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ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients

Esther Baena,1,2 Zhen Shao,1,2,3,9 Douglas E. Linn,4,9 Kimberly Glass,3 Melanie J. Hamblen,1,2,5 Yuko Fujiwara,1,2,5 Jonghwan Kim,1,2 Minh Nguyen,1,2 Xin Zhang,4 Frank J. Godinho,1,2,5 Roderick T. Bronson,6 Lorelei A. Mucci,7 Massimo Loda,8 Guo-Cheng Yuan,3 Stuart H. Orkin,1,2,5,10 and Zhe Li1,2,4,10

1Division of Hematology and Oncology, Boston Children’s Hospital, Boston, Massachusetts 02115, USA; 2Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA; 3Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, Massachusetts 02115, USA; 4Division of Genetics, Brigham and Women’s Hospital, Boston, Massachusetts 02115, USA; 5Howard Hughes Medical Institute, Boston, Massachusetts 02115, USA; 6Pathology, Harvard Medical School, Boston, Massachusetts 02115, USA; 7Department of Epidemiology, Harvard School of Public Health, Brigham and Women’s Hospital, Boston, Massachusetts 02115, USA; 8Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, Brigham and Women’s Hospital, Boston, Massachusetts 02115, USA

Distinguishing aggressive from indolent disease and developing effective therapy for advanced disease are the major challenges in prostate cancer research. Chromosomal rearrangements involving ETS transcription factors, such as ERG and ETV1, occur frequently in prostate cancer. How they contribute to tumorigenesis and whether they play similar or distinct in vivo roles remain elusive. Here we show that in mice with ERG or ETV1 targeted to the endogenous Tmprss2 locus, either factor cooperated with loss of a single copy of Pten, leading to localized cancer, but only ETV1 appeared to support development of invasive adenocarcinoma under the background of full Pten loss. Mechanistic studies demonstrated that ERG and ETV1 control a common transcriptional network but largely in an opposing fashion. In particular, while ERG negatively regulates the androgen receptor (AR) transcriptional program, ETV1 cooperates with AR signaling by favoring activation of the AR transcriptional program. Furthermore, we found that ETV1 expression, but not that of ERG, promotes autonomous testosterone production. Last, we confirmed the association of an ETV1 expression signature with aggressive disease and poorer outcome in patient data. The distinct biology of ETV1-associated prostate cancer suggests that this disease class may require new therapies directed to underlying programs controlled by ETV1.

[Keywords: ERG, ETS transcription factor, ETV1, Pten, androgen receptor, metabolism]

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Prostate cancer is a heterogeneous disease. Recent studies show little benefit from prostate-specific antigen (PSA) screening and radical prostatectomy for men with lower-risk disease [Wilt et al. 2012]. A central challenge in management is identification of those men with prostate cancer whose disease will eventually progress to the lethal castration-resistant stage. Understanding molecular events leading to castration-resistant prostate cancer (CRPC) is critical for the development of improved therapies for such patients.

Chromosomal rearrangements involving genes encoding ETS transcription factors [notably, ERG and ETV1] are found in ~50% of human prostate cancer cases and likely constitute the most frequent gene rearrangements in human malignancies [Tomlins et al. 2005; Gopalan et al. 2009]. The translocations place the coding regions of ERG or ETV1 under the control of androgen-responsive promoters, such as TMPRSS2, thereby activating expression in response to androgens. TMPRSS2 has been reported as the principal 5' fusion partner of ERG, whereas more heterogeneous 5' fusion partners, such as TMPRSS2, SCL45A3, or ACSL3, have been described for ETV1 [Tomlins et al. 2007; Attard et al. 2008b]. The majority of these 5' fusion
partners are also androgen-responsive genes. As ETS proteins, ERG and ETV1 are involved in regulation of cell growth, proliferation, differentiation, and apoptosis through activation or repression of target genes (Oikawa and Yamada 2003). Although functional overlap among different members of the ETS family exists, individual ETS factors also serve distinct roles. Thus, the expression pattern of ETS members through development varies, along with their repertoire of target genes, biological processes regulated, and oncogenic potentials (Seth and Watson 2005; Kunderfranco et al. 2010; Wei et al. 2010; Hollenhorst et al. 2011).

Clinical studies of the prevalence and prognostic significance of ETS fusions in prostate cancer have yielded discrepant results, possibly related to differences in the genetics of the evaluated populations and diversity in methods used. Several studies suggest that ETS fusions are associated with a worse prognosis (Demichelis et al. 2007; Nam et al. 2007; Attard et al. 2008a), whereas others have failed to confirm the correlation (Gopalan et al. 2009; Hermans et al. 2009; Minner et al. 2011). Cases with ETS fusions are generally grouped together for patient stratification. However, considering all ETS translocations as a single entity risks obscuring possible differences in the contribution of each to disease outcome. For example, effects of TMPRSS2-ERG, the most common translocation, may bias findings of aggregate studies. Studies to date have not specifically addressed the biology of individual ETS fusions and their associated outcomes.

In this study, we used knock-in mouse modeling and comprehensive genome-wide approaches to characterize the functional specificities of ERG and ETV1 in prostate cancer. Our data indicate that ERG and ETV1 regulate a common set of genes, such as androgen receptor (AR) target genes, but in an opposing direction. In particular, ETV1, but not ERG, up-regulates expression of AR target genes as well as genes involved in steroid biosynthesis and metabolism. This ETV1-driven oncogenic program predisposes prostate cells for cooperation with other oncogenic events, such as PTEN loss, leading to more aggressive disease in murine models and human patients. Our findings further establish different biological subtypes of human prostate cancer based on distinct ETS factor-driven signatures.

