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*Priority Report*

Cancer Research

# TMPRSS2–ERG-Mediated Feed-Forward Regulation of Wild-Type ERG in Human Prostate Cancers

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## Abstract

Recurrent gene fusions involving ETS family genes are a distinguishing feature of human prostate cancers, with *TMPRSS2–ERG* fusions representing the most common subtype. The *TMPRSS2–ERG* fusion transcript and its splice variants are well characterized in prostate cancers; however, not much is known about the levels and regulation of wild-type *ERG*. By employing an integrative approach, we show that the *TMPRSS2–ERG* gene fusion product binds to the *ERG* locus and drives the overexpression of wild-type ERG in prostate cancers. Knockdown of TMPRSS2–ERG in VCaP cells resulted in the downregulation of wild-type ERG transcription, whereas stable overexpression of TMPRSS2–ERG in the gene fusion-negative PC3 cells was associated with the upregulation of wild-type *ERG* transcript. Further, androgen signaling-mediated upregulation of TMPRSS2–ERG resulted in the concomitant upregulation of wild-type *ERG* transcription in VCaP cells. The loss of wild-type *ERG* expression was associated with a decrease in the invasive potential of VCaP cells. Importantly, 38% of clinically localized prostate cancers and 27% of metastatic prostate cancers harboring the *TMPRSS2–ERG* gene fusions exhibited overexpression of wild-type *ERG*. Taken together, these results provide novel insights into the regulation of ERG in human prostate cancers. *Cancer Res; 71(16); 5387–92. ©2011 AACR.*

## Introduction

Recurrent gene fusions involving ETS family genes are observed in a majority of human prostate cancers and have potential implications for diagnosis, prognosis, and therapy (1–3). These gene fusions typically result in the juxtaposition of the 5′-untranslated region of androgen-regulated genes to the oncogenic ETS family genes, resulting in their overexpression. The *TMPRSS2–ERG* fusions represent the most common subtype among ETS fusions, with a prevalence of approximately 50% in clinically localized prostate cancers (1, 4). Additional ETS family genes, which together account for approximately 10% of prostate cancer gene fusions, include *ETV1* (5), *ETV4* (6), and *ETV5* (7). *TMPRSS2* is an androgen-regulated gene located 3 Mb upstream of *ERG* in human chromosome 21q22.2, the upstream regulatory elements and promoter of which drive the overexpression of ERG upon the formation of gene fusion. Androgen signaling has been shown to induce the proximity of *TMPRSS2* and *ERG* locus in androgen responsive cells, and in combination with agents causing DNA double-strand breaks induces *TMPRSS2–ERG* gene fusions (8–10). In prostate cancer specimens, *TMPRSS2–ERG* gene fusions can be formed with the deletion of the intervening sequences (referred to as Edel) or without the deletion (11, 12). Castration-resistant metastatic prostate cancer sites harboring *TMPRSS2–ERG* are associated with Edel, suggesting that Edel is more aggressive (13). To date, multiple *TMPRSS2–ERG* isoforms and splice variants with variable biological activities have been described (14–17). For example, inclusion of a 72-bp exon has been reported to significantly enhance proliferation of prostate-derived cells (17). However, not much is known about the levels and regulation of wild-type ERG in prostate cancers. By employing an integrative approach, we show that the *TMPRSS2–ERG* gene fusion product binds to the *ERG* locus and drives the overexpression of wild-type ERG in human prostate cancers and suggest functional and clinical implications of this feed-forward regulation of ERG expression.

## Materials and Methods

### Samples

Prostate tissues were from the radical prostatectomy series and the Rapid Autopsy Program, which are both part of the University of Michigan Prostate Cancer Specialized Program of Research Excellence (SPORE) Tissue Core. Samples were collected with informed consent and prior institutional review board approval. Total RNA was isolated with Trizol (Invitrogen) according to the manufacturer's instructions.

