRSS2-ERG* gene rearrangements. However, mice with prostate-specific transgenic expression of PCa-associated ERG alone fail to develop PCa, highlighting that ERG requires other lesions to drive prostate tumorigenesis. Forkhead box (FOXO) transcription factor FOXO1 is a tumor suppressor that is frequently inactivated in human PCa. Here we demonstrate that FOXO1, but not other FOXO proteins (FOXO3 and FOXO4), binds and inhibits the transcriptional activity of PCa-associated ERG independently of FOXO1 transcriptional activity. Knockdown of endogenous FOXO1 increased invasion of TMPRSS2-ERG fusion positive VCaP cells, an effect completely abolished by ERG knockdown. Patient specimen

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Authors' Contributions

HH conceived the study. YY, AMB, DW, JA, YP, YYan, TM, YH, JD, XH, JZ, SJW, WGZ, YAW, and RAD generated mouse models, performed experiments, and analyzed the data. JZ, YY, YYan, and TM supervised and performed histological and IHC data analysis. HH, YY, AMB, and WX wrote the manuscript.

analysis demonstrated that FOXO1 and ERG protein expression inversely correlated in a subset of human PCa. Although human ERG transgene expression or homozygous deletion of *Foxo1* alone in the mouse prostate failed to promote tumorigenesis, concomitant ERG transgene expression and *Foxo1* deletion resulted in upregulation of ERG target genes, increased cell proliferation and formation of high-grade prostatic intraepithelial neoplasia (HGPIN). Overall, we provide biochemical and genetic evidence that aberrantly activated ERG cooperates with FOXO1 deficiency to promote prostate tumorigenesis and cell invasion. Our findings enhance understanding of PCa etiology and suggest that the FOXO1-ERG signaling axis can be a potential target for treatment of PCa.

Keywords

FoxO1; ERG; TMPRSS2-ERG; tumorigenesis; PIN; cell invasion; prostate cancer

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the third leading cause of cancer death in American men (1). Multiple genetic alterations including gene mutations, deletions and amplifications have been revealed by next generation high throughput sequencing in both primary and advanced PCa (2,3). However, the pathological consequence of many alterations detected in human PCa specimens has not been rigorously examined in cell culture systems and mouse models.

TMPRSS2-ERG gene rearrangements are one of the most frequent genetic alterations detected in human primary PCa (2,4). ERG is an oncogenic protein that belongs to the ETS transcription factor family (5). *TMPRSS2-ERG* gene fusions juxtapose the androgen-responsive *TMPRSS2* gene promoter with the *ERG* gene coding region. Such fusions result in aberrant overexpression of ERG in approximately 50% of both primary and advanced human PCa, suggesting a causal role of ERG in prostate tumorigenesis and progression.

Since the first report of the *TMPRSS2-ERG* gene fusion by Tomlins and colleagues (4), more than 20 different types of gene rearrangements have been identified in PCa patient samples (6,7). Among these, fusion of *TMPRSS2* exon 1 (a non-coding exon) to *ERG* exon 4 or 5 (designated as T1/E4 and T1/E5, respectively) are the two most frequently detected *TMPRSS2-ERG* rearrangements in patients (6,7). Notably, mice with prostate-specific transgenic expression of T1/E4 ERG develop very minor cancer precursor-like lesions, but not prostate neoplasia (8–11), suggesting that ERG requires other lesion(s) to drive prostate tumorigenesis. Indeed, further studies demonstrate that T1/E4 ERG cooperates with *Pten* deletion or AKT activation to induce PCa in mice (8,9,12). Conversely, ERG over-expression is also linked with aspects of later-stage tumors, such as cell invasion. ERG regulates expression of matrix metalloproteinase genes *MMP3*, *MMP9*, *ADAM19*, plasminogen activator pathway genes *PLAT* and *PLAU*, and chemokine receptor *CXCR4* in PCa (11,13). However, it remains unclear which other PCa-relevant lesions may cooperate with ERG over-expression to promote both prostate tumorigenesis and tumor progression.

Forkhead box transcription factors FOXO1, FOXO3, FOXO4, and FOXO6 (the human orthologs of *Caenorhabditis elegans* DAF-16 and *Drosophila melanogaster* dFOXO) are often recognized as tumor suppressors (14). Activation of these factors results in transcriptional upregulation of genes involved in apoptosis (e.g., *Bim* and *FasL*), cell cycle arrest (e.g., *p27^{KIP1}* and *p21^{CIP1}*), and oxidative stress detoxification (e.g., *MnSOD* and *Catalase*) (15–19). Mouse genetic studies demonstrate that somatic deletion of *Foxo* genes promotes formation of a cancer-prone condition characterized by thymic lymphomas and hemangiomas, suggesting that FOXOs are *bona fide* tumor suppressors (20). Moreover, FOXO1 and other FOXO proteins function as key downstream effectors of the PTEN tumor suppressor (21). Interestingly, the *PTEN* tumor suppressor gene is frequently deleted in human PCa and occurs concomitantly with ERG over-expression (9). PTEN loss leads to AKT and CDK1- or CDK2-mediated phosphorylation of FOXO1, exclusion of FOXO1 from the nucleus, and loss of the tumor suppressor functions in the nucleus (15,22–24). Increasing evidence suggests that AKT-phosphorylated FOXO1 also possesses tumor suppressor functions in the cytoplasm (25–27). In agreement with these findings, proteasome degradation of AKT-phosphorylated FOXO1 is critical for PI3K-AKT-mediated cell transformation and oncogenesis (28–30). In addition to PTEN defect-caused inactivation of FOXO1, the *FOXO1* gene is also frequently deleted at the genomic level or downregulated at the transcriptional level in human PCa (31).