**Results**

**Tmprss2-ETV1 cooperates with total Pten loss, leading to invasive adenocarcinoma**

As a step toward defining the roles of ETS fusions in prostate cancer, several groups have generated transgenic mouse strains that express ERG or ETV1 ectopically under the control of the Probasin (Pb) promoter (Pb-ERG or Pb-ETV1) (Tomlins et al. 2007, 2008; Klezovitch et al. 2008; Carver et al. 2009; King et al. 2009; Shin et al. 2009). Interpretation of results has varied. Prostatic intraepithelial neoplasia (PIN)-like lesions have been described in prostates of Pb-ERG and Pb-ETV1 transgenic males [Tomlins et al. 2007, 2008; Klezovitch et al. 2008; Shin et al. 2009]. However, others have reported that Pb-ERG transgenic males are normal [Carver et al. 2009; King et al. 2009]. Discrepant findings may be related to mouse strain differences, to different transgene integration sites, or in the precise portions of the ETS cDNAs that were expressed. We reasoned that mice engineered to express ETS factors from an endogenous promoter in the proper chromosomal configuration might provide a more relevant biological context. Moreover, prior transgenic models cannot address potential contributions of haploinsufficiency or loss of genes deleted between TMPRSS2 and ERG to prostate tumorigenesis, such as occurs in patients with a TMPRSS2-ERG fusion generated through an interstitial deletion of chromosome 21.

We engineered knock-in mouse models to recapitulate TMPRSS2-ETS fusions (with or without the interstitial deletion) in prostate cancer. We used two strategies. In the first strategy, we knocked in N terminus-truncated human ERG or ETV1 cDNA, together with an ires-GFP cassette, into exon 2 of the mouse Tmprss2 locus [referred to as T-ERG or T-ETV1 hereafter], which shares ~80% homology as well as at least two conserved AR-binding sites with those of the human TMPRSS2 [Fig. 1A; Supplemental Fig. S1; Jacquot et al. 2000]. The resultant fusion transcripts recapitulate the TMPRSS2-ERGa or TMPRSS2-ETV1a fusions in patients [Tomlins et al. 2005]. In the second strategy, we used sequential gene targeting to introduce loxP sites into the Tmprss2 and Erg loci on the same chromosome [Fig. 1A, Supplemental Fig. S2A,B]. Cre-mediated recombination deletes the ~3-Mb intragenic region and generates the Tmprss2-Erg fusion gene [Supplemental Fig. S2C,D], which approximates the TMPRSS2-ERGa fusion subtype [Tomlins et al. 2005]. Since most genes in this interstitial region are syntenic between humans and mice [Supplemental Fig. S2E], this unique knock-in model also permits assessment of the contribution of the interstitial deletion to prostate cancer development [referred to as T-3Mb-Erg or T-Δ-Erg before or after Cre-mediated excision of the interstitial region, respectively] [Fig. 1A]. In all three knock-in alleles [i.e., T-ETV1, T-ERG, and T-Δ-Erg] we confirmed expression of their corresponding fusion transcripts in prostates [Fig. 1B]. By immunohistochemistry (IHC), we detected moderate expression of Erg protein in the knock-in prostates [Fig. 1C]. Despite multiple efforts, we were unable to validate an antibody that faithfully detects ETV1 protein by IHC. Therefore, we used IHC staining for GFP as a surrogate for ETV1 expression, as the knock-in GFP reporter is under the same transcriptional control [Fig. 1A]. With this approach, we detected robust GFP [ETV1] expression in prostate epithelial cells but not in stromal cells [Fig. 1D]. In all three knock-in strains, prostates appeared largely normal, and we did not observe prostatic intraepithelial neoplasia (PIN) lesions or cancer [Fig. 1E]. However, in a portion of T-ETV1 males [four out of 11], in particular those at old ages [≥18 mo; three out of three], we observed varying degrees of inflammation [Fig. 1E]. In addition, pathological analysis in several exceptional T-Δ-Erg males [four out of 21 but in none of the T-ERG
males) revealed some hyperplasia and foci of cells with lightly stained cytoplasm and loss of polarity (Fig. 1E). Despite these minor phenotypes, we conclude that expression of ERG or ETV1 from the endogenous Tmprss2 promoter, even in the presence of the interstitial deletion (for Erg fusion), is insufficient to initiate prostate tumorigenesis.

