The transcription factor FOXF1 promotes prostate cancer by stimulating the mitogen-activated protein kinase ERK5

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Forkhead box F1 (FOXF1) is a stromal transcription factor that is not expressed in epithelial cells of normal prostate tissue. The role of FOXF1 in cancer is conflicting; its loss in some cancers suggests a tumor suppressive function, but its abundance in others is associated with protumorigenic and metastatic traits. Extracellular signal–regulated kinase 5 (ERK5) is associated with advanced-stage prostate adenocarcinoma (PCa) in patients. We detected a population of FOXF1-positive tumor cells in aggressive mouse and human PCAs. Using two murine orthotopic models of PCAs, we found that overexpression of FOXF1 in Myc-CaP and TRAMP prostate tumor cells induced tumor growth in the prostate and progression to peritoneal metastasis. Increased growth of FOXF1-positive prostate tumors was associated with increased phosphorylation of ERK5, a member of the mitogen-activated protein kinase (MAPK) family. FOXF1 transcriptionally induced and directly bound to promoter regions of genes encoding the kinases MAP3K2 and WNK1, which promoted the phosphorylation and activation of ERK5. Knockdown of ERK5 or both MAP3K2 and WNK1 in FOXF1-overexpressing PCa cells reduced cell proliferation in culture and suppressed tumor growth and tumor metastasis when implanted into mice. In human tumors, FOXF1 expression correlated positively with that of MAP3K2 and WNK1. Thus, in contrast to some tumors where FOXF1 may function as a tumor suppressor, FOXF1 promotes prostate tumor growth and progression by activating ERK5 signaling. Our results also indicate that ERK5 may be a new therapeutic target in patients with FOXF1-positive PCAs.

INTRODUCTION

Prostate adenocarcinoma (PCa) remains as one of the leading causes of cancer-related deaths for men in the western world. The National Cancer Institute estimates that 1 in 6 men in the United States will develop PCa in their lifetime, with an expected 1 in 36 men succumbing to the disease. Whereas organ-confined disease is often highly responsive to therapy, development of metastatic PCa reduces 5-year survival rates to only 28%. Several molecular mechanisms that govern the progression of PCa have been identified. Some of these include the loss of transcription factor NKX3.1 (1, 2), the increased expression of the gene encoding the oncogenic transcription factor MYC (3, 4); the presence of oncogenic gene fusions, including TMPRSS2-ERG translocations (5, 6); the loss of the phosphatase and tumor suppressor PTEN (7, 8); and enhanced mitogen-activated protein kinase (MAPK) signaling (9, 10). Although progress has been made clinically for patients with primary tumors, few therapeutic options are available for patients with metastatic disease. Thereby, uncovering more of the factors governing the development of invasive and ultimately metastatic PCa will present new opportunities for therapeutic intervention.

Extracellular signal–regulated kinase 5 (ERK5), also known as big mitogen-activated protein kinase-1 (B MK1), is the most recently discovered member of the MAPK family [reviewed in (11)]. ERK5 serves as an output for oncogenic receptor tyrosine kinase activity (12) and promotes osteosarcoma cancer cell invasion by inducing the expression of the genes encoding the transcription factor Slug and matrix metalloproteinase 9 (MMP9) (13). ERK5 promotes an inflammatory tumor microenvironment by inducing mRNA multiple inflammatory cytokines such as interleukin-1α (IL-1α), IL-1β, and IL-8; inhibiting ERK5 prevents the secretion of IL-1β (14). Increased ERK5 activity is associated with PCa progression in clinical specimens (15) and is shown to induce invasion by cultured PCA tumor cells through integrin–focal adhesion kinase signaling using electrode-based cell micromotion assays (16) and to promote metastasis in orthotopic models of PCa through the induction of multiple MMPs (17).

