TMPRSS2/ERG Promotes Epithelial to Mesenchymal Transition through the ZEB1/ZEB2 Axis in a Prostate Cancer Model

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Abstract
Prostate cancer is the most common non-dermatologic malignancy in men in the Western world. Recently, a frequent chromosomal aberration fusing androgen regulated TMPRSS2 promoter and the ERG gene (TMPRSS2/ERG) was discovered in prostate cancer. Several studies demonstrated cooperation between TMPRSS2/ERG and other defective pathways in cancer progression. However, the unveiling of more specific pathways in which TMPRSS2/ERG takes part, requires further investigation. Using immortalized prostate epithelial cells we were able to show that TMPRSS2/ERG over-expressing cells undergo an Epithelial to Mesenchymal Transition (EMT), manifested by acquisition of mesenchymal morphology and markers as well as migration and invasion capabilities. These findings were corroborated in vivo, where the control cells gave rise to discrete nodules while the TMPRSS2/ERG-expressing cells formed malignant tumors, which expressed EMT markers. To further investigate the general transcription scheme induced by TMPRSS2/ERG, cells were subjected to a microarray analysis that revealed a distinct EMT expression program, including up-regulation of the EMT facilitators, ZEB1 and ZEB2, and down-regulation of the epithelial marker CDH1 (E-Cadherin). A chromatin immunoprecipitation assay revealed direct binding of TMPRSS2/ERG to the promoter of ZEB1 but not ZEB2. However, TMPRSS2/ERG was able to bind to the promoters of the ZEB2 modulators, IL1R2 and SPINT1. This set of experiments further illuminates the mechanism by which the TMPRSS2/ERG fusion affects prostate cancer progression and might assist in targeting TMPRSS2/ERG and its downstream targets in future drug design efforts.

Introduction
Prostate cancer is one of the most frequent cancers in Men. Close to 30,000 patients are expected to die from the disease in the USA each year. A major advance in this research field is a recent discovery that frequent over-expression of E Twenty Six (ETS)-related proto-oncogenes may be driven by androgen receptor as a consequence of common genomic rearrangements. The predominant form of the aforementioned fusions with a frequency of ~85% [1], is the fusion between exon 1 from TMPRSS2 and exons 4–9 from the ERG gene, which occurs either by a deletion of 3 mega bases region separating these genes [2], or via an interchromosomal translocation [3,4]. As this fusion is already evident in Prostatic Intraepithelial Neoplasia (PIN) [5], investigating this fusion may hold the key towards understanding the mechanisms involved in early phases of prostate cancer.

Since its discovery [6], the TMPRSS2/ERG fusion has been extensively studied in several aspects, including early diagnosis, prognosis, contribution to cancer progression and even as a target for cancer therapy [7]. According to long term clinical studies performed on a large cohort of patients, it seems that TMPRSS2/ERG expression is associated with a more aggressive form of prostate cancer [8,9]. Further studies have shown a role for TMPRSS2/ERG fusion in tumorogenesis in terms of proliferation, invasion and cancer initiation and progression [10,11,12,13]. In general, it appears that cell proliferation is not necessarily promoted via TMPRSS2/ERG expression. As for tumorogenesis, the data is inconclusive. While knocking-down endogenous TMPRSS2/ERG in the VCaP prostate-derived cancer cells resulted in a reduction of both tumor uptake and volume [13,14], transgenic mice harboring TMPRSS2/ERG in their genome either developed PIN [10,15] or
reveal no histological evidence of PIN or invasive cancer [11,16]; depending on the specific model used in the study and the interpretation of the data. Despite the disagreement concerning the role of TMPRSS2/ERG in cancer initiation, cell invasion was suggested to be a consequence of TMPRSS2/ERG fusion both in vitro and in vivo [10,13,15]. Interestingly, an in silico study revealed that TMPRSS2/ERG co-expressed with histone deacetylase 1 (HDAC1) is coupled with down regulation of its known target [17]. This finding implies that TMPRSS2/ERG is associated with epigenetic reprogramming. Accordingly, in a follow-up study performed by the same group, HDACi, and HDAC specific inhibitors, compromised TMPRSS2/ERG expression or activity in ERG positive cells, in vitro [17,18]. In addition, recent findings demonstrated a cooperation between TMPRSS2/ERG fusion and deregulated activity of cancer-related pathways, such as PTEN [19], PI3-Kinase [16], and AKT or AR [20]. More recently, TMPRSS2/ERG was shown to mediate Epithelial to Mesenchymal Transition (EMT) through the induction of WNT signaling components [21]. Taken together, it could be surmised that other TMPRSS2/ERG-mediated pathways, might be converged at the same endpoint, namely, EMT and invasion; and therefore discovering new pathways through which TMPRSS2/ERG exert this effect is of great importance. The main motivation of this study is therefore to unravel such TMPRSS2/ERG related pathways in the context of prostate cancer. In a previous work we established immortalized and tumorigenic human prostate epithelial cells (PrECs) lines of defined genetic constitution [22]. Similarly, in the presented study, we generated genetically modified PrECs to serve as a background on which the effects of the TMPRSS2/ERG fusion could be genuinely studied. We found that TMPRSS2/ERG executes a distinct EMT expression program which is mainly governed by a direct activation of ZEB1 and an indirect induction of ZEB2 through SPINT1 and IL1R2 modulation, leading to an EMT phenotype in vitro and in vivo.