Overexpression of ERG or ETV1 from the Pb promoter or through lentiviral transduction in prostate cells has been previously reported to cooperate with activation of the PI3K pathway to drive a more aggressive prostate cancer phenotype (Carver et al. 2009; King et al. 2009; Zong et al. 2009). To determine whether this is also the case when ETV1 or ERG is expressed from the endogenous Tmprss2 promoter, we bred mice containing the knock-in alleles with Pten⁺⁻ mice. We found that within the time frame monitored (up to 15 mo of age), prostates of all aged T-ETV1;Pten⁺⁻, T-ERG;Pten⁺⁻, and T-Δ-Erg;Pten⁺⁻ double-mutant males developed PIN lesions that stain positive for phosphorylated AKT (pAKT), whereas prostates of Pten⁺⁻-alone mice appeared largely normal (Fig. 2A; Supplemental Fig. S3). In the above cohort, PIN lesions from double-mutant males maintained relatively uniform and high levels of ETV1 (GFP) or ERG expression (Fig. 2B); this is particularly notable for ERG, as in the prostates of ERG knock-in alone, ERG expression was

Figure 1. Tmprss2-ERG (with or without interstitial deletion) and Tmprss2-ETV1 expression are insufficient to initiate prostate tumorigenesis. (A) Targeting strategies for engineering Tmprss2-ERG and Tmprss2-ETV1 knock-ins. Strategy 1 is based on direct knock-in of N terminus-truncated human ERG or ETV1 cDNA [ΔN-hETS] into the murine Tmprss2 locus. Strategy 2 is based on the introduction of loxP sites to murine Tmprss2 and Erg loci by sequential gene targeting in mouse embryonic stem cells so that the 3-Mb interstitial region can be deleted by Cre-mediated recombination and meanwhile generate the Tmprss2-Erg gene fusion. Details of gene targeting are shown in Supplemental Figures S1 and S2. (B) RT–PCR showing expression of the Tmprss2-ETV1 fusion transcripts in T-ETV1 knock-in prostates and expression of the Tmprss2-ERG fusion transcripts in T-ERG and T-Δ-Erg knock-in prostates but not in wild-type [WT] prostates. (C) IHC staining showing moderate ERG expression (arrows) in the anterior lobes of a T-ERG knock-in male but not in the wild-type male. (D) IHC staining showing homogeneous GFP expression (as surrogate for ETV1) in the anterior lobes of a T-ETV1 knock-in male but not in the wild-type male. (E) Hematoxylin and eosin (H&E) staining showing normal prostate histology from all three knock-ins (showing ventral lobes except those of T-ETV1). Arrows in T-ETV1 pictures indicate inflammation in T-ETV1 knock-in males ([left] slight inflammation in the lateral lobe of a young knock-in male, [right] extensive inflammation in the anterior lobe of a 30-mo-old knock-in male). (Right) Arrows in the T-Δ-Erg picture indicate abnormal-looking (lightly stained "foamy"-looking cytoplasm, randomly distributed nuclei) prostate cells, observed in four out of 21 of T-Δ-Erg males. Bars, 100 μm (200 μm in top right picture). All animals analyzed in C–E were ~10 mo of age unless otherwise indicated.
initially relatively weak and heterogeneous (Fig. 1C). Thus, overexpression of ETV1 or ERG correlates with the observed localized premalignant phenotype.

To test cooperation of Tmprss2-ETS with total loss of Pten, we used Pb-Cre [Wu et al. 2001] to inactivate a conditional knockout allele of Pten [Lesche et al. 2002] and generated Pb-Cre;T-3Mb-Erg;PtenL/L males and Pb-Cre;T-ETV1;PtenL/L males. Prostate cancer development in these models was tracked by pAKT expression (Supplemental Fig. S5). Under our housing and genetic background (mixed C57/BL6-129), Pb-Cre;PtenL/L males developed localized PIN lesions that slowly progressed to prostate adenocarcinomas. In contrast, the Pten loss-driven prostate cancer phenotype in Pb-Cre;T-ETV1;PtenL/L males was markedly enhanced. The majority of Pb-Cre;T-ETV1;PtenL/L males died before 1 yr of age, possibly due to large prostatic cyst formation [Supplemental Fig. S5]. In contrast, the majority of Pb-Cre;PtenL/L and Pb-Cre;T-3Mb-Erg;PtenL/L males survived to at least 1 yr of age (Fig. 2C). On histology, we observed aggressive GFP+ (from the T-ETV1 allele) prostate adenocarcinoma cells invading into stroma in Pb-Cre;T-ETV1;PtenL/L prostates [Fig. 2D, panels c,d]. Prostate cancer cells metastasized locally to the urogenital area [e.g., in lymphatic vessel]...
cDNA carrying a substrate tag that permits in vivo targeting of prostate epithelial cells, RWPE-1 cells. Thus, RWPE-1 cells ectopically expressing ETV1 or ERG in immortalized human nontumorigenic prostate cancer cells were engineered to express full-length ETV1 or ERG, respectively, in immortalized prostate cells (Dvorak et al. 1986). To gain mechanistic insights into this difference, we performed an integrated genomic analysis to identify their respective target genes. First, we set up a series of experiments to demonstrate that ETV1 and ERG target genes are associated with cell proliferation and lipid metabolism (Supplemental Fig. S9). As revealed by quantitative RT–PCR of select common genes, ETV1 expression induced up-regulation of genes involved in AR signaling (TMPRSS2 and SOX9) or invasion and lipid metabolism (VIMENTIN, ADRB2, and ACSL3) as well as down-regulation of cell cycle genes (E2F1 and BRCA1) (Fig. 3C). In contrast, these genes exhibited largely an opposite expression pattern in ERG-expressing RWPE-1 cells (Fig. 3C). Thus, these data point to distinct regulatory programs driven by ERG and ETV1 in nontumorigenic prostate cells.