In the present study, we demonstrate that FOXO1, but not FOXO3 or FOXO4, binds to PCa-associated ERG and inhibits ERG-mediated gene transcription, as well as subsequent ERG-mediated invasion of human PCa cells. Importantly, we also find that concomitant deletion of the *Foxo1* gene and transgenic expression of PCa-associated ERG promotes prostate tumorigenesis in mice, while each individual lesion alone has no such effect.

Materials and Methods

Plasmids and antibodies

Mammalian expression vectors for HA-tagged full-length wild-type ERG, HA-tagged T1/E4 ERG (39), and HA-tagged T1/E5 ERG (99) were described previously (32). Mammalian expression vectors for FLAG-FOXO1, FLAG-FOXO1-HR-537, HA-AKT-CA, PTEN WT, PTEN C142S, PTEN G129R, PTEN G129E, and GST-FOXO1 fusion protein vectors were described previously (33,34). GST-ERG fusion protein vectors were also described previously (32). Empty vector pcDNA3.1 was purchased from Thermo Fisher Scientific. *MMP9* promoter-based firefly luciferase reporter was acquired from Addgene. Antibodies used for Western blotting were: Santa Cruz Biotechnology: anti-ERG (sc353 and sc354), anti-ERK2 (sc1647); Sigma-Aldrich: anti-FLAG M2; Covance: anti-HA 1.1; Cell Signaling Technology: anti-FOXO1 (9462L), anti-AKT (9272S), anti-PTEN (9559L); Jackson Immuno Research Laboratories: anti-light chain specific rabbit IgG secondary antibody (211-032-171); GE Healthcare UK Limited: ECL anti-rabbit (or anti-mouse) IgG horseradish peroxidase linked whole antibody. Antibodies used for co-IP were: Vector Laboratories, Inc.: mouse IgG and rabbit IgG; Cell Signaling Technology: anti-Myc (2276), anti-FOXO1 (9462L). Antibodies used for ChIP were: Vector Laboratories, Inc.: rabbit IgG; Abcam: anti-ERG (ab92513).

Cell lines, cell culture, and transfection

The cell lines VCaP, PC-3, and LAPC-4 were purchased from ATCC (Manassas, VA) and authenticated via STR profiling. VCaP cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning cellgro) supplemented with 13% fetal bovine serum (FBS) (Thermo Fisher Scientific 10437028). PC-3 cells were cultured in RPMI 1640 medium (Corning cellgro) supplemented with 10% FBS (Thermo Fisher Scientific 10437028). LAPC-4 cells were cultured in Iscove's modified Dulbecco's medium (Corning cellgro) supplemented with 15% FBS (Thermo Fisher Scientific 10437028). Potential contamination mycoplasma was often checked using the Lookout Mycoplasma PCR Detection Kit purchased from Sigma-Aldrich. Cell culture medium was routinely supplied with Plasmocin (InvivoGen) to prevent mycoplasma contamination. Transfections were performed following manufacturer's instructions with Lipofectatmine2000 (Thermo Fisher Scientific) or by electroporation using Electro Square Porator ECM 830 (BTX) with Mirus Ingenio solution. Approximately 75–90% transfection efficiencies were achieved.

RNA interference

Cells were transfected with siRNA following manufacturer's instructions, by electroporation. Non-specific control siRNA was purchased from RIBOBIO (siN05815122147). siRNA for PTEN was purchased from Thermo Fisher Scientific (M00302302), for ERG was purchased from Dharmacon (M003886010005), and for FOXO1 was purchased from Dharmacon (D003006060020).

Cell invasion assay

Cell invasion was quantified by crystal violet staining using the Corning matrigel invasion chamber assay according to manufacturer's instructions (Corning). Cells were transfected with indicated plasmids or siRNA and cultured in medium with normal serum concentrations before plating in Corning matrigel invasion chambers in 24-well plates. Once in the invasion chambers, cells were cultured in serum-free medium inside the chamber, with medium containing 10% FBS outside the chambers. After 24 h, cells were fixed in methanol for 15 min and then stained with 1 mg/ml crystal violet in 10% ethanol for 30 min. After rinsing with water 3 times, the membranes of the chambers were mounted and covered on slides and observed using a light microscope. Eight fields of each view from three independent replicates were recorded and analyzed.

Cell migration assay

Migration assays were performed using a 24-well Transwell chamber system (Corning Inc). Cells were transfected with indicated plasmids and cultured in medium with normal serum concentrations before plating in Corning migration chambers in 24-well plates. Cells were seeded in the upper chamber at 1.5×10^4 cells/ml in 0.1 ml serum-free culture media. Media supplemented with 10% FBS was placed in the bottom well in a volume of 0.8 ml (used as a chemoattractant). After incubation for 24 h at 37°C in an atmosphere containing 5% CO₂, migrated cells on the lower surface were stained with crystal violet stain and counted under a light microscope. All experiments were repeated six times over the days.