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### Cell culture and cloning

VCaP cells were cultured in Dulbecco's modified Eagle's medium high glucose medium containing 10% FBS; LNCaP cells, PC3 cells stably overexpressing TMPRSS2–ERG (PC3-TMPRSS2–ERG), and PC3 cells stably overexpressing LacZ (PC3-LacZ) were cultured in RPMI 1640 medium containing 10% FBS in a 5% $CO_2$ humidified incubator. Parental VCaP, LNCaP, and PC3 cells were obtained from American Type Culture Collection and were passaged for fewer than 6 months. Lentiviral LACZ and TMPRSS2:ERG-3×FLAG overexpression vectors were created by cloning into the pLL_ires_GFP backbone available from the University of Michigan vector core. Briefly, the coding sequence was amplified by PCR, digested with Xho1 and Xba1, and ligated into the pLL vector. The TMPRSS2–ERG variant used is the most prevalent gene fusion product [*TMPRSS2* exon 1 fused to *ERG* exon 2 (NM_182918.3)]. Lentivirus was created at the University of Michigan virus core and used to transduce PC3 cells in the presence of 4μg/mL polybrene (Sigma). PC3-LACZ and PC3-TMPRSS2–ERG cells were then sorted for green fluorescent protein (GFP) expression (top 25%) and used for subsequent assays. Genetic identity of the cells was confirmed by genotyping. GFP expression was monitored every 3 days, and cells were kept in culture for no more than 4 weeks. The adenoviral constructs for ERG (exons 2–10) and LacZ are described elsewhere (4).

### Chromatin immunoprecipitation and chromatin immunoprecipitation-seq analysis

Published chromatin immunoprecipitation (ChIP)-seq sequence data were downloaded from the following GEO datasets: VCaP H3K36me3 (GSM353624), LNCaP H3K36me3 (GSM353627), VCaP ERG (GSM353647), LNCaP ERG (GSM353648), and VCaP Pan-H3 (GSM353622; ref. 18). LNCaP Pan-H3 ChIP, VCaP Input DNA, and LNCaP Input DNA sequence data were prepared into libraries and sequenced by using the Genome Analyzer (Illumina) following manufacturer's protocols. The detailed ChIP procedure is described elsewhere (18). Reads were mapped to the human genome (hg19) using the BWA v0.5.8 (http://bio-bwa.sourceforge.net) short read aligner (19). Only reads with a single best alignment were considered for further analysis. Enriched DNA–protein binding sites were determined by using the MACS (version 1.4.0beta) peak-calling software using a threshold $P$ value of $1e^{-5}$ and default settings for all other parameters (20). Input DNA was used as a control for the ERG peak-calling analyses, and the Pan-H3 antibody ChIP-seq was used as a control for the H3K36me3 analyses. The ChIP quantitative real-time PCR primers for amplifying wild-type *ERG* (NM_182918.3) promoter are: forward: GTGCTTGCAGCCCGTGTGAA and reverse: TGTCCAGCCCAAAGAAACAGGATA. The antibodies for ChIP assays are rabbit IgG (Santa Cruz Biotechnology, #sc-2027), rabbit α-FLAG (Sigma, #F7425), rabbit α-ERG (Epitomics, #2805-1).

## Results and Discussion

We first studied the *ERG* locus in the androgen-sensitive VCaP and LNCaP cell lines by using our previously published ChIP-seq compendium (18). Specifically, we analyzed the landscape of trimethylated lysine 36 of histone H3 (H3K36me3) enrichment and ERG binding in these cell lines. The genes actively transcribed by RNA polymerase II (Pol II) are marked by H3K36me3 enrichment along the length of the