**ERG and ETV1 have shared and distinct chromatin targets**

To interrogate further similarities and differences of ERG and ETV1 transcriptional programs, we performed ChIP-on-chip analysis. We identified ERG ChIP targets in VCaP cells (harboring TMPRSS2-ERG) using a ChIP-seq approach (Supplemental Table S1), consistent with reported ChIP-seq data in RWPE-1 cells (Hollenhorst et al. 2011). Bound regions for each factor were typically in close proximity to the transcription start sites (TSSs) (Supplemental Fig. S10C,D). We confirmed that ERG–ETV1 common targets, ERG-only targets, and ETV1-only targets were largely an opposite expression pattern in ERG-expressing RWPE-1 cells (Fig. 3C). Thus, these data point to distinct regulatory programs driven by ERG and ETV1 in nontumorigenic prostate cells.

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The genetically engineered knock-in mice distinguished ETV1 from ERG in supporting invasive adenocarcinoma. To gain mechanistic insights into this difference, we performed an integrated genomic analysis to identify their respective target genes. First, we ectopically expressed ETV1 or ERG in immortalized human nontumorigenic prostate epithelial cells, RWPE-1 cells. Thus, RWPE-1 cells were engineered to express full-length ETV1 or ERG cDNA carrying a substrate tag that permits in vivo targeting of prostate epithelial cells, RWPE-1 cells. Thus, RWPE-1 cells ectopically expressing ETV1 or ERG in immortalized prostate cells (Dvorak et al. 1986). To gain mechanistic insights into this difference, we performed an integrated genomic analysis to identify their respective target genes. First, we set up a series of experiments to demonstrate that ETV1 and ERG target genes are associated with cell proliferation and lipid metabolism (Supplemental Fig. S9). As revealed by quantitative RT–PCR of select common genes, ETV1 expression induced up-regulation of genes involved in AR signaling (TMPRSS2 and SOX9) or invasion and lipid metabolism (VIMENTIN, ADRB2, and ACSL3) as well as down-regulation of cell cycle genes (E2F1 and BRCA1) (Fig. 3C). In contrast, these genes exhibited largely an opposite expression pattern in ERG-expressing RWPE-1 cells (Fig. 3C). Thus, these data point to distinct regulatory programs driven by ERG and ETV1 in nontumorigenic prostate cells.

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Interestingly, IPA analysis indicated that nuclear receptor signaling pathways, including those associated with estrogen, androgen, and glucocorticoid receptor signaling, were significantly enriched in ERG–ETV1 common targets (Fig. 4C). In contrast, the ERG-only subset correlated with the cell cycle network. Intriguingly, the lipid metabolism biological network as well as the Oncostatin M and IL-3 signaling pathways, which have been correlated with increased cell motility and invasiveness (Dentelli et al. 1999; Holzer et al. 2004), were enriched in the ETV1-only subset (Fig. 4C). Taken together, our combined gene expression and ChIP-on-chip analyses argue that ERG and ETV1 control distinct transcriptional programs in prostate cells.

**ERG and ETV1 interact differentially with the AR signaling pathway**

The AR pathway is a critical driver of tumorigenic prostate development in both androgen-dependent (AD) and castration-resistant stages (Wang et al. 2009). Our data suggest that genes associated with AR signaling belong to the ERG–ETV1 common target category (Fig. 4C, Supplemental Fig. S11A). To address potential cross-talk of ERG and ETV1 common targets with the AD program, we defined an improved androgen-driven signature from AD VCaP and LNCaP cell lines that mitigates differences among increased cell motility and invasiveness (Dentelli et al. 1999; Holzer et al. 2004), were enriched in the ETV1-only subset (Fig. 4C). Taken together, our combined gene expression and ChIP-on-chip analyses argue that ERG and ETV1 control distinct transcriptional programs in prostate cells.

To determine whether ERG and ETV1 also regulate AR signaling differentially in vivo, we examined GFP expression in 

**Figure 3.** ERG and ETV1 regulate a common program in immortalized nontumorigenic RWPE-1 prostate cells but in an opposing fashion. (A) Expression profiling of ERG-overexpressing [R.ERG] and ETV1-overexpressing [R.ETV1] BWPE-1 cells compared with BirA-expressing controls [CTL]. Heat map generated by hierarchical clustering and by applying Pearson correlation and the complete linkage rule. The heat map shows differentially expressed genes [fold change, >1.5; false discovery rate [FDR], <0.05]. (Red) Highest expression; (blue) lowest expression. (B) Bidimensional plot comparing expression profiles of genes differentially expressed [fold change, >1.5] in R.ERG versus R.CTL and in R.ETV1 versus R.CTL RWPE-1 cells. The red line represents the distribution of genes. The dotted line corresponds to a gene density fold change of 1. (C) RT–PCR analysis of select genes associated with prostate cancer pathways upon ERG or ETV1 overexpression in RWPE-1 cells. n = 3 per group. Error bars, SEM; t-test: (** P < 0.01. If no P-value is indicated, P > 0.05.)
active in the ventral prostate lobes [Han et al. 2005]. In accord with this, we observed stronger staining for ERG in the ventral lobes of Pb-AR;T-ERG prostates, compared with barely detectable ERG staining in T-ERG prostates (Fig. 5F). Furthermore, we measured expression levels of select AR targets in mouse prostates. In the Pb-AR background, most AR targets were down-regulated in T-ERG males, whereas AR targets were typically up-regulated in T-ETV1 males, thus illustrating the opposite regulation of AR signaling by ERG and ETV1 in vivo (Fig. 5G). Despite elevated AR signaling, prostates of Pb-AR-alone as well as those of Pb-AR;T-ERG and Pb-AR;T-ETV1 males appeared largely normal. Taken together, human prostate cancer cell and mouse model data indicate that differential regulation of the AR pathway by ETV1 and ERG occurs not only in vitro, but, importantly, also under the physiological setting.