Generation of prostate-specific *Foxo1* deletion and *ERG* over-expression mice

Foxo1 loxp/loxp (*Foxo1^{fl/p}*) conditional mice were originally generated in the laboratory of Dr. Ronald DePinho and reported previously (20). Transgenic *ERG* mice were purchased from The Jackson Laboratory (010929), originally generated in the laboratory of Dr. Valeri Vasioukhin at Fred Hutchinson Cancer Research Center, Seattle, WA (10). Probasin (*Pb*)-*Cre4* transgenic mice were acquired from the National Cancer Institute (NCI) Mouse Repository, originally generated in the laboratory of Dr. Pradip Roy-Burman at University of Southern California, Los Angeles, CA (35). *Foxo1^{pc-/-}*; *Pb-ERG* mice were obtained by cross breeding *Pb-Cre4* males with *Foxo1^{pc-/-}* and *Pb-ERG* females. All mice were maintained under standard conditions of feeding, light, and temperature with free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic.

PCR-based genotyping of mice

Genotyping of wild-type and conditional alleles of *Foxo1* genes as well as the *Cre* and *ERG* transgenes was performed following standard PCR protocol with primers listed in Supplementary Table S1.

Mouse prostate tissue section and Hematoxylin and Eosin (H&E) staining

Four-micron thick sections were cut from formalin-fixed paraffin-embedded (FFPE) mouse prostate tissues and mounted onto slides. Slides were deparaffinized with xylene and rehydrated through graded ethanol washes. Slides were then stained with hematoxylin, and washed with water followed by ethanol before counterstaining with 1% eosin. Lastly, slides were dehydrated through graded ethanol washes and xylene washes before coverslips were sealed over the tissue sections.

Immunohistochemistry (IHC)

Four-micron thick sections were cut from FFPE tissues and mounted onto slides. Antigen retrieval and immunostaining was performed as described previously (34). Antibodies used for IHC were: Abcam: anti-Ki-67 (ab15580), anti-ERG (ab92513); Cell Signaling Technology: anti-FOXO1 (29H4). Hematoxylin was applied for counterstaining. Cleaved Caspase 3 IHC detection was performed as described previously using Cleaved Caspase-3 IHC Detection Kit (Cell Signaling Technology).

Human PCa specimens and IHC scoring

Prostate cancer TMAs were purchased from US Biomax, Inc. As indicated by US Biomax, Inc (<https://www.biomax.us/FAQs#q10>), all tissues were collected under the highest ethical standards with the donors being formally informed and with their consent obtained. Given that US Biomax, Inc has the patient consent form in place already, the investigators of the current study did not acquire additional informed written consent from the subjects. However, the patient studies were conducted in accordance the ethical guidelines of Declaration of Helsinki and approved by Mayo Clinic Institutional Review Board (IRB). TMA specimens were used for antigen retrieval and immunostaining as described previously (36). Primary antibodies used were anti-FOXO1 (Bethyl) and anti-ERG (Abcam). Staining

intensity and staining percentage for each tissue was graded using a set of criteria. Intensity was graded 0 to 3, with 0 being no staining, 1 low staining, 2 medium staining, and 3 strong staining. A final staining index (SI) score for each staining was obtained by multiplying values obtained from staining percentage and intensity and used for correlation analysis.

Generation of graphs and statistical analysis

Cell culture experiments were carried out with three or more replicates. Statistical analyses were performed by the Student *t* test for cell culture and mouse tissue studies. Heat map and correlation for ERG and FOXO1 IHC were generated by R software (version 2.15.0 from <http://www.r-project.org>).

Results

FOXO1 inhibits transcriptional activity of PCa-associated *TMPRSS2-ERG* fusions

Because ERG cooperates with PTEN loss in prostate tumorigenesis (8,9,12,37) and FOXO1 is a key downstream effector of PTEN (21,38,39), we first examined whether the transcriptional activity is regulated by FOXO1. To this end, we performed *MMP9* promoter-based luciferase assay because *MMP9* is a well-studied ERG transcriptional target gene (40). As expected, expression of wild-type ERG (ERG-WT) and T1/E4 and T1-E5, two most frequently detected *TMPRSS2-ERG* rearrangements (Fig. 1A), increased the activity of the *MMP9* reporter gene in PC-3 cells, a *TMPRSS2-ERG* fusion-negative PCa cell line (40) (Fig. 1B). Importantly, ectopic expression of wild-type FOXO1 (FOXO1-WT) inhibited the transcriptional activity of ERG-WT, T1/E4 and T1/E5 ERG (Fig. 1B). This observation is consistent with previous reports that while a significant portion of ectopically expressed FOXO1-WT proteins retain in the cytoplasm of PTEN-deficient PCa cells like PC-3, some FOXO1 proteins are localized and functional in the nucleus (33). The ERG inhibitory activity of FOXO1 was largely enhanced by wild-type PTEN, but not tumor-associated, lipid phosphatase-deficient mutants (C124S, G129R and G129E) in PTEN-null PC-3 cells (Fig. 1C), which is consistent with the previous findings that restored expression of PTEN largely increased nuclear localization and activity of FOXO1 in PTEN-deficient PCa cells (21,33). Moreover, expression of the constitutively active FOXO1-A3, in which three AKT phosphorylation sites (Thr24, Ser256 and Ser319) were converted to alanine residues, largely inhibited ERG transcriptional activity and importantly, this effect was not enhanced by PTEN (Fig. 1D). Conversely, ectopic expression of constitutively active AKT (AKT-CA) in PC-3 cells completely reversed ERG inhibition induced by FOXO1-WT expression in a dose-dependent manner, but not by FOXO1-A3 expression (Fig. 1E and F). Next, we examined the effect of endogenous FOXO1 on expression of transcriptional target genes of endogenous *TMPRSS2-ERG* fusions. We demonstrated that knockdown of endogenous *FOXO1* (by a pool of three independent siRNAs) increased expression of ERG target genes (e.g. *PLAT*, *PLAU*, *ADAM19*, *MMP3*, and *MMP9* (11)) in T1/E4 ERG fusion-positive human PCa cell line VCaP (4), and similar results were obtained in PTEN knockdown cells (Fig. 1G and H). Notably, *FOXO1* and *PTEN* co-knockdown failed to further increase the expression of these genes (Fig. 1G and H), suggesting that PTEN and FOXO1 regulate ERG activity through the same pathway. Taken together, these data identify FOXO1 as a negative