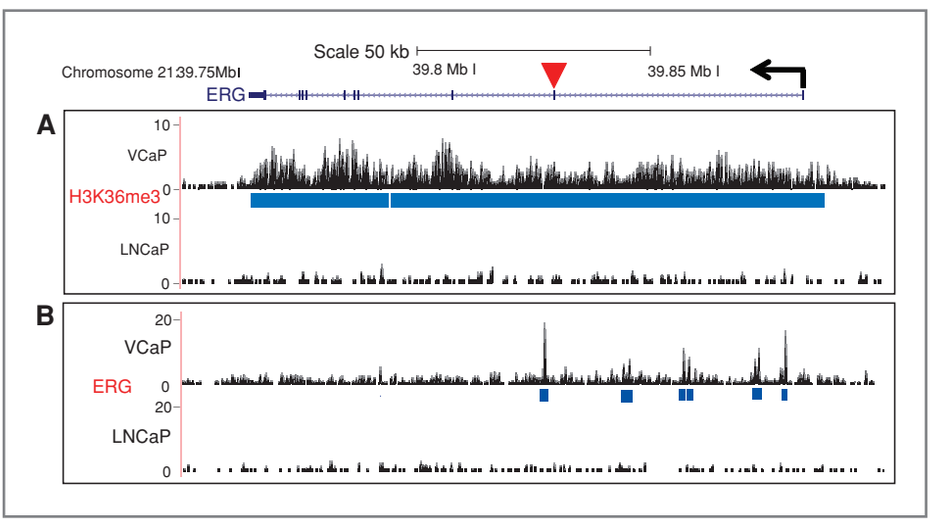


Figure 1. ChIP-seq coverage data showing the H3K36me3 chromatin mark and the ERG transcription factor binding at the *ERG* locus in VCaP and LNCaP cells. An arrow denotes the direction of *ERG* transcription; vertical bars represent exons. In each coverage plot, the *y*-axis represents the number of overlapping reads at each position, normalized to the reads per kilobase per million metric. Significant enrichment ($P < 10^{-5}$) is denoted by blue boxes below the raw data. A, the transcriptionally active *ERG* locus in VCaP cells is marked by significant enrichment of the H3K36me3 mark. The TMPRSS2–ERG gene fusion results in upregulation of *ERG* exon 2 (denoted by the red arrowhead) and beyond in VCaP cells. However, the H3K36me3 domain extends upstream of *ERG* exon 2, suggesting that the entire *ERG* locus is transcriptionally active (and not just the exons driven by gene fusion). By contrast, LNCaP cells lack H3K36me3 enrichment. B, the ERG protein binds to multiple locations within the *ERG* locus in VCaP cells but not LNCaP cells.

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transcribed region (21). As VCaP cells endogenously harbor the *TMPRSS2–ERG* gene fusion which produces a chimeric transcript comprising of *TMPRSS2* (exon 1) and *ERG* (exons 2–10), the *ERG* locus is enriched for the H3K36me3 mark in this cell line (Fig. 1A). By contrast, the LNCaP cells do not harbor the *TMPRSS2–ERG* gene fusion, lack ERG expression, and do not exhibit H3K36me3 enrichment at the *ERG* locus. Interestingly, the H3K36me3 epigenetic mark spanned the entire *ERG* locus (exons 1–10) in VCaP cells, even though gene fusion drives the overexpression of *ERG* exon 2 and beyond, indicating that the entire *ERG* locus is transcriptionally active in these cells. High expression levels of *ERG* exon1 was observed in VCaP cells, suggesting that in addition to the *TMPRSS2–ERG* chimeric transcript, these cells also express wild-type *ERG* (Supplementary Fig. S1). The results of 5′ rapid amplication of cDNA ends experiments indicated that Exon 1 of *ERG* represents bona fide wild-type *ERG*, and its expression is not driven by fusion to any other gene (Supplementary Fig. S2). In addition, we observed strong binding of the ERG protein to the *ERG* locus in VCaP cells, but not in the LNCaP cells (Fig. 1B). The majority of the ERG enrichment peaks clustered near exon 1 of wild-type *ERG*. Taken together, these observations suggest that in addition to the *TMPRSS2–ERG* transcript, VCaP cells have high levels of wild-type *ERG* transcript, the expression of which is presumably regulated by ERG protein.