ETV1 directs androgen metabolism in prostate epithelial cells

In addition to the opposing regulation of common targets by ETV1 and ERG, we hypothesized that unique targets controlled by ETV1 might contribute to the aggressive phenotype seen in association with ETV1 expression. To gain mechanistic insights into programs selectively regulated by ETV1 we sorted GFP + (thus, ETV1-expressing) prostate luminal cells from T-ETV1 knock-in males and compared them with luminal cells from wild-type prostates by microarray expression profiling (Fig. 6A,B). We confirmed the luminal cell expression pattern in both sorted samples (Supplemental Fig. S14). By GSEA, we identified several cancer-associated metabolic pathways that were enriched in T-ETV1 luminal cells (Supplemental Fig. S15A). Of note, cholesterol and steroid biosynthesis pathways, both of which are strongly related to prostate tumorigenesis [Twiddy et al. 2010; Zadra et al. 2010], were most highly enriched (Fig. 6C,D). On analysis of a patient cohort with CRPCs metastatic to bone [Stanbrough et al. 2006], we observed that genes associated with the steroid hormone biosynthesis pathway and androgen and estrogen metabolism are significantly enriched in tumors with higher ETV1 expression (Supplemental Fig. S15A). HSD17B7, a gene shared by steroid biosynthesis and steroid hormone biosynthesis pathways, was up-regulated in both T-ETV1 luminal cells and ETV1-high bone metastases (Supplemental Fig. S15B). HSD17B7 as well as other HSD17B enzyme genes (HSD17B4 and HSD17B10) are ChIP targets of ETV1 in prostate cancer cells and are components of the lipid metabolism

Figure 4. ERG and ETV1 drive specific transcriptional programs. (A) Venn diagram of targets occupied by ERG and ETV1. The intensity of binding at each probe was calculated by model-based analysis of tiling array (MAT) [Johnson et al. 2006]. MAT scores were then normalized by quantile–quantile normalization [Bolstad et al. 2003] between ETV1 and ERG ChIP-on-chip experiments. Target loci were defined as the peaks associated with P-value < 10^{-4}. (B) Enrichment of ETS-binding motifs and other indicated motifs in all ChIP target subsets. The Fisher exact test was applied. (C) IPA analysis of ChIP-defined target gene sets implicating common target genes in nuclear receptor signaling pathways and ETV1 unique targets in lipid metabolism network. The significance of enrichment of each gene set is shown as –Log(P-value).
network enriched in ETV1-only ChIP targets (Fig. 4C; Supplemental Fig. S15C). By independent ChIP analysis, we validated ETV1, but not ERG, binding to the HSD17B7 and HSD17B4 promoters (Fig. 6E, Supplemental Fig. S15D). Expression of HSD17B7 in LNCaP cells was reduced upon ETV1 depletion (Fig 6F). In ETV1-overexpressing RWPE-1 cells, HSD17B7 expression trended upward (although not statistically significant) (Supplemental Fig. S16A). We also confirmed higher Hsd17b7 expression in lineage-depleted T-ETV1 prostate cells (Fig. 6G). Since HSD17B7 is critical in converting less active forms of estrogen and androgen to more active forms (Fig. 6H; Krazeisen et al. 1999), and Tmprss2 is an androgen and estrogen dual-responsive gene, we reasoned that up-regulation of the steroid biosynthesis pathway by ETV1 may provide prostate cells with an intrinsic source of steroids. If this supposition were correct, such cells might be intrinsically castration-resistant. To test this prediction, T-ETV1 knock-in males as well as T-ERG and wild-type control males were castrated. We observed that almost half of prostate cells from the castrated T-ETV1 mice were GFP+. In addition, we also detected a small population of GFPlow prostate cells from the castrated T-ERG males (Supplemental Fig. S16B), consistent with a recent study and possibly reflecting the existence of a subpopulation of Tmprss2+ prostate cells that are intrinsically castration-resistant.

Figure 5. ERG and ETV1 regulate AR signaling in an opposite manner. (A) Androgen-induced genes are depleted in ETV1-silenced LNCaP cells upon 16-h androgen stimulation [left] no androgen stimulation; [right] with androgen stimulation. The androgen-induced signature was obtained from the common AR ChIP targets in LNCaP and VCaP cells that were up-regulated in them upon androgen stimulation. (B) Androgen-induced genes are significantly enriched in ERG-silenced VCaP cells upon 16-h androgen stimulation compared with controls [left] no androgen stimulation; [right] with androgen stimulation. (C,D) ETV1 silencing specifically decreases expression of AR-associated genes (C), whereas ERG silencing increases their expression (D). Mean, n = 3; error bars, SEM; t-test: (*) P < 0.05; (**) P < 0.01. If no P-value is indicated, P > 0.05. (E) Flow cytometry analysis demonstrating robust GFP+ population in the T-ETV1 prostates but not in the T-ERG prostates. However, in the presence of the Pb-AR transgene, GFP expression can be readily detected in Pb-AR;T-ERG prostates, in addition, GFP expression in Pb-AR;T-ETV1 prostates appear to be further elevated. (F) IHC staining showing weak ERG staining in the ventral lobe of a T-ERG knock-in male [blue arrow; compared with strong Erg staining in the endothelial cells [black arrow]] but much stronger ERG staining in the ventral lobe of a Pb-AR;T-ERG male [blue arrow; almost comparable with ERG staining in endothelial cells in the same section [black arrow]]. Bars, 50 μm. (G) Real-time PCR quantification showing up-regulation of most AR target genes in Pb-AR;T-ETV1 prostates and slight down-regulation of them in Pb-AR;T-ERG prostates in relation to those of Pb-AR-alone prostates. Mean, n = 3; error bars, SEM; t-test: (*) P < 0.05, (**) P < 0.01. If no P-value is indicated, P > 0.05.
resistant (Casey et al. 2012). As expected, the castration-resistant GFP+ cells in T-ETV1 males exhibited higher Hsd17b7 levels than controls (Supplemental Fig. S16C).