regulator of ERG transcriptional activity, and this effect is inhibited due to PTEN loss or AKT activation.

FOXO1 directly interacts with ERG

To elucidate how FOXO1 regulates ERG transcriptional activity, we next explored whether ERG and FOXO1 can bind one another. Co-immunoprecipitation (co-IP) assays demonstrated that endogenous FOXO1 forms a protein complex with endogenous T1/E4 ERG in VCaP cells (Fig. 2A), indicating an interaction at the physiological condition. Ectopically expressed FOXO1 also interacted with expressed T1/E4 ERG, the most commonly occurring TMPRSS2-ERG rearrangement, in ERG-fusion negative PC-3 cells (Fig. 2B). This interaction is specific to FOXO1, as there was no detectable interaction of T1/E4 ERG with FOXO3 and FOXO4, two other FOXO family members expressed in PCa (23,41) (Fig. 2B). In vitro protein binding assays with purified GST-tagged fragments of ERG and endogenous FOXO1 from LAPC-4 cell lysate revealed that FOXO1 binds to the N-terminus of ERG (amino acids 1–200) (Fig. 2C and D). Conversely, using GST-tagged fragments of FOXO1 purified from bacteria and in vitro transcribed and translated ERG proteins, in vitro protein binding assays demonstrated that ERG binds directly to a central region of FOXO1 (amino acids 149–419) (Fig. 2E and F). Given that FOXO1 interacts with T1/E4 ERG, which lacks N-terminal 39 amino acids (Fig. 1A and 2A, B), these data indicate that FOXO1 binds to the region of ERG constituting of amino acids 40–200.

Both FOXO1 and ERG are highly expressed in normal endothelial cells (42,43). Endothelial cells are useful to determine whether FOXO1 interacts with ERG under physiological conditions in cells that normally express both proteins. Endothelial cells also express androgen receptor (AR) (44). Similar to the finding in PCa cells (45), co-IP assays demonstrated that ERG interacts with AR in HUVEC cells (Supplementary Fig. S1A). In contrast, no FOXO1-ERG interaction was detected under the same condition in HUVEC cells (Supplementary Fig. S1A). Although the exact molecular basis underlying the binding difference in different cell types is unclear at present and warrants further investigation, these findings are highly significant because it suggests that due to the presence of their interaction ERG functions are constrained to certain degree by FOXO1 in prostatic cells, but not in endothelial cells. As a result, FOXO1 deletion can be oncogenic in the prostate, but not in endothelial cells since loss of FOXO1 inhibition of ERG occurs specifically in prostatic cells.

FOXO1 inhibits ERG target gene expression independently of transcriptional activity

FOXO1-HR-537 is a DNA binding- and transactivation-dual deficient mutant of FOXO1, in which histidine 215 (a key residue for DNA binding) is mutated to arginine (H215R) and the transactivation domain (TAD, amino acids 538–655) is deleted (33,34) (Fig. 3A). To determine whether FOXO1 inhibition of ERG activity is mediated through protein-protein or the transcriptional activity of FOXO1, PC-3 cells were co-transfected with T1/E4 ERG in combination with FOXO1-WT or FOXO1-HR-537. Ectopic expression of each construct inhibited expression of ERG target genes *PLAU*, *ADAM19*, and *MMP9* to a similar extent (Fig. 3B). Luciferase assays also showed that ectopic expression of FOXO1-HR-537 inhibited the transcriptional activity of T1/E4 ERG to the same extent as FOXO1-WT (Fig.

3C). These data suggest that FOXO1 inhibits T1/E4 ERG in a manner independent of its DNA binding and transactivation functions. We further demonstrated that FOXO1 inhibition of ERG-mediated transcription does require the ERG-binding region in FOXO1 encompassing amino acids 167–354 (Fig. 2E, F and 3D). Thus, FOXO1 inhibits ERG-mediated gene expression via protein-protein interaction, but independently of FOXO1 DNA binding and transactivation functions.