## Results

### Establishment of immortalized PrECs cultures

In order to investigate the impact of TMPRSS2/ERG in a genetically modified environment we sought to establish an immortalized PrECs culture. Normal prostate epithelial cells were produced from a human prostatectomy specimen and were subsequently grown in culture. To induce immortalization, cells were introduced with the telomerase catalytic subunit hTERT, and both the p53 and pRB pathways were perturbed using p53 knockdown and over-expression of cyclinD/CDK4, respectively, giving rise to an immortal cell line designated as EP (Figure 1A and B). Next, the immortalized cells were infected with retroviruses encoding either TMPRSS2/ERG or empty-vector control (Figure 1C). ERG protein level was comparable with its previously reported expression level in cell lines and cancer samples [23,24]. Notably, TMPRSS2/ERG alone or in combination with hTERT and/or p53 knockdown was not sufficient to

![Figure 1. Prostate epithelial cells immortalization.](https://www.plosone.org/figure1.png)
induce immortalization (data not shown). Finally, following a previous report that the combination of Androgen Receptor (AR) and high levels of ERG promotes the development of a more poorly differentiated, invasive adenocarcinoma than either gene alone [20]; AR was introduced into the poorly differentiated, invasive adenocarcinoma than either gene and high levels of ERG promotes the development of a more previous report that the combination of Androgen Receptor (AR) induce immortalization (data not shown). Finally, following a mirror image was evident in EP-AR (EP cells) did not form tumors (Data not shown), while EP-AR (EP cells) demonstrated an enhanced migratory capacity. The experiment was repeated using matrigel-coated wells in order to examine the cells ability to penetrate and invade a dense surface. Once again, invasion ability was significantly more discernible in the TMPRSS2/ERG-expressing cells (Figure 2C). The loss of CDH1 (E-Cadherin) is considered to be the most fundamental event during EMT [27]. We therefore measured the levels of CDH1 mRNA and protein using QRT-PCR and immunofluorescence staining, respectively. Indeed, EP-AR TMPRSS2/ERG cells demonstrated a marked reduction in the levels of CDH1 mRNA (Figure 2D) and protein (Figure 2E). Additionally, VIM (Vimentin), a known mesenchymal marker was found to be elevated in the TMPRSS2/ERG-expressing cells (Figure 2F). In sum, our data suggest that TMPRSS2/ERG overexpression provokes an epithelial to mesenchymal transition in vitro.