Forkhead box F1 (FOXF1) is a member of the winged helix family of transcription factors with a known role in embryonic development (18, 19). Postnatal expression of FOXF1 is confined to mesenchyme-derived cells, such as endothelium, fibroblasts, and smooth muscle cells, but not normal epithelial cells (20). Although FOXF1 is implicated in epithelial carcinogenesis, its functional role is controversial. In breast cancer cell lines, FOXF1 has been shown to function as a tumor suppressor and is inactivated through hypermethylation of its promoter (21). Hypermethylation of FOXF1 promoter was shown in a subpopulation of invasive ductal carcinomas (21). In colon and breast cancer cell lines, FOXF1 protects cells from DNA re-replication (22). Genomic analysis has shown that some human PCa cells have a loss of the 16q24 chromosome region, which contains several genes including FOXF1 (23). On the basis of these tumor suppressor properties of FOXF1 in breast and colon cancers, it was proposed that FOXF1 is most probably a tumor suppressor in PCa cells that contain genomic deletions of 16q24,
but this hypothesis had never been confirmed experimentally. In contrast, high amounts of FOXF1 are found in 78% of Hedgehog (HH)-positive non–small cell lung cancers, and this high abundance is positively correlated with metastasis (24). Increased expression of FOXF1 is also found in basal cell carcinoma, medulloblastoma, and rhabdomyosarcomas (25, 26). In addition, FOXF1 promotes a stem-like state in lung cancer cells (27) and activates epithelial-to-mesenchymal transition (EMT) in breast cancer cells (28), raising the possibility that FOXF1 can function as an oncogene. These conflicting findings in different cancer types suggest that the role of FOXF1 in carcinogenesis is complex and depends on tissue specificity.

Here, we investigated the role of FOXF1 in prostate cancer using two orthotopic mouse models of PCa and found that FOXF1 drives PCa tumor cell proliferation and invasion through transactivation of the ERK5 pathway. New-generation ERK5 inhibitors are currently in development (29); our findings suggest that ERK5 inhibitors may be therapeutic in patients with FOXF1-positive PCa.

RESULTS

FOXF1 is not detected in normal prostate epithelium, but its abundance is increased in a subset of cells in prostate tumors

Immunohistochemistry demonstrated that FOXF1 protein was present in the stromal cells of the normal mouse prostate but was undetectable in the prostate epithelium (Fig. 1A). However, during spontaneous prostate cancer development in SV-40 (simian virus 40) large and small T antigen–driven TRAMP (transgenic adenocarcinoma of mouse prostate) transgenic mice (30) and in Myc oncogene–driven ARR2PB-c-Myc/Hi-Myc-CaP transgenic mice (31), FOXF1 protein was detected in a subset of epithelial-derived tumor cells. Likewise, FOXF1 was detected in a subset of cells in human PCa (Fig. 1A).

Overexpression of FOXF1 in PCa tumor cells increases the growth of orthotopic xenografts in mice

To examine the role for FOXF1 in PCa, we used lentiviral vectors to overexpress murine Foxf1 and express the luciferase reporter in Myc-CaP murine PCa cells that have extremely low amounts of Foxf1 on the mRNA level that is undetectable on the endogenous protein level (Fig. 1B). The tumor cells were grafted orthotopically into the anterior prostate lobes of syngeneic male mice (32). Luciferase-transfected Myc-CaP cells containing the empty vector instead of the FOXF1 vector were used as controls. Overexpression of FOXF1 in Myc-CaP cells increased the mass of primary tumors (Fig. 1C, upper panel), increased luciferase intensity in the lower abdomen (Fig. 1C, bottom panel), and induced metastatic dissemination of Myc-CaP cells into the peritoneal cavity (Fig. 1D). About 90% of mice with FOXF1-positive tumor engraftments developed metastasis compared to only 10% of control mice (Fig. 1D). To ensure that the role of Foxf1 is not limited to the Myc-CaP model of PCa, we examined the effects of Foxf1 in TRAMP orthotopic grafts by transducing TRAMP cells with Foxf1-expressing lentivirus. Similar to the Myc-CaP tumor model, we observed larger tumor sizes in the TRAMP Foxf1-OE orthotopic engraftments (Fig. 1E). More than 75% of mice with TRAMP Foxf1-OE tumors had peritoneal metastasis, whereas no metastases were found in the control mice (Fig. 1F). These results demonstrate that FOXF1 promotes PCa growth and metastasis in two orthotopic mouse models of prostate cancer.