FOXO1 inhibits ERG recruitment to target genes

Because protein-protein interaction inhibited ERG target gene expression, we examined the effect of FOXO1-ERG interaction on ERG recruitment to chromatin. ChIP assays in VCaP cells demonstrated that ectopic expression of either FOXO1-WT or transactivation-deficient mutant FOXO1-HR-537 invariably inhibited ERG recruitment to target genes *PLAU*, *MMP3*, and *MMP9* to a similar degree (Fig. 3E and F). Conversely, knockdown of endogenous *FOXO1* by a pool of three independent siRNAs in VCaP cells increased endogenous ERG binding to its target genes (Fig. 3G and 3H). These data further support our hypothesis that FOXO1 can inhibit ERG recruitment to target genes through protein-protein interaction. Given that FOXO1 does not directly binds to the DNA binding motif of ERG, the exact mechanism by which FOXO1 interaction reduces ERG recruitment to its downstream targets is unclear. However, our data cannot rule out the possibility that FOXO1 interaction with the N-terminal half of ERG may affect ERG DNA binding by causing overall conformation changes in ERG protein as demonstrated in IQGAP1, another FOXO1 interacting protein (27).

FOXO1 inhibits ERG-mediated PCa cell invasion and migration

Many ERG target genes of interest in this study regulate cellular invasion and migration in PCa (5), including *PLAT*, *PLAU*, *MMP3*, *MMP9*, and *ADAM19*. Previous reports have demonstrated that ERG over-expression in immortalized, nonmalignant *TMPRSS2-ERG* fusion negative prostatic epithelial cells (e.g. BPH-1 and RWPE cells) promotes cell invasion (8,10,46). We further explored how knockdown of endogenous *FOXO1* or *ERG* in *TMPRSS2-ERG* fusion positive VCaP cells affected cell invasion. Knockdown of *FOXO1* by a pool of three independent RNAs increased invasion of VCaP cells (Fig. 4A–C). This effect was reversed by co-knockdown of endogenous *ERG* (Fig. 4A–C). Similarly, the increase in cell invasion observed after ectopic expression of ERG in ERG fusion-negative PC-3 cells was abrogated by co-expression of either FOXO1-WT or transactivation-deficient mutant FOXO1-HR-537 (Fig. 4D and E). These results are consistent with previous findings from us and others that *TMPRSS2-ERG* is a known predominant factor in control of cellular invasion of fusion-positive cells such as VCaP (11), and that FOXO1 can inhibit ERG-mediated cell invasion independently of FOXO1 transcriptional activity. Next, we examined the effect of the ERG-binding region (amino acids 167–354) in FOXO1 on ERG-induced PCa cell migration and metastasis. Similar to the effect on cell invasion, we demonstrated that ERG expression in ERG fusion-negative PC-3 cells increased cell migration (Fig. 4F and G). This effect was reversed by co-expression of constitutively active FOXO1-A3, but not the ERG binding region-deletion mutant FOXO1-A3 167-354 (Fig. 4F and G). This data suggests that the ERG binding region in FOXO1 is important for FOXO1 inhibition of ERG-induced PCa cell migration.

Expression of FOXO1 and ERG inversely correlates in PCa patient specimens

It has been shown previously that expression of FOXO1 is often completely or partially lost due to genomic deletion, promoter methylation or transcriptional downregulation in human prostate cancer cell lines and patient specimens (31,41,47). These observations are further supported by the result obtained from The Cancer Genome Atlas (TCGA) data set (2) (Supplementary Fig. S1B). In contrast, ERG proteins are frequently upregulated through mechanisms of gene fusions and posttranslational modifications. We employed a tissue microarray (TMA) strategy to examine expression of these two proteins in a cohort of prostate adenocarcinoma specimens (n = 184 TMA elements) obtained from 92 patients. IHC was performed using protein-specific antibodies and staining was evaluated by measuring both staining intensity and percentage of positive cells. Representative IHC images showing high and low/no staining of FOXO1 and ERG and corresponding H&E staining are shown in Fig. 5A. In this cohort, 80 out of 184 (43.5%) TMA specimens were ERG positive, suggesting that the rate of ERG alterations is similar to that reported previously (2). By excluding the cases that were negative for both ERG and FOXO1 staining (n = 66 TMA elements), our further analysis showed that FOXO1 protein expression inversely correlated with ERG expression in the rest of specimens examined (Spearman's correlation $r = -0.3425$, $P = 0.000147$) (Fig. 5B and C). In support of this finding, TCGA data analysis showed that approximately 46% (21 out of 46) FOXO1-deleted specimens harbor ERG fusions (Supplementary Fig. S1B). Thus, both IHC and genomic data indicate that FOXO1 loss and ERG over-expression co-occurred at least in a subset of human prostate cancers.

Concomitant *Foxo1* deletion and *TMPRSS2-ERG* over-expression induces PIN formation and proliferation in mice