The effect of TMPRSS2/ERG on tumorigenesis

In an attempt to extend the previous observation to an in vivo model; we either injected the genetically-modified cell lines subcutaneously or implanted them orthotopically into the prostate of nude mice. Sixty eight days following the implantation, tumors were removed, sectioned and stained for EMT markers. Comparing the orthotopic implantation sites of the distinct cell lines revealed that hTERT/shp33/CDK4-immortalized PrECs (EP cells) did not form tumors (Data not shown), while EP-AR formed discrete nodules interspersed throughout the murine prostate (Figure 2F, indicated by blue arrows). Notably, EP-AR TMPRSS2/ERG cells formed large malignant tumors, which surrounded the normal murine prostate nodules (Figure 2F, black arrowheads). Moreover, EP-AR-derived nodules demonstrated positive staining for the epithelial marker CDH1, and failed to stain for the mesenchymal marker VIM (Figure 2F, blue arrows). A mirror image was evident in EP-AR TMPRSS2/ERG-derived tumors, which expressed high levels of VIM and were negative for CDH1, further corroborating the in vitro observation that TMPRSS2/ERG induces EMT. Staining for MKI67 (Ki-67), a known proliferation marker, revealed an extensive expression in the EP-AR-derived nodules (37% ± 2 positive cells) compared to the EP-AR-derived nodules (8% ± 2). This indicates that the EP-AR-derived nodules are less proliferative and may account for their latent nature.

The results described thus far suggest that TMPRSS2/ERG facilitates EMT and, consequently, the formation of more aggressive and proliferative tumors. Several studies demonstrated that compared to PIN lesions, TMPRSS2/ERG rearrangement frequency in localized invasive prostate cancers, is doubled [5,29,39,50]. This observation implies that TMPRSS2/ERG requires additional modifications in order to be positively selected as the disease progresses. To test this hypothesis, we utilized a previously generated, Ras-transformed PrECs culture [22]. These cells harbor ectopically-expressed hTERT, the viral oncogenes SV40 small and large T antigens, oncogenic H-RasV12 and Androgen Receptor. An empty vector or a TMPRSS2/ERG-encoding vectors were introduced into these cells, to generate two distinct cell lines, LHSR and LHSR TMPRSS2/ERG, respectively (Figure 3A). In agreement with the results obtained with EP-AR cells, CDH1 was down-regulated in LHSR cells expressing TMPRSS2/ERG (Figure 3B). Next, cells were orthotopically injected into nude mice prostates, as well as sub-cutaneously. As expected, following merely 28 days, both cell lines gave rise to tumors with no significant differences in size (Tumor incidence is presented in Table S1). Accordingly, MKI67 staining revealed no differences between the cell lines in regards to proliferation rate (Data not shown). Interestingly, the TMPRSS2/ERG-expressing tumors demonstrated a marked up-regulation of VIM and a noticeable down-regulation of CDH1 compared to the control tumors (Figure 3C), further validating the facilitation of EMT by the TMPRSS2/ERG in an additional, and a more aggressive, in vivo model. Since the LHSR cell lines are highly aggressive, they are not suitable to study the effect of TMPRSS2/ERG on metastases formation, as the mice had to be sacrificed within a short period following the injections. Nevertheless, in one case, TMPRSS2/ ERG-expressing tumor metastasized into the murine lung. As shown in Figure S1, this metastasis originated from the LHSR TMPRSS2/ERG primary tumor, as it stained positive with human-specific anti-AR antibody. It is tempting to speculate that EMT induced by the TMPRSS2/ERG granted cells with migratory and invasive capacities and eventually enabled them to home and proliferate at a distant site. Thus, given a highly transformed genetic background, TMPRSS2/ERG-induced EMT might facilitate invasion and metastasis.

EP-AR and LHSR expressing TMPRSS2/ERG are not contaminated with cells of mesenchymal lineage