FOXF1 promotes tumor cell proliferation in two mouse models of prostate cancer

Increased tumor weights in FOXF1-overexpressing (herein called “Foxf1-OE”) Myc-CaP and TRAMP tumors were associated with the increased expression of proliferation-specific mRNAs, as shown by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), including mRNAs encoding cyclin B1, cyclin D1, CDC25b, Aurora kinase B, FoxM1, and cyclin E1 (Fig. 2 and A and B). Because these genes induce cellular proliferation in various tumor models (30, 33), immunohistochemistry was used to visualize cells undergoing cell cycle. The number of Ki-67– and phosphorylated histone H3–positive tumor cells was greater in Foxf1-OE Myc-CaP and TRAMP tumors than in controls (Fig. 2C). In vitro analysis of Foxf1-OE tumor cells confirmed the in vivo findings, showing significantly higher proliferation of Foxf1-OE Myc-CaP cells compared to control tumor cells containing empty vector (fig. S1, A to D). Staining for cleaved caspase-3, a marker of apoptosis (33), showed no significant difference in the number of apoptotic cells between control and Foxf1-OE Myc-CaP or TRAMP tumors (Fig. 2C). Together, these data suggest that Foxf1 induces prostate tumor growth by increasing the proliferation of tumor cells.

FOXF1 induces prostate cancer cell invasion and metastasis

As described above, one of the more prominent effects of FOXF1 was the induction of local metastasis to the seminal vesicles and throughout the peritoneal cavity. Histology [hematoxylin and eosin (H&E)] staining demonstrated the invasive phenotype in the Foxf1-OE Myc-CaP tumors (Fig. 3A), as well as a significantly higher number of peritoneal metastases detected per animal (Fig. 3B). The expression of mRNAs encoding known mediators of prostate cancer invasion and progression (34–37), including the matrix metalloproteinases MMP2, MMP9, MMP10, and MMP13, the transcriptional activator ETS translocation variant 1, and S100 calcium-binding protein A8, was significantly increased in Myc-CaP tumor cells expressing Foxf1 (Fig. 3C). There was no significant change in mRNA encoding stathmin 1, previously shown to be a prometastatic mechanism in PCa (Fig. 3C). Similar to Foxf1-OE Myc-CaP tumors, the TRAMP Foxf1-OE xenografts had high frequency of local metastases per animal (Fig. 3D) and exhibited a similar mRNA signature for genes involved in the tumor invasion and metastasis (Fig. 3E). Analysis of Foxf1-OE tumor cells in culture assays confirmed the in vivo findings, showing significantly higher invasion by Foxf1-OE Myc-CaP and Foxf1-OE TRAMP cells than control tumor cells expressing an empty vector (fig. S1, E to H). Together, these results demonstrate that PCa tumors expressing Foxf1 are more aggressive and have a greater capacity to invade surrounding tissues and metastatize to the peritoneal cavity.

FOXF1 increases the expression of EMT-associated genes in PCa grafts

Considering the evidence that Foxf1-OE tumors were more aggressive, we next examined the expression of known markers of EMT, which is often associated with PCa aggressiveness (38, 39). Within Foxf1-OE Myc-CaP orthotopic tumors, there was an overall decrease in the abundance of mRNA encoding the epithelial marker E-cadherin and a gain in that encoding mesenchymal marker N-cadherin (fig. S2A). The abundance of mRNA encoding the EMT-associated transcription factor SNAIL2 was increased in Foxf1-OE Myc-CaP tumors compared to control tumors (fig. S2A). TRAMP Foxf1-OE prostate tumors showed a similar pattern with the loss of epithelial E-cadherin and gain of mesenchymal markers N-cadherin and vimentin (fig. S2B). Furthermore, TRAMP Foxf1-OE tumors had increased abundance of mRNA encoding SNAIL1 as well as mRNAs encoding several other EMT transcription factors, including SNAIL2, TWIST1, and ZEB2 (fig. S2B). Immunohistochemical staining confirmed increased abundance of N-cadherin at the protein level as well in both Myc-CaP and TRAMP Foxf1-OE prostate tumors (fig. S2, C and D). These results suggest that Foxf1 promotes cellular reprogramming toward a

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mesenchymal identity in PCA tumor cells, likely contributing to the invasive phenotype.