We next conducted gene silencing experiments to determine the role of *TMPRSS2–ERG* and wild-type *ERG* transcripts in VCaP cells. We designed siRNAs that specifically target (i) *TMPRSS2–ERG* transcript, (ii) wild-type *ERG* transcript, or (iii) both *TMPRSS2–ERG* and wild-type *ERG* transcripts. Specific knockdown of *TMPRSS2–ERG* transcript resulted in a reduction in levels of wild-type *ERG* transcript in addition to total *ERG* transcription as represented by ERG-ALL primers (Fig. 2A). However, specific knockdown of wild-type *ERG* transcript resulted in reduction of total *ERG* transcription without affecting the levels of *TMPRSS2–ERG* transcript. These results indicate that ERG protein can upregulate the levels of wild-type *ERG* transcription, but not the levels of *TMPRSS2–ERG* transcript. Further support is obtained by the observation that PC3 cells stably overexpressing TMPRSS2–ERG show higher transcript levels of wild-type *ERG* as compared with LacZ controls (Fig. 2B). This upregulation is associated with the binding of FLAG-tagged ERG protein to the promoter of wild-type *ERG* (Fig. 2B). Similar results were obtained upon

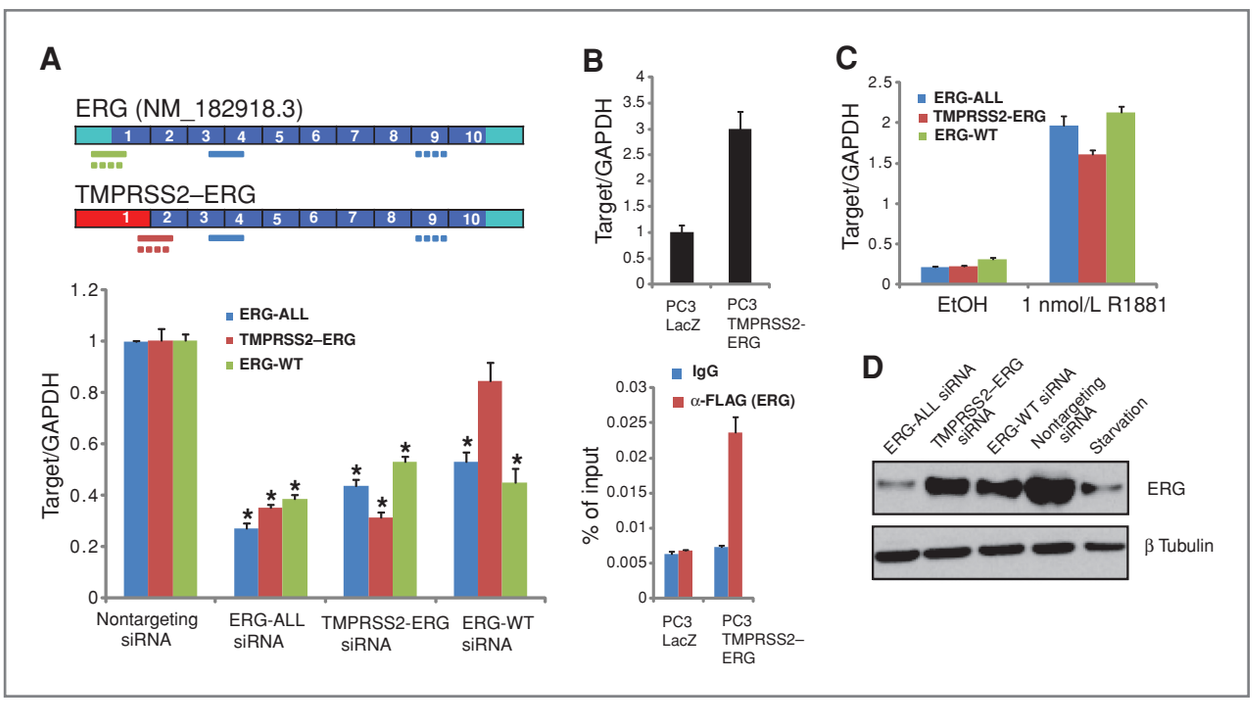


**Figure 2.** TMPRSS2–ERG-mediated feed-forward regulation of ERG. A, schematic representation of the wild-type *ERG* (ERG-WT, accession: NM_182918.3) and *TMPRSS2–ERG* transcript (top). The locations of primers for gene expression analysis (solid line) and siRNA sequences (dotted line) are shown. The TMPRSS2–ERG siRNA and ERG-WT siRNA specifically target the *TMPRSS2–ERG* and wild-type *ERG* transcripts, respectively. The ERG-ALL siRNA targets both these transcripts. The TMPRSS2–ERG and ERG-WT primers specifically detect *TMPRSS2–ERG* and wild-type *ERG* transcripts, respectively; the ERG-ALL primers detect both these transcripts. Targeted knockdown of *TMPRSS2–ERG* transcript is associated with the downregulation of wild-type *ERG* transcript. However, targeted knockdown of wild-type *ERG* does