To exclude the possibility that the reported EMT stems from a cross contamination of mesenchymal cell cultures we performed Short Tandem Repeat (STR) based fingerprinting. STR loci are repetitive sequence elements, 3 to 7 base pairs in length, which are abundantly distributed throughout the human genome. PCR-based STR analysis is increasingly being used as a means for human identification for forensic and linkage studies [31,32, 33,34]. We analyzed both EP and LHSR cultures based on allele assignment for each of the STR loci tested. EP-AR and EP-AR TMPRSS2/ERG were found to be identical with respect to the 16 STR loci (Table 1). LHSR and LHSR TMPRSS2/ERG were also found to be identical to each other, however not to EP-AR or EP-AR TMPRSS2/ERG. To corroborate this observation we also performed spectral Karyotyping (SKY) analysis, in order to detect unique recurrent chromosomal features, specifically appearing in the two isogenic cell cultures. As shown in Figure S2, EP-AR and EP-AR TMPRSS2/ERG have additional material in chromosome 11, while the LHSR and LHSR TMPRSS2/ERG exhibit 3 specific chromosomal translocations, again indicating that each
Figure 2. **TMPRSS2/ERG promotes EMT in prostate epithelial cells.** (A) For morphological comparison, cells were photographed using a light microscope. (B) Cells were seeded in transwells and their migratory capacity towards FCS was measured by counting the migrating cells. (C) The same setup as in (B) was used with matrigel-coated transwells in order to compare cell invasiveness. (D) Cells were analyzed for CDH1 (E-Cadhein) expression using QRT-PCR. The results are presented as mean ± SD of a triplicate from a representative experiment. * denotes a significant differential expression of the gene compared to the control. (E) Cells were plated on slides and stained for CDH1 and Vimentin. DAPI was used to visualize nuclei. (F) Cells were implanted into murine prostate glands. Glands were removed 68 days after implantation, sectioned and either stained with Hematoxilin and Eosin (H&E) or with antibodies against human AR, CDH1 and Vimentin. (X400 Magnifications). Note that EP-AR (Control) cells formed discrete prostate nodules (Blue arrows), which are positively stained with the human specific anti-AR antibody (α-hAR). In contrast, EP-AR TMPRSS2/ERG-derived tumors, which are positively stained with α-hAR antibody, engulfed the α-hAR-negative murine nodules (Black arrowheads).

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type of culture, stems from the same origin. These results suggest that the EMT reported herein is a genuinely induced by TMPRSS2/ERG rather than by cross-contamination.

**TMPRSS2/ERG**-induced EMT is mediated by the ZEB1/ZEB2 axis

Numerous pathways are known to converge in **CDH1** repression during epithelial to mesenchymal transition [27]. Thus, we sought to measure the expression levels of several transcription factors which were reported to facilitate EMT by either direct or an indirect repression of **CDH1** [27]. The expression levels of SNAI1 (Snail), SNAI2 (Slug), FOXC2, GSC (Goosecoid), TWIST1, TCF4 (E2.2), TCF3 (E47) and KLF8 were measured and found to be either low or equally expressed in both EP-AR and EP-AR TMPRSS2/ERG cell lines (Figure 4A). Remarkably, the expression of ZEB1 and ZEB2, two known direct repressors of **CDH1** [27], were dramatically up-regulated in the **TMPRSS2/ERG**-expressing cells (Figure 4A). **ZEB1** induction by **TMPRSS2/ERG** was further validated at the protein level by immunostaining (Figure 4B). To test whether **ZEB1** has an effective role in promoting the EMT process in our model, its expression was stably knocked-down using short-hairpin RNA (shRNA) and migration assay was performed. As shown in Figure 4C, **ZEB1** levels declined dramatically following **ZEB1** knockdown, resulting in a significant attenuation of the migratory capacity of the **TMPRSS2/ERG**-expressing cells (Figure 4C, lower panel).

Both **ZEB1** and **ZEB2** promoter regions consist of putative **TMPRSS2/ERG** binding motifs (Figure S3), implying that **TMPRSS2/ERG** might directly bind their promoters and augment their expression. To test this hypothesis, we conducted a Chromatin Immuno-Precipitation (ChIP) assay using an α-**ERG** antibody. Interestingly, as depicted in figure 5A, **TMPRSS2/ERG** seems to directly bind **ZEB1** promoter, but not **ZEB2**. As a negative control we used a promoter region from **CDH1**, which is a part of the **ZEB1**/**ZEB2** axis, but does not harbor an **ERG** binding site. This result implies that **TMPRSS2/ERG** might indirectly induce **ZEB2** via the mediation of **ZEB1** up-stream effectors. To investigate this conjecture, and to better understand the mechanism by which **TMPRSS2/ERG** executes the EMT program at large; we undertook a genome-wide approach and