**FOXF1 induces ERK5-MAPK signaling**

To determine molecular mechanisms underlying FOXF1-induced prostate carcinogenesis, we performed RNA sequencing analysis on RNA isolated from TRAMP control and TRAMP FoxF1-OE prostate tumors. Outputs were analyzed and categorized on the basis of functional annotation using ToppGene Suite software (40). This approach revealed a significant up-regulation of several components of the ERK5-MAPK signaling pathway (Fig. 4, A to C). These include the expression of Map3k2 and Wnk1, which encode mitogen-activated protein kinase kinase 2 (MAP3K2, also known as MEKK2) and an upstream kinase WNK lysine-deficient protein kinase 1 (WNK1), which are critical upstream activators of ERK5 (41, 42). Incidentally, Mmp9, a downstream transcriptional target of ERK5-MAPK signaling, was also induced in TRAMP and FoxF1-OE Myc-CaP prostate tumors, as assessed by qRT-PCR (presented above; Fig. 3, C and E). Other genes with increased expression were Mmp10, Mmp13, EphA7, and Cbl (Fig. 4C); these genes encode proteins that are involved in cell invasion, cell-cell communication, and aggressive tumor phenotypes, respectively. Changes in mRNAs were confirmed by qRT-PCR in both TRAMP and Myc-CaP xenografts (Figs. 4D and 5A and fig. S3A). Consistent with activation of ERK5 signaling, Western blot and immunohistochemistry showed increased phosphorylation of ERK5 protein in FoxF1-OE tumors (Fig. 5, B and C, and
Fig. 2. FoxF1 induces cellular proliferation in Myc-CaP and TRAMP orthotopic tumors. (A and B) qRT-PCR analysis of the expression of mRNAs encoding Foxf1 and cell cycle–associated genes in control and FoxF1-OE Myc-CaP tumors (A) and TRAMP (B), normalized against the expression of mRNA encoding β-actin. Total mRNA was isolated from prostate tumors 4 weeks after orthotopic inoculation of Myc-CaP or TRAMP cells, respectively. Data are means ± SEM from 10 tumors per group. (C) Immunohistochemical staining for FoxF1, Ki-67, phosphorylated histone 3 (PH3), and cleaved caspase-3 in control and FoxF1-OE Myc-CaP and TRAMP tumors harvested at 4 weeks after orthotopic inoculation of cells. Data are means ± SEM from 10 random microscope fields in tumors from 8 to 10 mice per group. Images are magnified at ×200; scale bar, 50 μm. *P < 0.05, unpaired Student’s t test.

Fig. 3. FoxF1 induces prostate cancer invasion and metastasis. (A) H&E staining of prostates bearing control or Foxf1-overexpressing (FoxF1-OE) Myc-CaP tumor cells. Images are magnified at ×100; scale bar, 100 μm. (B) Peritoneal metastatic lesions per mouse bearing control or FoxF1-overexpressing Myc-CaP tumors. Data are means ± SEM from 10 mice per group. (C) qRT-PCR assessing the expression of mRNAs of invasion-associated genes (Mmp2, Mmp9, Mmp10, Mmp13, Etv1, S100A8, and Stmn1) in control and FoxF1-overexpressing Myc-CaP tumors. mRNA abundance was normalized to that encoding β-actin. Data are means ± SEM from 10 mice per group. (D and E) As in (B) and (C), respectively, for control or FoxF1-overexpressing TRAMP tumors. *P < 0.05, unpaired Student’s t test.
Because mRNA expression of Wnk1 and Map3k2 kinases, both the upstream activators of ERK5 (41, 42), was increased in FoxF1-OE murine tumor cells and was associated with increased ERK5 signaling, we examined the possibility that FOXF1 regulates the transcription of Wnk1 and Map3k2 genes. Several potential FOXF1-binding sites were found in Wnk1 and Map3k2 promoters (Fig. 5, D and E). Chromatin immunoprecipitation (ChIP) assays in Myc-CaP cells demonstrated that FOXF1 directly bound to the −5667/−5680 but not to the −1623/−1636 region of Map3k2 promoter (Fig. 5F). Likewise, FOXF1 bound to the −4458/−4471 but not to the −2957/−2970 region of Wnk1 promoter (Fig. 5G). Promoters of both Wnk1 and Map3k2 were cloned into luciferase-expressing vectors and then cotransfected with a FOXF1 vector into human embryonic kidney (HEK) 293T cells. Relative luciferase signal indicated that FOXF1 increased the transcriptional activation of both Wnk1 and Map3k2 promoters, indicating that Wnk1 and Map3k2 genes are direct transcriptional targets of FOXF1 (Fig. 5, H and I). Immunohistochemistry of Myc-CaP xenografts supported these data, in that MAP3K2 staining was substantially increased in FoxF1-OE tumors and correlated with increased staining for phosphorylated ERK5 (Fig. 5C). Thus, FOXF1 transcriptionally activates Map3k2 and Wnk1 promoters and increases the phosphorylation of ERK5.