t-test: (***) P < 0.001. If no P-value is indicated, P > 0.05. (H) Schematic diagram showing the key role of 17-β HSD enzymes, including HSD17B7, in converting androgen and estrogen from their less active forms to active forms. (I) ETV1 overexpression in RWPE-1 cells promoted the elevation of the endogenous testosterone level, while no changes were observed upon ERG overexpression [mean, n = 4; error bars, SEM]. Testosterone levels per 10^6 cells (R.ETV1 mean = 642.16 pg/μL; R.ERG mean = 0.49 pg/μL; R.CTL mean = 1.89 pg/μL). t-test: (***) P < 0.001. (J) Testosterone levels were reduced in androgen-deprived [charcoal-treated] LNCaP cells upon stable ETV1 silencing [k/d] as compared with controls [mean, n = 3; error bars, SEM]. [NSC] Nonsilencing shRNA control. Testosterone levels per 10^6 cells [NSC mean = 74.69 pg/μL; R.ERG mean = 0.49 pg/μL; ETV1k/d mean = 0.56 pg/μL]. t-test: (***) P < 0.001.

Figure 6. ETV1 regulates steroid metabolism in prostate cells. (A, right plot) Flow cytometry profiles and gating strategies showing GFP+ prostate luminal cells [Lin Sca-1 CD49frmed] sorted from T-ETV1 knock-in males used for microarray analysis. (Left plot) The Lin Sca-1 CD49frmed prostate luminal cells sorted from wild-type (WT) control males were used as the control. (B) Real-time PCR quantification confirming ectopic ETV1 expression in sorted GFP+ prostate luminal cells from T-ETV1 knock-in mice [mean, n = 3 samples per group; error bars, SEM]. (C,D) Steroid and cholesterol biosynthesis pathways are the top pathways significantly enriched in T-ETV1 knock-in prostate luminal cells compared with controls. Note that a critical enzyme in the steroid biosynthesis pathway, HSD17B7, is also a key enzyme in the steroid hormone biosynthesis pathway, which is enriched in metastatic prostate cancers (Supplemental Fig. S15). (E) ChIP-PCR validation of ETV1 binding to HSD17B7 [pB7], HSD17B4 [pB4], and HSD17B10 [pB10] promoters [mean, n = 5; error bars, SEM]. [pCTL1 and pCTL2] Nonspecific promoter control regions. (F) Only HSD17B7 levels significantly decreased upon knockdown of ETV1 [k/d] [mean, n = 3, error bars, SEM] under both the androgen-deprivation condition [CH-T] charcoal-treated] and the regular condition in the presence of serum [FBS]. Conversely, HSD17B7 expression increased upon ETV1 overexpression in RPWE-1 cells [Supplemental Fig. S16A]. t-test: (**) P < 0.01. If no P-value is indicated, P > 0.05. (G) T-ETV1 knock-in prostate cells exhibit increased Hsd17b7 expression levels compared with wild-type controls [mean, n = 3; error bars, SEM]. t-test: (*) P-value < 0.05. (H) Schematic diagram showing the key role of 17-β HSD enzymes, including HSD17B7, in converting androgen and estrogen from their less active forms to active forms. (I) ETV1 overexpression in RWPE-1 cells promoted the elevation of the endogenous testosterone level, while no changes were observed upon ERG overexpression [mean, n = 4; error bars, SEM]. Testosterone levels per 10^6 cells (R.ETV1 mean = 642.16 pg/μL; R.ERG mean = 0.49 pg/μL; R.CTL mean = 1.89 pg/μL). t-test: (***) P < 0.001. (J) Testosterone levels were reduced in androgen-deprived [charcoal-treated] LNCaP cells upon stable ETV1 silencing [k/d] as compared with controls [mean, n = 3; error bars, SEM]. [NSC] Nonsilencing shRNA control. Testosterone levels per 10^6 cells [NSC mean = 74.69 pg/μL; R.ERG mean = 0.49 pg/μL; ETV1k/d mean = 0.56 pg/μL]. t-test: (***) P < 0.001.
of endogenous testosterone in control, ETV1-expressing, and ERG-expressing human RWPE-1 cells. Remarkably, ETV1-expressing RWPE-1 cells showed much higher levels (>300-fold higher) of endogenous testosterone compared with ERG-expressing and control RWPE-1 cells [Fig. 6f, Supplemental Fig. S17A,B]. Conversely, ETV1 knockdown in LNCaP cells reduced testosterone production under conditions of androgen deprivation [Fig. 6f]. To further characterize this observation, we quantified the intraprostatic levels of testosterone in wild-type, T-ETV1, and T-ERG mice under noncastrated and castrated conditions. As expected, noncastrated mice showed very low concentrations of testosterone in their prostate cells. Interestingly, castrated T-ETV1 mice indeed exhibited higher testosterone levels than those of castrated wild-type and T-ERG prostate samples [Supplemental Fig. S17C]. These results indicate that ETV1 expression directly regulates androgen production in prostate cells.