![Figure 3. **TMPRSS2/ERG** modulates EMT markers in Ras-transformed prostate cells.](https://www.plosone.org/doi/fig/10.1371/journal.pone.0021650.g003)
conducted an expression micro-array-based comparison between EP-AR and EP-AR TMPRSS2/ERG cells. A total of 1215 annotated genes were differentially expressed between the two cell lines ([13 up-regulated and 402 down-regulated], from which we retrieved the ones that were both associated with ZEB1 or ZEB2, and implicated in EMT in the literature (For the detailed filtering method refer to legend of figure 5B). To verify the authenticity of this set of genes we also validated their microarray-derived differential expression patterns using QRT-PCR (Data not shown). As depicted in figure 5B, seven genes matched the filtering criteria. Two of them, IL1R2 and SPINT1 (Figure 5C, validated by QRT-PCR), were reported to encode upstream effectors of ZEB2 expression; the former was shown to elevate ZEB2 expression levels [35], while the latter attenuates its expression [36], suggesting that they might be the mediators of TMPRSS2/ERG dependent ZEB2 elevation. Both IL1R2 and SPINT1 promoter regions consist of putative TMPRSS2/ERG binding motifs (Figure S2), and indeed TMPRSS2/ERG exhibited a significant binding to their promoters in a ChIP assay (Figure 5D). To further corroborate SPINT1 and IL1R2 effect on ZEB2 expression in our system, we knocked-down their expression using small-interfering RNA (siRNA). As shown in figure 5E, SPINT1 and IL1R2 levels were effectively reduced upon siRNA transfection, resulting in ZEB2 elevation and reduction, respectively. In sum, TMPRSS2/ERG seems to directly bind and trans-activate ZEB1 while indirectly inducing ZEB2 via trans-activation and trans-repression of its effectors, SPINT1 and IL1R2.

**Discussion**

In this study we provide substantial evidence to support the notion that TMPRSS2/ERG assumes an active role in epithelial to mesenchymal transition via the activation of the ZEB1/CDH1 pathway, both in vitro and in vivo. These findings shed light on the mechanism by which the TMPRSS2/ERG fusion exerts its oncogenic effect.
Figure 4. TMPRSS2/ERG-induced EMT is mediated by the ZEB1/ZEB2 axis. (A) EMT-associated transcription factors expression. Cells were analyzed for the expression of the specified genes using QRT-PCR. The results are presented as mean ±SD of a triplicate from a representative experiment. ND = Not detected. * denotes a significant differential expression (P-value < 5.6 × 10^{-2}). (B) Cells were plated on slides and stained with α-ZEB1 antibody. Nuclei were visualized by DAPI. (C) Prostate glands were injected with EP-AR (Control) and EP-AR TMPRSS2/ERG as described, removed, sectioned and stained with an α-ZEB1 antibody. The blue arrow denotes a human nodule. The black arrow denotes mouse nodules with a negative staining for AR. (X400 Magnification). (D) ZEB1 was knocked-down and its mRNA levels were measured (upper panel). * denotes a significant differential expression (P-value = 8 × 10^{-4}). Cells were subjected to migration assay as described (lower panel). ** denotes a significant differential expression (P-value = 4 × 10^{-3}).

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TMPRSS2/ERG Promotes EMT via ZEB1/2

A. Chromatin IP

B. EMT related genes = n

1178 TMPSRSS2/ERG differentially expressed gene (Microarray)

300 EMT 7 Zeb1 / Zeb2 associated genes (Genomatix)

30 SPINT1, IL1R2

LLGL2, CLDN4

MMP7, CDH2

WNT7A

C. Relative expression

D. Chromatin IP

E. siRNA experiments

Relative expression

Control SPINT1 siRNA

Control SPINT1 siRNA

Control IL1R2 siRNA

Control IL1R2 siRNA

Control ZEB2 siRNA

Control ZEB2 siRNA
Retroviral infections

Retroviral infections were performed serially and polyclonal-infected populations were drug-selected after each infection. Amphotropic retroviruses were produced by transfection of 293T cells with amphotropic packaging plasmid pCL-10A1, and a retroviral vector encoding the gene of interest. Culture supernatants containing retrovirus were collected 48 hours post-transfection.