not lead to the downregulation of the *TMPRSS2–ERG* transcript. *, $P < 0.01$ by Student's *t*-test. B, lentivirus-mediated stable overexpression of FLAG-tagged TMPRSS2–ERG fusion product in PC3 cells results in the upregulation of wild-type *ERG* transcript (top). ChIP assay with α-FLAG antibody shows binding of ERG protein to the promoter of wild-type *ERG* locus (bottom). C, stimulation with 1 nmol/L R1881, a synthetic androgen agonist, for 24 hours resulted in the upregulation of both the *TMPRSS2–ERG* and wild-type *ERG* transcripts. D, knockdown of *TMPRSS2–ERG* or wild-type *ERG* transcripts individually results in reduced ERG protein levels. Maximal reduction of ERG protein levels is observed with the ERG-ALL siRNA that targets both the *TMPRSS2–ERG* and wild-type *ERG* transcripts. Starvation or androgen deprivation also results in a significant reduction in ERG protein levels.

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ectopic overexpression of the TMPRSS2–ERG gene fusion product in LNCaP cells (Supplementary Fig. S3).

We next determined if androgen signaling could affect the levels of *TMPRSS2–ERG* and wild-type *ERG* in VCaP cells. Administration of synthetic androgen (R1881) resulted in robust upregulation of both *TMPRSS2–ERG* and wild-type *ERG* transcription (Fig. 2C). Given the observation that *ERG* was not androgen responsive in a TMPRSS2–ERG negative setting (e.g., LNCaP cells; ref. 2), we hypothesized that upregulation of wild-type *ERG* by androgen is mediated by TMPRSS2–ERG. To study the effects of siRNA against different *ERG* transcripts and androgen depletion (starvation) in regulating ERG protein levels, immunoblot analysis was conducted (Fig. 2D). Although siRNA against *TMPRSS2–ERG* or wild-type *ERG* reduced the levels of ERG protein, maximal reduction of ERG protein was obtained with ERG-ALL siRNA that targets all the *ERG* transcripts and also starvation or androgen deprivation. The proteins encoded by wild-type *ERG*