Among human PCa cell lines, we identified 22RV1 and C4-2B cells as expressing high endogenous FOXF1 at both the mRNA and protein levels (fig. S4, A and E). In both human PCa cell lines, knocking down FOXF1 decreased tumor growth in orthotopic mouse models (fig. S4B), reduced the amount of MAP3K2 and WNK1 at both the mRNA and protein levels (fig. S4, C to E), and reduced the amount of ERK5 phosphorylation in tumor cells (fig. S4E). These results indicate that FOXF1 promotes growth and ERK5 signaling in 22RV1 and C4-2B human prostate tumors.

### Inactivation of ERK5 or WNK1 and MAP3K2 Reduces Tumor Growth and Metastasis in FOXF1-Positive Prostate Tumors

We next examined whether FOXF1 promotes prostate cancer growth and progression through the ERK5 signaling pathway. Considering that upstream activators of ERK5 pathway are transcriptional targets of FOXF1, targeting ERK5 itself represents a point where the two pathways converge, likely interfering with any potential compensatory mechanisms that might exist in the absence of either MAP3K2 or WNK1. Myc-CaP cells stably expressing FOXF1 were transduced with either lentivirus containing short hairpin RNA (shRNA) against Erk5 or control shRNA. Transduced Myc-CaP cells were then orthotopically transplanted into syngeneic mouse prostate. Depletion of ERK5 in FoxF1-OE tumors decreased tumor size and mass to those similar to control tumors (Fig. 6, A to C) and prevented the development of peritoneal metastasis in these mice (Fig. 6, A)

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and D), indicating that FOXF1 induced prostate cancer metastasis through ERK5. Treating cultured cells with an ERK5-specific pharmacological inhibitor, XMD8-92 (29), decreased their proliferation (Fig. S5, A and B), invasion (Fig. S5, C and D), and migration (Fig. S5, E and F). In addition, depletion of ERK5 decreased the abundance of cell proliferation markers in tumor cells to that observed in control tumors (Fig. 6E) and decreased FOXF1-induced changes in the expression of genes associated with cell cycle (Fig. 6F), EMT (Fig. 6G), and extracellular matrix degradation (Fig. 6H), supporting the notion that FOXF1 induces an invasive phenotype in PCa tumors through activation of ERK5. Thus, FOXF1 induces prostate tumor cell proliferation, invasion, and metastasis through ERK5.

Consistent with above data suggesting a role for MAP3K2 and WNK1, depletion of both MAP3K2 and WNK1 from Myc-CaP tumor cells before orthotopic implantation into mice decreased tumor growth (Fig. 7A), inhibited peritoneal metastasis (Fig. 7B), and reduced the abundance of phosphorylated ERK5 (Fig. 7, C and D). Furthermore, in human PCa tumors, the expression of FOXF1 was increased in a subset of the 58 patient samples (Fig. 7E and fig. S6), and the expression of FOXF1 positively correlated with that of WNK1 and MAP3K2 promoters relative to control Ig. Together, our data indicate that FOXF1 stimulates prostate tumor growth and progression by inducing ERK5 signaling in tumor cells.

**DISCUSSION**

Previous studies have implicated FOXF1 as an oncogene (28), whereas others have reported it as a tumor suppressor (21, 43). Although these studies appear to be conflicting, it is possible that FOXF1 has different functions depending on cell or tissue specificity. It is also possible that mutation status of tumor cells determines whether FOXF1 functions as an oncogene or tumor suppressor. Although the regulation of FOXF1 in tumor cells is largely unknown, tissue-specific contexts may account for activation of its expression in a subset of mouse and human PCa. To understand the context and effects of FOXF1 within the tumor cells in this study, we used orthotopic transplantation models for PCa. We found that neither c-Myc nor T antigen was sufficient to induce
FOXF1 in prostate tumor cells because neither Myc-CaP nor TRAMP-C2RE3 cells expressed Foxf1 in cell culture conditions. By forcing ectopic expression of FOXF1 in these cell lines and subsequently transplanting these cells into the prostates of mice, we have uncovered a mechanism by which FOXF1 promotes PCa tumor growth and progression, that is, through activation of the ERK5-MAPK signaling pathway.