ETV1-only gene sets associate with an aggressive phenotype in patients

Data to this point suggest that ETV1 and its unique oncogenic program contribute to invasive prostate cancer. To relate these findings to patients, we analyzed data from a patient cohort that includes 22 primary localized and 29 metastatic samples, of which ~50% carried the TMPRSS2-ERG fusion [ETV1 rearrangement status not characterized] [Stanbrough et al. 2006]. In this cohort, samples exhibiting higher ERG expression correlated with localized tumors, whereas high ETV1 expression enriched for metastases [Fig. 7A]. We repeated the analysis with an independent cohort of 150 prostate tumor samples from patients at Memorial Sloan-Kettering Cancer Center [MSKCC] [Taylor et al. 2010]. A correlation between high ETV1 expression and metastases and the presence of more localized prostate tumors in the high ERG expression group were confirmed in this second cohort [Fig. 7B]. Samples with high ERG or high ETV1 expression did not overlap, consistent with a strong tendency to mutual exclusivity in both primary and metastatic samples [Supplemental Fig. S18A,B]. We next interrogated whether ERG and ETV1 cooperate similarly with PTEN deletion in the MSKCC cohort by analyzing the outcome of patients carrying deletion of PTEN and overexpression of ERG or ETV1. High ERG expression with PTEN loss failed to correlate with the worse outcome [Fig. 7C]. In contrast, high ETV1 expression cooperated with PTEN loss, as shown by much poorer disease-free survival [Fig. 7D]. These data are consistent with a previous report correlating greater disease recurrence with high ETV1 levels [Shin et al. 2009].

We next evaluated for the first time whether ERG- and ETV1-specific gene signatures serve as tumor biomarkers or as a predictor of aggressive behavior. We analyzed ERG and ETV1 signatures defined in our analysis of ERG and ETV1 ChIP and expression profiling data in the Swedish watchful waiting and MSKCC cohorts [Setlur et al. 2008; Taylor et al. 2010], including 362 localized prostate cancer samples and 150 patients with localized and metastatic prostate cancer, respectively. Of note, ETV1-specific signatures, comprised of genes directly bound by ETV1 and up-regulated upon ETV1 expression, are associated with a high Gleason score (>7) in both cohorts and with lethality in the MSKCC cohort, again highlighting a correlation between ETV1 expression and a worse disease prognosis [Fig. 7E; Supplemental Fig. S18C]. Taken together, data from three independent patient cohorts concur in validating that ETV1 drives a transcriptional program in prostate cells that is distinct from that of ERG. Moreover, our data suggest for the first time that the ETV1-driven program dictates a poorer outcome in patients with prostate cancer.

Discussion

Our multidisciplinary studies reveal distinct transcription programs regulated by ERG and ETV1 in prostate cells. In particular, we show that they control overlapping gene targets but largely in an opposing fashion, they also control unique targets and pathways. Overall, the networks regulated by ERG are associated with cell cycle and DNA replication, whereas those controlled by ETV1 are related to synthesis of lipids and other metabolic pathways. These networks are distinct and contribute to different pathogenic consequences. These conclusions are validated by findings in novel knock-in mouse models and by patient outcome analysis.

AR signaling is a common pathway regulated by ERG and ETV1 but in an opposite manner

AR signaling is central to prostate development and tumorigenesis. Indeed, AR has recently been implicated in double-strand breaks that favor the formation of translocations involving androgen-driven promoters and ETS family members [Lin et al. 2009; Haffner et al. 2010]. We observed a complex relationship between ERG- or ETV1-regulated networks and AR signaling. In agreement with prior findings, we observed negative regulation of AR signaling by ERG [Yu et al. 2010]. In contrast, ETV1 cooperates with AR signaling by favoring activation of the AR transcriptional program [Fig. 5]. Upon androgen stimulation, ETV1 recruitment to the established PSA and TMPRSS2 regulatory elements correlates with AR binding, suggesting coordinate regulation of androgen-driven genes by ETV1 and AR.

We validated divergent regulation of AR signaling by ERG and ETV1 in vivo in knock-in mice. As Tmprss2 is an AR target, the transcriptional output from the Tmprss2 promoter serves as a reporter for AR activity. In T-ERG knock-in mice, ERG expression appears to down-regulate AR target genes, including Tmprss2, which would lead to down-regulation of its own expression, thus forming a negative regulatory loop, consistent with prior findings [Yu et al. 2010]. In contrast, in T-ETV1 knock-in males, ETV1 expression positively cooperated with AR signaling, leading to further enhancement of expression of AR targets, including Tmprss2, which would then support robust expression of ETV1 and the GFP reporter, reflecting a...
positive regulatory loop. These mechanistic differences account for the striking difference in GFP intensity in the two knock-in strains, which were identically engineered (Fig. 5E). Of note, the negative loop in the T-ERG knock-in is overridden by elevated AR signaling in vivo. Indeed, the only consistent association between the TMPRSS2-ERG fusion and clinical parameters identified thus far is the association of this fusion (in particular when ERG expression was measured by IHC) with a high level of AR signaling (Minner et al. 2011; Hoogland et al. 2012). Last, in human prostate tumors with ETV1 fusions, several 5’ fusion partners other than TMPRSS2 are also involved.