Because FOXO1 knockdown increased the transcriptional activity of ERG (Figs. 1 and 3) and FOXO1 loss and ERG protein over-expression correlated in human PCa specimens (Fig. 5 and Supplementary Fig. S1B), we generated *Foxo1* deletion and *TMPRSS2-ERG* transgenic compound mice to determine the role of the combination of *Foxo1* deletion and *TMPRSS2-ERG* over-expression in prostate tumorigenesis. *Foxo1* deletion and transgenic expression of ERG were confirmed by IHC in prostate tissues from double mutated mice (*Foxo1*^{pc-/-}; *Pb-ERG*) and their littermate controls: "Wild-type" (*Pb-Cre* negative), prostate-specific *Foxo1* deletion *Foxo1*^{pc-/-} (*Pb-Cre*⁺; *Foxo1*^{p/p}); prostate-specific transgenic T1/E4 *ERG* (*Pb-ERG*) (Supplementary Fig. S2). IHC for ERG also demonstrated expected nuclear expression of transgenic ERG and loss of Foxo1 in the appropriate mice (Supplementary Fig. S2). Importantly, H&E staining for all four lobes of the mouse prostate revealed the development of HGPIN in the double mutant *Foxo1*^{pc-/-}; *Pb-ERG* mice at 10 months of age, but not in the single mutant or "wild-type" mice (Fig. 6A). It is worth noting that even up to 12 months of age, only double mutant *Foxo1*^{pc-/-}; *Pb-ERG* mice displayed HGPIN (Fig. 6B). IHC for the cell proliferation marker Ki-67 demonstrated that *Foxo1* knockout alone moderately (three- or four-fold) increased the number of proliferating cells compared to "wild-type" in mice at 10 months of age (Fig. 6C and D). However, concomitant *Foxo1* knockout and T1/E4 ERG over-expression largely (approximately 15-fold) increased the number of proliferating cells compared to "wild-type" (Fig. 6C and D), which is consistent with the observed HGPIN formation and tumorigenesis (Fig. 6A). Additional IHC for

cleaved caspase 3, a marker of apoptosis in mice at 10 months showed no overt change in the number of apoptotic cells among all groups of mice examined (Supplementary Fig. S3), further supporting the idea that cell proliferation is the major contributor to PIN formation.

Previous studies show that the periacinar fibroblast layers appear to be wider in PIN lesions, implying a local activation of periacinar fibroblasts in response to PIN development (48). IHC for smooth muscle actin (SMA) demonstrated that the prostate acini in “wild-type”, *Foxo1* deletion and ERG transgene mice were surrounded by a highly condensed layer of SMA-positive stroma, which represents a natural barrier blocking the invasion of tumor cells into the adjacent stromal tissue (Fig. 6E). In contrast, loosened layers of SMA-positive stroma were detected in the prostates of *Foxo1* knockout and ERG transgenic compound mice (Fig. 6E). These data suggest that loss of FOXO1 and gain of ERG over-expression cooperate to drive PIN formation and a potential invasive phenotype in mice as early as 10 months of age.

Loss of FOXO1 abolishes its target gene expression, but enhances ERG target gene expression in the mouse prostate

We next examined the effect of *Foxo1* deletion on expression of the target genes of its own and ERG in the prostates of double mutant mice. FOXO proteins are implicated as tumor suppressors by transcriptionally upregulating cyclin-dependent kinase inhibitor *Cdkn1b* (called *p27^{KIP1}*) and pro-apoptotic gene *BCL2L11* (also called *BIM*). As shown in Fig. 7A, homozygous deletion of *Foxo1* largely diminished expression of *p27^{KIP1}*, which is consistent with increased cell proliferation in *Foxo1*-deleted prostates (Fig. 6C and D). As expected, *Foxo1* deletion also reduced expression of *Bim* although there was no overt difference in apoptosis between *Foxo1* intact and deleted mice (Fig. 7B and Supplementary Fig. S3). One plausible explanation is that the basal level of *Bim* expression in the prostates of “wild-type” mice is much lower than other genes such as *p27^{KIP1}* (Fig. 7C and D), and therefore may not be insufficient to trigger apoptosis. Intriguingly, it seems that expression of transgenic ERG increased expression of both *p27^{KIP1}* and *Bim* mRNAs, but the data varied greatly among replicates and they were not statistically significant (Fig. 7A and B). This result was further confirmed by RNA high throughput sequencing (RNA-seq) data obtained from independent mice (Fig. 7C and D). Moreover, in agreement with the development of loosen stroma layers in the prostates of *Foxo1* deletion and *ERG* overexpression compound mice (Fig. 6E), ERG target genes, known to be involved in cell invasion such as *Plau* and *Mmp3*, were up-regulated in the prostates of double mutant mice (Fig. 7E and F). This finding supports our human PCa cell line data that FOXO1 loss relieved inhibition of expression of ERG target genes linked with cell invasion (Figs. 3 and 4). Together, these findings provide an explanation for the increased proliferation of prostatic cells in double mutant mice. More interestingly, these findings also suggest that ERG-positive, FOXO1-negative tumors may be more invasive than ERG-negative counterparts.

Discussion

Large human PCa datasets have revealed that PCa is often characterized by the presence of multiple lesions (2,3). However, many of these combinations of lesions and the role they may play in PCa initiation and progression have not been functionally tested. This study is the first report to explore a role for concomitant FOXO1 inactivation and ERG over-expression in prostate tumorigenesis and cancer progression. Through a combination of PCa cell culture models and a mouse model with prostate-specific *Foxo1* knockout and transgenic *TMPRSS2-ERG* expression, we have demonstrated that ERG over-expression cooperates with loss of Foxo1 tumor suppressive activity and loss of Foxo1-mediated inhibition of ERG to increase cell proliferation, leading to HGPIN formation in mice (Fig. 7E). Additionally, increased expression of ERG target genes important for cell invasion in both the cell systems and double mutant mouse model further suggest that FOXO1 loss and ERG over-expression are also important for tumor progression (Fig. 7E). This study highlights that the FOXO1-ERG axis may be a valuable therapeutic target for PCa, and expands our knowledge of how these two overlapping lesions function to promote prostate tumorigenesis.