FOXF1 appears to be important for multiple steps in PCa tumor progression, including tumor cell proliferation, invasion, and metastasis. Ectopic FOXF1 expression promoted tumor growth and metastatic dissemination to seminal vesicles and the peritoneal cavity in a manner dependent on the ERK5 pathway. Whereas aberrant ERK5 signaling has been shown to induce prostate tumor progression (14–17), factors that transcriptionally regulate this pathway are largely unknown. We found that FOXF1 transcriptionally activates Wnk1 and Map3k2, both of which are involved in the phosphorylation and activation of ERK5. Given that novel ERK5 inhibitors are in development (29) and that FOXF1 directly targets this pathway, it is possible that ERK5 may be an effective target in FOXF1-positive prostate cancers. It is also possible that the increased amount of FOXF1 in tumor cells within human PCa clinical specimens may be a predictive marker of ERK5 activation and consequently of tumors that are more likely to respond to ERK5 inhibitors.

Previous studies demonstrated that a rare subset of human PCa contains genomic deletion in 16q24 locus, which includes the FOXF1 gene (23). These studies indicate that human 16q24 chromosome region likely contains a tumor suppressor. We found that suppression of WNK1 and MAP3K2 protein abundance inhibited the activation ERK5 and reduced the growth of FOXF1-positive tumors, suggesting that FOXF1 activates ERK5 signaling through WNK1 and MAP3K2. Given these results, it is unlikely that FOXF1 is the 16q24 tumor suppressor as was originally proposed. The 16q24 region contains several important genes, including two forkhead transcription factors, FOXC2 and FOXL1, and the noncoding RNA Fender, all of which have been implicated in regulating cell proliferation during embryonic development, vascular growth, and intestinal morphogenesis (44–46). Therefore, it is possible that one of these...
MATERIALS AND METHODS

Mouse cell lines and orthotopic and transgenic tumor models
Foxf1-expressing plasmid pGIPZ-VSVG-GFP-Foxf1 and the control plasmid pGIPZ-VSVG-GFP were used to generate lentiviruses at Cincinnati Children's Hospital Viral Vector Core. TRAMP-C2RE3 PCa cells [named TRAMP herein; (30)] and Myc-CaP PCa cells (47) were transduced with lentiviruses. After 2 days, green fluorescent protein (GFP)-expressing cells were sorted using flow cytometry. FOXF1 expression was confirmed using qRT-PCR and Western blot. Subconfluent cell cultures were trypsinized, counted, and resuspended to a density of 200,000 cells/ml for TRAMP and 50,000 cells/ml for Myc-CaP in either phosphate-buffered saline (PBS) or type I collagen (BD Biosciences). A total of 10 µl of cell suspensions was injected directly into the anterior prostatic lobe of wild-type 9- to 11-week-old male mice (C57BL/6 for TRAMP cells and FVBN for Myc-CaP cells). Orthotopic tumors were harvested and microdissected at 5 weeks (TRAMP) and 4 weeks (Myc-CaP) after implantation. For the in vivo tumor imaging, firefly luciferase was cloned into a pPRLL-SIN-cPPT-MNDU3-WPRE (pTMWL) lentiviral construct (gift from P. Malik, Cincinnati Children's Hospital Medical Center). Lentiviral particles, generated as previously described (30), were used to induce luciferase expression and subsequently orthotopically injected into the prostates of mice. Tumors were imaged in vivo using the IVIS Spectrum Imaging System (PerkinElmer). Briefly, mice were sedated using 1 to 2% constant isoflurane flow. Mice were injected intraperitoneally with potassium luciferin salt (150 mg/kg; Gold Biotechnology) dissolved in PBS and imaged about 15 min after injection by the IVIS system according to the manufacturer's instructions. The spontaneously developed tumors from the TRAMP (32, 33) (23 weeks old) and Hi-Myc/ARR2PB-c-Myc (30) (30 weeks old) transgenic mice were dissected, fixed, sectioned, and used for immunohistochemistry. All animal studies for this study were approved by the Animal Care and Use Committee of Cincinnati Children's Hospital Research Foundation.

Human prostate carcinoma cell lines and orthotopic models
LNCaP, DU145, PC3, 22RV1 (purchased from the American Type Culture Collection; gift from S. Kasper, University of Cincinnati) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (Gibco). Plasmid constructs were obtained from the CCHMC shRNA Screening Core. Cells were transduced with lentivirus produced and purified by the CCHMC Viral Core Facilities. Cells were selected 2 days after transduction with puromycin. Knockdown was confirmed using qRT-PCR before tumor experiments. Subconfluent cell cultures were trypsinized, counted, and resuspended in type I collagen (BD Biosciences) to a density of 500,000 cells per injection. Suspended cells (10 µl) were injected directly into the anterior prostatic lobe of NOD-SCID-γ (nonobese diabetic males of about
9 to 11 weeks old). Orthotopic tumors were harvested and microdissected at 4 weeks after implantation.