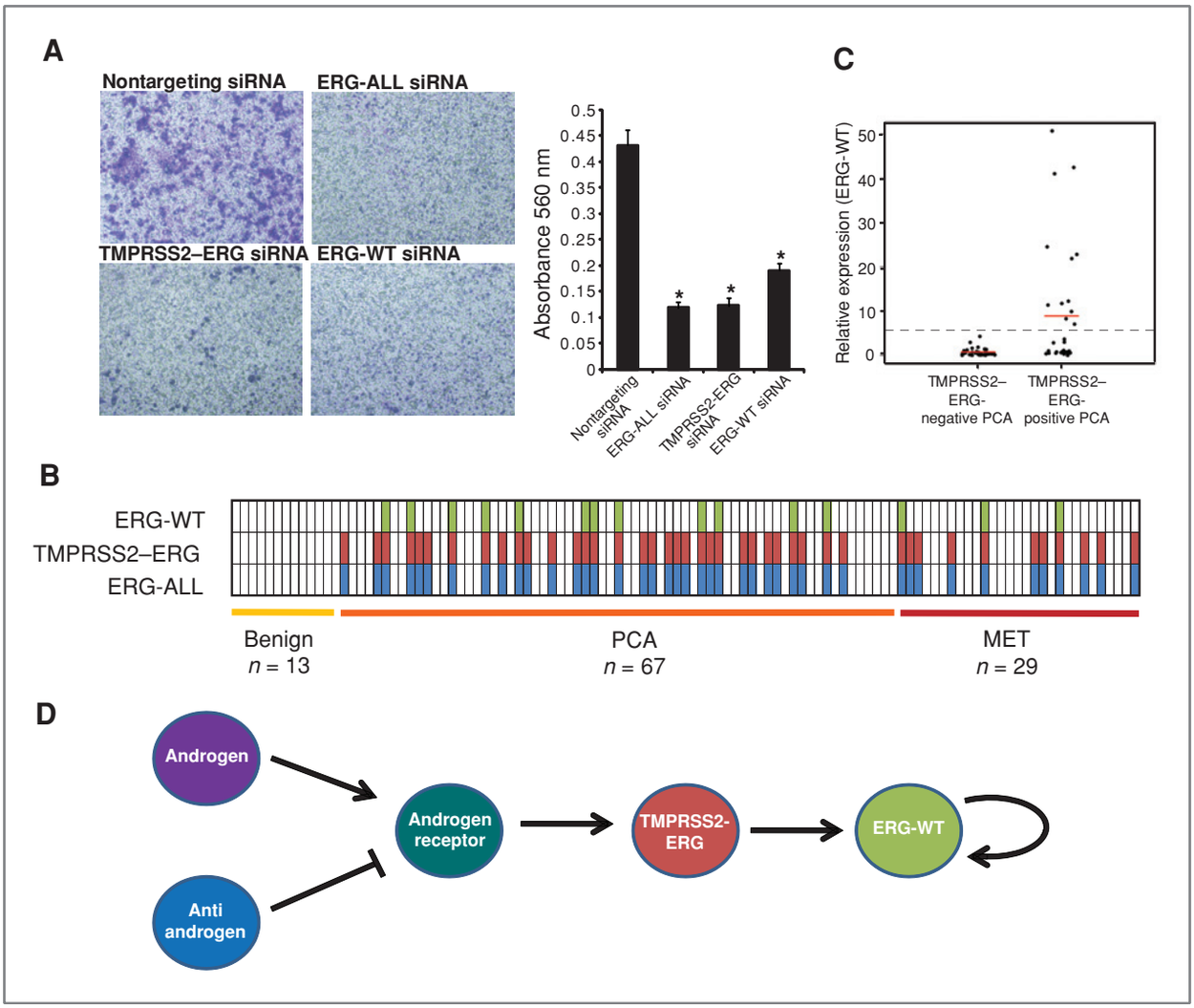


Figure 3. Functional studies and clinical relevance of TMPRSS2–ERG-mediated feed-forward regulation of ERG. A, a reconstituted basement membrane invasion chamber assay (Boyden chamber assay) was used to assess the invasive potential of VCaP cells treated with siRNA's against ERG isoforms. Representative fields of invaded and stained cells are shown (left). Invasion was quantified using colorimetry (absorbance at 560 nm, right). *, $P < 0.01$ by Student's *t*-test (right). B, matrix representation of the transcript status of ERG isoforms in benign prostate, clinically localized prostate cancer (PCA), and metastatic prostate cancer (MET) as measured by quantitative reverse transcription PCR. Each column represents 1 case; the top, middle, and bottom rows represent the status of wild-type *ERG* transcript (ERG-WT), *TMPRSS2–ERG* fusion transcript, and all *ERG* transcripts (ERG-ALL), respectively. A colored cell indicates that the tissue is positive for the corresponding transcript. C, scatter plot representing the relative expression levels of wild-type *ERG* in TMPRSS2–ERG negative and positive clinically localized prostate cancers. Vertical axes represent the relative expression levels; the red bars represent the mean, dashed line represents the threshold used to classify samples as either wild-type ERG positive or negative. For clarity, points have been horizontally displaced within each sample class. D, proposed model for TMPRSS2–ERG-mediated feed-forward regulation of ERG. Androgen signaling can increase the levels of *TMPRSS2–ERG* transcript, whereas antiandrogens have the opposite effect. The presence of *TMPRSS2–ERG* transcript results in the production of ERG protein, which then binds to the promoter of wild-type *ERG* to drive its transcription resulting in the production of wild-type ERG protein, which is functionally identical to the TMPRSS2–ERG gene product. This creates a feed-forward loop that upregulates wild-type *ERG* transcription.