In particular, the data presented emphasize that FOXO1 loss and concomitant ERG expression play bifunctional roles in PCa. While FOXO1 inhibited ERG-mediated cell invasion, likely through decreased ERG chromatin-binding and decreased expression of ERG gene targets, the increased proliferation of prostatic cells in *Foxo1^{pc-/-};Pb-ERG* mice also contributes to HGPIN formation. These two phenomena (cell proliferation and HGPIN formation versus cell invasion) could contribute to both initiation and progression of prostate tumors. One remaining question that warrants further investigation is whether ERG controls a currently uncharacterized transcriptional program important for cell proliferation, which is held in check by FOXO1 in prostatic cells under normal conditions. While loss of FOXO1 tumor suppressive activity is expected to increase cell proliferation, there may be additional ERG-mediated regulation of the cell cycle. This idea is supported by the finding that ERG over-expression and FOXO1 loss have an additive effect on proliferation in the mouse prostate, greater than the increase in proliferation observed with FOXO1 loss alone.

Our findings also highlight a previously well-characterized functional link between PTEN, downstream AKT signaling, and FOXO1 activity. We demonstrated that both PTEN and FOXO1 have an inhibitory effect on ERG-mediated transcription, which can be rescued by expression of constitutively active AKT. Here, AKT activation through PTEN loss is thought to trigger FOXO1 phosphorylation and exclusion from the nucleus where it may no longer inhibit ERG simply due to spatial separation. This result suggests our findings are not only relevant to ERG-positive, *FOXO1* deletion tumors, but that a similar mechanism may occur in tumors with ERG over-expression and *PTEN* deletion.

The data presented in this study emphasize the importance of understanding how multiple lesions cooperate in PCa initiation and progression. With the advent of 'omics' data, researchers within the PCa field have rapidly expanded the list of lesions that may contribute to PCa development. Potential PCa therapeutics must account for the network of lesions and unique molecular interplay that occurs in any one patient. Thus, studies such as ours that

reveal additive, synergistic, or even antagonistic relationships between PCa lesions are extremely valuable to the understanding of the origins and drivers of PCa as well as of potential future therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

FOXO1 inhibits transcriptional activity of PCa-associated TMPRSS2-ERG fusions. **A**, The indicated are the ERG exons with the coding region highlighted in yellow and wild-type (WT), T1/E4 and T1/E5 truncated ERG proteins. NTD, N-terminal domain; PNT, pointed domain; CAE, central alternative exons; ETS, DNA binding domain; TAD, transactivation domain; Met, Methionine. **B**, *MMP9* luciferase reporter assay. PC-3 cells were transfected with the ERG reporter gene *MMP9*-Luc, a renilla luciferase reporter, and plasmids as indicated, followed by Western blot (WB) and luciferase activity measurement. ERK2 was used as loading control. * $P < 0.01$. **C**, *MMP9* luciferase reporter assay and WB, as

described in **B** in PC-3 cells transfected with indicated plasmids. **D**, *MMP9* luciferase reporter assay and WB, as described in **B** in PC-3 cells transfected with indicated plasmids. **E**, *MMP9* luciferase reporter assay and WB, as described in **B** in PC-3 cells transfected with indicated plasmids. **F**, *MMP9* luciferase reporter assay and WB, as described in **B** in PC-3 cells transfected with indicated plasmids. **G**, Western blot. VCaP cells were transfected with indicated siRNAs and harvested 48 h later for subsequent analysis. **H**, RT-qPCR. mRNA harvested from VCaP cells as in **G** was converted to cDNA for RT-qPCR analysis of ERG target gene expression. *GAPDH* was used as an internal control. * $P < 0.01$ compared to siControl.

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Figure 2. FOXO1 interacts directly with ERG. **A**, Western blot analysis of endogenous FOXO1 and ERG proteins in VCaP cells immunoprecipitated by indicated antibody or non-specific IgG. **B**, Western blot analysis of ectopically expressed proteins immunoprecipitated by indicated antibody or non-specific IgG in PC-3 cells transfected with indicated Flag- and Myc-tagged plasmids. **C**, Schematic depicting a set of GST-ERG recombinant protein constructs. **D**, Coomassie Blue staining of GST and GST-ERG recombinant proteins purified from bacteria (bottom), and Western blot analysis of FOXO1 proteins in LAPC-4 cells pulled down by GST recombinant proteins (top). Red arrow, non-specific band. * in red, unexpected, but

reproducible band shift. * in black, denotes expected fragment size. **E**, Schematic depicting a set of GST-FOXO1 recombinant protein constructs. **F**, GelCode Blue staining of GST and GST-FOXO1 recombinant proteins purified from bacteria (bottom), and Western blot analysis of in vitro transcribed and translated ERG proteins pulled down by GST recombinant proteins (top).

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Figure 3.