**Immunohistochemistry**

Immunostaining was performed as described (32). The following antibodies were used for immunohistochemistry: FOXF1 (19), Ki-67 (Thermo Scientific, RM-9106-S0), phosphorylated histone H3 (Santa Cruz, 8656-R), cleaved caspase-3 (R&D Systems, AF835), ERK5 (Cell Signaling, 3552S), phosphorylated ERK5 (Cell Signaling, 3371S), and MAPSK2 (Abcam, ab33918).

**Chromatin immunoprecipitation**

Myc-CaP PCa cells stably expressing either FOXF1 or control vector were harvested, cross-linked using formaldehyde, sonicated to produce fragments of about 500 to 1000 base pairs (bp) in size, and immunoprecipitated using FOXF1 antibody (19) as previously described (48, 49). Reverse cross-linked ChIP DNA samples were subjected to qPCR using oligonucleotides specific to promoter regions of mouse Map3k2 (−5667/−5680 (5′-TAGTAGGAGGACAGACAGGG-3′) and 5′-GCTTGACACTAAGGCCACA-3′) and −1623/−1636 (5′-GGCAACCCACACTGTTT-3′ and 5′-CACCACATGGCCTCTGTTCCA-3′) or mouse Wnk1 (−4458/−4471 (5′-CACACAGAGAGAGGTCAAA-3′ and 5′-GCTCCTTGTGTCAGATC-3′) and −2957/−2970 (5′-CTTGGTGGCGGAGTTTGT-3′ and 5′-CAAGGGTCTCAAAGCATTTGCAGT-3′)).

**Quantitative RT-PCR**

Total RNA was prepared from mouse prostates and analyzed by qRT-PCR using the StepOnePlus Real-Time PCR system (Applied Biosystems) as described (30). RNA was amplified with TaqMan Gene Expression Master Mix (Applied Biosystems) combined with inventoried mouse gene expression assays. Invariant TaqMan mouse gene assays are summarized in table S1.

**Cell growth assay**

Control or FoxF1-expressing TRAMP and Myc-CaP cells were plated in triplicate. Cells were counted at 1, 2, 3, and 4 days using a hemocytometer. The WST-1 assay (Roche) was used according to the manufacturer’s instructions as described (50). Experiments were performed in triplicates and presented as average numbers of cells ± SEM of three independent determinations.

**Transwell invasion assay**

Porous membranes (BD Biosciences) were coated with 100 μl of a 1:50 dilution of Matrigel (BD Biosciences) and allowed to dry overnight at room temperature. The following day, 100,000 control or FoxF1-expressing TRAMP or Myc-CaP cells were cultured on membranes in serum-free DMEM containing 0.1% bovine serum albumin. Cell invasion was induced by adding 5% serum to the lower chamber and was allowed to proceed at 37°C for 24 to 48 hours. Subsequently, Matrigel-invading cells were fixed with 95% ethanol. Cells remaining in the upper chamber were removed with a cotton swab, whereas those remaining in the lower chamber were stained with crystal violet. Invasion was quantified by counting the invading cells.

**Scratch migration assay**

Control and FoxF1-OE TRAMP or control and FoxF1-OE Myc-CaP cells were plated onto six-well plates and allowed to grow into confluent monolayers. Scrape “wounds” were generated using a 20-μl pipette tip, and the cell medium was replaced. Phase-contrast images of the cells were taken at 0, 4, 8, 12, and 24 hours after wounding, and the average scratch width was quantified using ImageJ (National Institutes of Health). The relative migration distance was calculated by dividing the width of the scratch at each time point by the width of the scratch at time 0. The relative distance was then converted to a percentage by multiplying by 100%. Experiments were done in triplicate.