TMPRSS2/ERG plasmid

TMPRSS2 (exon-1) and ERG (exons 4-9) fusion-encoding pBabe-Hygro plasmid, was kindly provided by Dr. Jan Trapman (Erasmus University Medical Center, Rotterdam, the Netherlands).

Orthotopic implantation of tumor cells

The protocol for in vivo experiments was approved by the Sheba Medical Center Institutional Animal Care and Use Committee (Permit No. 468–2008). Mice were anesthetized prior to injections and sacrificed when tumor size reached 1 cm³. All efforts were made to minimize animals’ suffering. Immunodeficient mice (Harlan Laboratories, Israel) were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg Xylazin 2:1 (Ketaset). A lower midline incision was made; and 1 × 10⁵ cells (in 100 µl PrEGM:matrigel (BD Bioscience) 1:1 mix), were implanted into the ventral prostate lobes using a 30-gauge needle and a 0.1 mL syringe. Testosterone pellets (Innovative Research of America, Sarasota, FL) were implanted under the skin. Two Subcutaneous injections were placed for each mouse as well. Mice were sacrificed at 28 or 68 days, as indicated, after the intraprostatic implantation of tumor cells. A table summarizing tumor incidence is presented as Table S1.

Migration and invasion assays

Cells were plated at a cell density of 1.5 × 10⁵ per well in 8 μm transwells (Co-Star) in triplicates and incubated for 24 hours. Then, cells that were attached to the outer part of the wells were removed by incubation with trypsin for 30 minutes and counted. For invasion assays, the transwells were coated with matrigel diluted in cell media 1:5. Cells were seeded in the presence of basal medium and migrated towards 10% FCS.

Western blot analysis

Total cell lysates were fractionated by SDS-gel electrophoresis. Proteins were transferred to nitrocellulose membranes, and immunoblotted with the indicated antibodies. Rabbit α-p53 (produced in Rotter’s lab); human specific α-Androgen Receptor (α-hAR N-20, Santa-Cruz Biotechnology); α-ERG (SC-354, Santa-Cruz Biotechnology); α-H-ras (C-20, Santa-Cruz Biotechnology); α-hTERT (H-231, Santa-Cruz Biotechnology); α-cyclin D2 (C-17, Santa-Cruz Biotechnology); α-actin (I-19, Santa-Cruz Biotechnology). Bands were detected by horseradish

Materials and Methods

Cell lines propagation

Human PrECs were obtained from BioWhittaker (Rockland, ME) and propagated in Prostate Epithelial Growth Medium (Lonza, Walkersville, MD) as previously described [22]. Cells were maintained in a humidified incubator at 37°C and 5% CO2.

Figure 5. TMPRSS2/ERG induces an EMT transcription program. (A) Chromatin-IP assay was performed in EP-AR TMPRSS2/ERG cells using α-ERG antibody and IgG as a control. The results are presented as mean ± SD of a triplicate from a representative experiment. * denotes P-value < 3 × 10⁻². (B) The list of 1215 differentially expressed genes was intersected with a list of genes associated with ZEB1 or ZEB2, which was obtained from the “Genomatix” software [47], resulting in 37 shared genes. The 37-genes list was filtered for genes associated with EMT according to the literature (n = unknown sample size), leaving the 7 genes that are listed in the box below. (C) Cells were analyzed for SPINT1 and IL1R2 expression using QRT-PCR. The results are presented as mean ± SD of a triplicate from a representative experiment. * denotes P-value < 5 × 10⁻³. (D) Same set-up as (A) was used for IL1R2 and SPINT1 promoters (E) Left panels: EP-AR cells were transfected with siRNA targeting SPINT1 and mRNA expression of the designated genes was measured by QRT-PCR. The results are presented as mean ± SD from two experiments utilizing two different siRNA oligonucleotides. * denotes P-value = 7 × 10⁻⁴, ** denotes P-value = 1 × 10⁻². Right panels: the same experimental set-up was used with siRNA targeting IL1R2 in EP-AR TMPRSS2/ERG. * denotes P-value = 2 × 10⁻⁴, ** denotes P-value = 1 × 10⁻².