FOXO1 inhibits ERG target gene expression independently of FOXO1 transcriptional activity. **A**, Diagram depicting wild-type FOXO1 (FOXO1-WT) and DNA binding- and transactivation-deficient mutant FOXO1 (FOXO1-HR-537). ERG binding region is also shown. FKH (forkhead), DNA binding domain; NLS, nuclear localization sequence; NES, nuclear export sequence; TAD, transactivation domain. **B**, RT-qPCR analysis of expression of indicated ERG target genes in VCaP cells at 24 h after transfected with indicated plasmids. *GAPDH* was used as an internal control. * $P < 0.01$. **C**, *MMP9* luciferase reporter assay. PC-3 cells were transfected with the ERG reporter gene *MMP9*-Luc, a renilla

luciferase reporter, and plasmids as indicated, followed by Western blot and luciferase activity measurement. ERK2 was used as loading control. * $P < 0.01$. **D**, RT-qPCR analysis, as described in **B** in VCaP cells transfected with indicated plasmids. **E**, ChIP-qPCR. VCaP cells were transfected with indicated plasmids and then harvested after 24 h for ChIP using anti-ERG antibody or non-specific IgG. ERG binding to the *PLAU*, *MMP3* and *MMP9* loci was normalized to input. * $P < 0.01$. **F**, Western blot of lysates from VCaP cells transfected as in **E**. ERK2 was used as loading control. **G**, ChIP-qPCR. VCaP cells were transfected with indicated siRNAs (control or FOXO1-specific) and then harvested after 48 h for ChIP using anti-ERG antibody or non-specific IgG. ERG binding to the *PLAU*, *PLAT* and *MMP9* loci was normalized to input. * $P < 0.01$. **H**, Western blot of lysates from VCaP cells transfected as in **G**.

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Figure 4. FOXO1 inhibits ERG-mediated PCa cell invasion and migration. **A**, Western blot analysis of lysates from VCaP cells transfected with the indicated siRNAs. ERK2 was used as loading control. **B**, Cell invasion assay. VCaP cells transfected as in **A** were cultured in Corning matrigel invasion chambers for 24 h and then stained with crystal violet. Representative fields of view from invasion assay were shown. **C**, Quantification of results from cell invasion assay in **B**. * $P < 0.01$. **D**, Western blot and quantification of results of invasion assay in PC-3 cells transfected with indicated plasmids. * $P < 0.01$. **E**, Representative fields of view from invasion assay in **D**. **F**, Western blot and quantification of results of migration

assay in PC-3 cells transfected with indicated plasmids. * $P < 0.05$, ** $P < 0.001$, NS, not significant. **G**, Representative fields of view from migration assay in **F**.

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Figure 5. Expression of FOXO1 and ERG proteins inversely correlate in a subset of human PCa specimens. **A**, Representative images of H&E and IHC staining of FOXO1 and ERG in prostate adenocarcinoma patient tissues. **B**, Heat map showing the IHC staining index (SI) of FOXO1 and ERG proteins in TMA specimens in which both FOXO1 and ERG SIs were greater than 0 ($n = 118$). The scale bar indicates IHC SI. Each line in the heat map represents each TMA element. **C**, Correlation analysis of expression of FOXO1 and ERG proteins in TMA specimens in which both FOXO1 and ERG SIs were greater than 0 ($n = 118$). Non-parameter Spearman correlation co-efficiency and the P value are also shown.

Figure 6.

Foxo1 loss in combination with *TMPRSS2-ERG* over-expression promotes formation of prostatic intraepithelial neoplasia (PIN) in mice. **A**, H&E staining of mouse prostate sections from mice with the indicated genotypes at 10 months of age. DLP, dorsolateral prostate. VP, ventral prostate. AP, anterior prostate. Representative images were taken from 10 mice per group (n = 10/group). **B**, Summary of incidence of PIN lesions in genotypes of mice at 10 and 12 months of age. **C**, IHC for Ki-67 in prostate sections from mice with indicated genotypes at 10 months of age. Representative images were taken from 3 mice per group (n = 3/group). **D**, Quantification of Ki-67 positive cells from the tissue sections in C. * $P < 0.05$, ** $P < 0.01$, NS = Not Significant.

0.05, ** $P < 0.001$, NS, not significant. **E**, IHC for smooth muscle actin (SMA) in prostate sections from mice with indicated genotypes at 10 months of age. Representative images were taken from 3 mice per group ($n = 3/\text{group}$).

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Figure 7.

Expression of FOXO1 and ERG target genes in the prostates of Foxo1 deletion/ERG overexpression mice. **A** and **B**, RT-qPCR analysis of expression of FOXO1 target genes *Cdkn1b* (**A**) and *Bim* (*Bcl2l11*) (**B**) in the prostate of mice (n = 3) with the indicated genotypes at 10 months of age. * *P* < 0.05; NS, not significant. **C** and **D**, Expression of *Cdkn1b* (**C**) and *Bim* (*Bcl2l11*) (**D**) in wild-type (WT) and *ERG* transgenic (*Pb-ERG*) mice revealed by RNA-seq analysis (n = 3/group). **E** and **F**, RT-qPCR analysis of expression of ERG target genes *Plau* (**E**) and *Mmp3* (**F**) in the prostate of mice (n = 3) with the indicated genotypes at 10 months of age. * *P* < 0.05; ** *P* < 0.01. **G**, Hypothetical model depicting the

role of FOXO1 inactivation plus aberrant activation of over-expressed ERG due to genetic loss or function loss of FOXO1 in PCa initiation and progression. *, PCa-associated ERG fusion (N-terminally truncated). #, FOXO1 inactivation includes gene deletion and AKT and CDK1/2-mediated phosphorylation/nuclear exclusion of FOXO1 due to PTEN loss.

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