**Cloning of Map3k2 and Wnk1 promoters and the luciferase assay**

Mouse genomic DNA was used to amplify the −6372/−1 bp region of the Wnk1 promoter [National Center for Biotechnology Information (NCBI) reference no. NC_000084.6] using the primers 5′-TGTTTCC-TGGATGCCTTGTCACA-3′ and 5′-AAATGCGCCGAGTCTGAAGC-3′. The same method was used to amplify the −5787 bp region of the +22 to +22 region of Map3k2 promoter (NCBI reference no. NC_000084.6) using the primers 5′-TCTGGGCTCTGAAAGGAGGAGCTTG-3′ and 5′-TGTACACAGCCCCGATGTC-3′. PCR products were cloned into a pGL2 firefly luciferase reporter plasmid (Promega) and verified by DNA sequencing. HEK293T cells were transfected with CMV-FoxF1 or CMV-empty plasmids, as well as with luciferase reporter driven by the −5.7-kb Map3k2 promoter region (Map3k2-LUC) or by the −6.4-kb Wnk1 promoter region (Wnk1-LUC). CMV-Renilla was used as an internal control to normalize transfection efficiency. A dual luciferase assay (Promega) was performed 24 hours after transfection, as described previously (30).

**Human patient data set analysis**

Human patient expression data were obtained through the Gene Expression Omnibus (GEO; GSE2109). Bioinformatics analysis was done using R with the Bioconductor suite. The “affy” package was used for normalization (51). Expression values were normalized to endogenous housekeeping controls and compared to generate relative expression. GraphPad Prism was used on normalized expression values to perform Pearson’s correlation and to evaluate statistical significance.

**Statistical analysis**

Microsoft Excel was used to calculate SD and statistically significant differences between samples using the Student’s t test. P < 0.05 was considered statistically significant.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Overexpression of Foxf1 increases cellular proliferation, invasion, and migration of PCa tumor cells in culture.

Fig. S2. Overexpression of Foxf1 induces activation of the MAPK pathway in TRAMP orthotopic tumors.

Fig. S4. Depletion of FOXF1 reduces the growth of human PCa xenografts.

Fig. S5. Pharmacological inhibition of ERK5 in Foxf1-OE PCa cells decreases tumor cell proliferation, invasion, and migration in culture.

Fig. S6. FOXF1 expression is increased in a subset of human prostate cancers. Table S1. TaqMan probes.

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Editorial retraction

After an investigation by the Cincinnati Children's Hospital Medical Center, the research integrity officer of Cincinnati Children's Hospital released a finding recommending the retraction of the Research Article “The transcription factor FOXF1 promotes prostate cancer by stimulating the mitogen-activated protein kinase ERK5” by L. Fulford, D. Milewski, V. Ustiyan, N. Ravishankar, Y. Cai, T. Le, S. Masineni, S. Kasper, B. Aronow, V. V. Kalinichenko, T. V. Kalin, published in Science Signaling on 10 May 2016 (1). Science Signaling ran an Editorial Expression of Concern regarding this Research Article on 18 July 2017 (2). Correspondence from the research integrity officer, dated 21 March 2018, stated that the investigation found that it was more likely than not that data were falsified in Fig. 2C and in figs. S3B and S4E. It was further stated that Cincinnati Children's Hospital supported the authors’ request for the retraction of the Research Article. Science Signaling has contacted the authors of the Research Article and confirmed that they all concur with the retraction. In light of the investigation and request from the authors to retract the paper, Science Signaling is retracting the paper in full.

John F. Foley
Editor

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The transcription factor FOXF1 promotes prostate cancer by stimulating the mitogen-activated protein kinase ERK5

Logan Fulford, David Milewski, Vladimir Ustiyan, Navin Ravishankar, Yuqi Cai, Tien Le, Sreeharsha Masineni, Susan Kasper, Bruce Aronow, Vladimir V. Kalinichenko and Tanya V. Kalin

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Out-FOXing prostate cancer

Some types of prostate cancer can be aggressive, and these metastatic forms of the disease have few treatment options. Fulford et al. found that prostate tumors from some patients express the gene encoding the transcription factor forkhead box F1 (FOXF1), which is not expressed in normal prostate epithelium. Overexpressing FOXF1 increased tumor growth and metastatic progression when tumor cells were implanted into mice. FOXF1 bound to and increased the expression of genes encoding the kinase ERK5 and two upstream kinases, MAP3K2 and WNK1. Pharmacologically inhibiting ERK5 or knocking down FOXF1, ERK5, or both MAP3K2 and WNK1 suppressed prostate tumor growth and metastasis. The findings indicate targets for personalizing therapeutic intervention in prostate cancer patients with FOXF1-positive tumors.