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Figure 6. A schematic model describing the proposed mechanism by which TMPRSS2/ERG induces EMT. Black lines represent novel data; arrowheads represent activation; bar-headed lines represent repression; grey lines represent literature-based data.

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resulted in a more aggressive phenotype which included enhanced motility and invasiveness [45]. Finally, SPINT1 knockdown in the pancreatic cancer cell line SUTT-2, induced EMT and invasion which were accompanied by ZEB2 elevation and CDHI reduction [36]. Collectively these data suggest that SPINT1 acts as a tumor suppressor in Prostate cancer. We were able to show that SPINT1 partially exerts its effect by reducing the levels of ZEB2 and therefore it is repressed by TMPRSS2/ERG.

Recently, reports which focus on the cooperation of TMPRSS2/ERG with different partners in the cancerous process are emerging [16,19,20]. Our findings (Depicted in figure 6) extend our knowledge as to the identity of these partners and their mechanisms of action in promoting prostate cancer.
peroxidase–conjugated secondary antibodies and enhanced SuperSignal west pico chemiluminescent substrate (Thermo-scientific).

Quantitative Real-Time PCR (QRT-PCR)

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was generated from a 2-μg aliquot of the RNA using MMLV reverse transcriptase, amplification grade DNase I, random hexamer primers, RNaseOUT, and dinucleotide triphosphates (all from Invitrogen), according to the manufacturer’s instructions. QRT-PCR was performed using SYBR-Green Master Mix (Applied-Biosystems, CA, USA) on a 7500 Real-Time PCR system (Applied-Biosystems, CA, USA). Gene expression was normalized to GAPDH. Primers sequences are listed in Table S2.

Immunohistochemistry

Xenografts were fixed in formalin, embedded in paraffin and sectioned at 4 μm. The slides were incubated at 60°C for one hour. After sections were dewaxed and rehydrated, a CC1 Standard Benchmark XT pretreatment for antigen retrieval was selected (Ventana-Medical Systems). α-Vimentin (NCL-L-VIM-572, Leica Novocastra) was diluted 1:100. α-Ki67 antibody (MU297-UC, Biogenex), was diluted 1:50. α-CDH1 antibody (18-0223, Zymed) was diluted 1:25. Antibodies were incubated for 40 minutes at 37°C. Detection was performed with iView detection kit (Ventana-Medical Systems) and counterstained with hematoxylin (Ventana-Medical Systems). Then, slides were dehydrated in 70% ethanol, 95% ethanol and 100% ethanol for 10 seconds each. Before coverslipping, the sections were cleared in xylene for 10 seconds and mounted with Entellan.

For AR staining, antigen retrieval was performed using a pressure cooker (Milestone, Microwave-Laboratory Systems) at 120°C for 5 minutes in citrate buffer pH 6, cooled for 10 minutes, and rinsed with TBS buffer. Subsequently, an endogenous peroxidase block was performed for 10 minutes in 3% H2O2/PBS. After TBS rinsing, sections were blocked with 10% goat serum for 30 minutes and incubated with the α-hAR primary antibody (N-20, Santa-Cruz Biotechnology, 1:50) overnight in 4°C. Detection was performed with the Histostain SP Broad Spectrum kit (Zymed Laboratories, Invitrogen, U.S.A.). Briefly, sections were incubated with a biotinylated secondary antibody and subsequently, after TBS rinse, with HRP-streptavidin, for 30 minutes at room temperature. The antibody was visualized with the substrate-chromogen AEC, counterstained with hematoxylin and coverslipped with an aqueous mounting fluid (glycergel).

SKY analysis

Described in detail in [46].