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and *TMPRSS2–ERG* transcripts are indistinguishable in terms of molecular weight. These results suggest that (i) both *TMPRSS2–ERG* and wild-type *ERG* transcripts contribute toward total ERG protein levels in VCaP cells, and (ii) the protein product encoded by TMPRSS2–ERG upregulates the levels of wild-type ERG.

To determine the functional consequence of the presence of wild-type *ERG* and *TMPRSS2–ERG* transcripts in VCaP cells, invasion assays were conducted. Knockdown of *TMPRSS2–ERG* and wild-type *ERG* individually or in a combined fashion with the ERG-ALL siRNA resulted in a significant loss of cell invasion (Fig. 3A). Although TMPRSS2–ERG siRNA and wild-type ERG siRNA individually do not result in maximal loss of ERG protein unlike ERG-ALL siRNA (Fig. 2D), the results of invasion assay indicate that VCaP cells are ultrasensitive to ERG levels. Given that wild-type *ERG* has functional relevance in cell-based assays, we next determined if TMPRSS2–ERG gene fusion upregulates wild-type *ERG* expression in clinical samples of prostate cancer. The expression levels of *TMPRSS2–ERG*, wild-type *ERG*, and total *ERG* were measured in a panel of benign prostates ($n = 13$), clinically localized prostate cancers ($n = 67$), and metastatic prostate cancers ($n = 29$; Fig. 3B). None of the benign prostate tissues expressed either *TMPRSS2–ERG* or wild-type *ERG* transcripts. In clinically localized prostate cancers, 37.5% (12/32) of TMPRSS2–ERG positive cases and none (0/35) of the TMPRSS2–ERG negative cases exhibited high levels of wild-type *ERG* (Fig. 3C, and Supplementary Table S1). Similarly, 27.3% (3/11) of TMPRSS2–ERG positive metastatic prostate cancers expressed high levels of wild-type *ERG*, whereas none (0/18) of the TMPRSS2–ERG negative metastatic prostate cancers showed high wild-type *ERG* expression. By analyzing published prostate cancer array CGH datasets (22, 23), we could rule out ERG locus amplification as a possible cause of increased wild-type *ERG* expression (Supplementary Fig. S4). Taken together, these results suggest that TMPRSS2–ERG-mediated upregulation of wild-type *ERG* transcription occurs in a subset of gene fusion positive prostate cancers.

*TMPRSS2–ERG*, the first recurrent gene fusion to be identified in human prostate cancers, is also the most prevalent. Although it is apparent that the androgen-responsive *TMPRSS2* promoter drives the overexpression of ERG, we identified a novel TMPRSS2–ERG-mediated feed-forward loop that upregulates wild-type *ERG*, thereby, increasing the steady-state levels of ERG protein (Fig. 3D). We have further subclassified TMPRSS2–ERG-positive prostate cancers in to 2 groups based on wild-type ERG expression. In the future, it will be interesting to determine if the 2 groups have distinguishing clinical features that are prognostically or therapeutically relevant. Why TMPRSS2–ERG-mediated upregulation of ERG gets selected in human prostate cancers is not very clear, however, some possibilities do exist. Because ERG is a key driver of cancer progression, any change in cellular circuitry that increases the steady-state ERG protein levels may be positively selected. This may also result in oncogene addiction, and it will be interesting to test if prostate cancers with TMPRSS2–ERG and wild-type ERG represent a subtype that is ultrasensitive to ERG inhibition, as this appears to be the case with VCaP cells. Thus, by employing an integrative approach, we have identified a novel mechanism of regulation of *ERG* transcription that holds functional significance and clinical relevance.

## Disclosure of Potential Conflicts of Interest

A.M. Chinnaiyan serves on the advisory board of Gen-Probe, and is a coinventor on a patent filed by the University of Michigan covering the diagnostic and therapeutic field of use for ETS fusions in prostate cancer. The diagnostic field of use has been licensed to Gen-Probe, Inc. Gen-Probe did not play a role in the design and conduct of this study, in the collection, analysis, or interpretation of the data, or in the preparation, review, or approval of the article. The other authors disclosed no potential conflicts of interest.

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