Expression micro-arrays

Experiments were performed using Affymetrix GeneChip Human Gene 1.0 ST Arrays according to manufacturer’s recommendations. Briefly, 100-600 ng of total RNA was used to generate first-strand cDNA using random hexamers primer. After second-strand synthesis, in vitro transcription was performed. The resulting cRNA was then used for a second cycle of first-strand cDNA synthesis with UTP resulting in single-stranded DNA which was used for fragmentation and terminal labeling. cDNA generated from each sample was processed as per manufacturer’s recommendation using an Affymetrix GeneChip Instrument System manual [https://www.affymetrix.com/support/downloads/manuals/wt_sensetarget_label_manual.pdf].

Micro-array data analysis

EP-AR and EP-AR TMPRSS2/ERG expression profiles were analyzed on duplicate arrays. Gene level RMA sketch algorithm (Affymetrix Expression Console and Partek Genomics Suite 6.2) was used for crude data generation. A t-test with an uncorrected P-value was used to identify significantly differentially expressed genes (P-value < 0.05), with a threshold of at least two-fold change. This analysis yielded a set of 1215 differentially expressed genes.

Chromatin immunoprecipitation

Cells underwent cross-linking (1% formaldehyde, room temperature, 10 minutes) followed by quenching (glycine 0.125 M). Cells were rinsed with cold PBS, incubated with 20% trypsin (Gibco), washed with PBS, scraped and centrifuged. Cells were lysed (5 mM PIPES pH 8.0, 33 mM KCl, 0.5%NP40, 1%protease inhibitors) on ice for 20 minutes. Nuclei were collected by centrifugation (4,000 rpm), resuspended in nuclear lysis buffer (50 mM Tris–Cl, pH 8.1, 10 mM EDTA, 1%SDS, 1%protease inhibitors) and incubated on ice for 10 min. Samples were sonicated to an average DNA fragment length of 500 bp and then centrifuged (20,000 g). The chromatin solution was pre-cleared by adding protein A beads (2 hours, 4°C) (Santa Cruz Biotechnology). Immunoprecipitation of chromatin was done for 12 hours, in 4°C, using 1 μl antibody (α-ERG SC-354, Santa-Cruz Biotechnology and IgG I-2511, Sigma), followed by incubation with 50 μl protein A beads (2 hours). Immunoprecipitates were consecutively washed with dilution buffer (100 mM Tris–Cl, pH 9.0, 500 mM LiCl, 1%NP-40, 1%Deoxycholic acid, 1% protease inhibitors), TSE150, TSE500 and TE pH = 8. Samples were treated with 10 μg RNase A (30 minutes), followed by 30 μg of proteasome K treatment (2 hours, 50°C) and incubation at 65°C overnight. DNA samples were extracted using QiAquick PCR Purification Kit (Qiagen). QRT-PCR was performed as described above with each sample containing 2 μl of immunoprecipitated DNA. Primers sequences are listed in Table S2.

Short tandem repeat (STR) based fingerprinting

DNA was amplified by PCR using the reagents supplied in the AmpFSTR® Identifiler Plus (Applied Biosystems, Foster City, 94404 Ca., USA) for the following STR loci: D8S1179, D21S11, D7S820, CSF1PO D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA TPOX, D18S51, Amelogenin, D5S818, and FGA. The products were separated on an Applied Biosystems, 3130 genetic analyzer and analyzed using the software supplied by the manufacturer.

Supporting Information

Figure S1 The formation of tumor metastasis. LHSR T/ERG tumor metastasized into the murine lung and stained for AR (right hand side) compared to the normal lung of the LHSR mouse (left hand side) (X400 Magnification). Arrow in the LHSR T/ERG panel indicates lung metastasis. (TIF)

Figure S2 EP-AR and LHSR exhibit identical chromosomal characteristics as their TMPRSS2/ERG expressing counterparts. The designated cell cultures were subjected to SKY analysis. (A) Most recurrent features are shown in a table. (B) Representative images of the chromosomal features, recurrent features are circled in white. (TIF)
Table S1 Tumor incidence summary.

Table S2 Primers list.

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