**Figure 7.** ETV1, rather than ERG, expression and the program it drives are associated with advanced prostate cancer in multiple patient cohorts. (A) Heat map showing ERG and ETV1 expression pattern in localized and bone metastatic prostate cancer samples using the Beth-Israel (BI) cohort data set (Stanbrough et al. 2006). Heat map generated by hierarchical clustering and by applying Pearson correlation and the complete linkage rule. Heat map showing differentially expressed select genes (fold change, >2; FDR, <0.05). (B) Graph showing ERG and ETV1 expression along prostate cancer progression from localized to metastatic samples in the MSKCC cohort (Taylor et al. 2010). The graph reveals that the number of patients carrying ETV1 overexpression (fold change, >3), PTEN deletion, and AR alterations (amplification and expression fold change, >3) increased in metastatic samples compared with localized prostate tumors, while patients carrying high levels of ERG (fold change, >3) did not increase over time (also in Supplemental Fig. S18B). t-test: (*) P-value < 0.05; (**) P-value < 0.01. If no P-value is indicated, P > 0.05. (C) Disease-free survival plot showing that among all patients in the MSKCC cohort with PTEN deletion (n = 21), those with ERG overexpression (n = 4) exhibited no survival difference from the rest of patients with PTEN deletion. P = 0.553 by log-rank test. (D) Disease-free survival plot showing that among all patients in the MSKCC cohort with PTEN deletion (n = 21), those with ETV1 overexpression (n = 8) exhibited much worse survival compared with remaining patients with PTEN deletion. (*) P = 0.015 by log-rank test. (E) Correlation between ERG- and ETV1-associated gene sets with patient prognosis. Overlap between genes enriched in patient samples associated with indolent or aggressive prostate cancer from the MSKCC cohort (Taylor et al. 2010) and ERG or ETV1 gene sets defined in Figures 3 and 4. ETV1-associated genes are enriched in patients with a higher Gleason score in the Swedish cohort (also in Supplemental Fig. S18C; Sethur et al. 2008). "UP" represents those genes up-regulated in the shown category with a fold change of >1.5. The significance of overlap of these gene sets was calculated by the Fisher exact test and visualized as connecting line width (cutoff, P = 0.01). (Red) Aggressive prostate cancer-associated; (green) ETV1-associated gene sets; (blue) TMPRSS2-ERG fusion-associated gene sets; (purple) ERG signature-associated gene sets; (yellow) AR-associated gene sets; (orange) common targets of ERG and ETV1. (F) Model illustrating the differential contribution by ERG and ETV1 to prostate tumorigenesis under the PTEN loss background. See the text for details.
Since the majority of these 5′ fusion partners are also androgen-responsive genes (Tomlins et al. 2007; Attard et al. 2008b), we reason that a similar positive regulatory loop may be operative in such tumors to enhance AR signaling and ETV1 expression. In addition, AR has been reported to regulate the endogenous ETV1 locus as well (Cai et al. 2007), suggesting a positive feedback between both the endogenous and rearranged ETV1, thus predisposing affected prostate cells for prostate cancer development.

ETV1, but not ERG, reprograms metabolic pathways in normal prostate epithelial cells

Among the unique genes up-regulated by ETV1, those encoding for enzymes involved in cholesterol and steroid biosynthesis and in cancer-associated metabolic pathways (e.g., glycolysis) (Supplemental Fig. S15A) are of particular interest. In particular, we were able to demonstrate increased production of endogenous testosterone upon ETV1 expression in both human prostate cells and T-ETV1 knock-in mice (upon castration) (Fig. 6I,J; Supplemental Fig. S17C). Due to the increased testosterone level, we anticipated observing higher levels of androstenedione or dihydrotestosterone as well; however, none were detected, possibly due to technical limitations. However, decreased levels of estrone were detected in ETV1-expressing RWPE-1 cells, probably due to the promotion of testosterone synthesis (Supplemental Fig. S17B).

Although ETV1-expressing prostate cells appear ostensibly normal (Fig. 1E), their metabolic programs resemble those of cancer cells. Increased aerobic glycolysis has been observed only in advanced disease, whereas increased sterol and protein synthesis are common features of both primary and advanced prostate cancer (Swinnen et al. 2000; Rossi et al. 2003; Ettinger 2004; Bauer et al. 2005). In particular, activation of lipid metabolism has been described in most localized and metastatic prostate tumors, underscoring its potential role in prostate cancer progression (Tewdwydd et al. 2010; Zadra et al. 2010). Arachidonic acid metabolism and Toll-like receptor signaling inflammatory pathways (De Marzo et al. 2007) are enriched in both CRPCs with higher ETV1 expression and T-ETV1 mouse prostate cells, correlating with the extended areas of inflammation observed in T-ETV1 prostates (Fig. 1E). Thus, we speculate that this unique ETV1-controlled program, in concert with positive cooperation with AR signaling, may reprogram prostate cells for malignant progression in association with additional oncogenic events (Fig. 7F).

TMPRSS2-ETV1 and TMPRSS2-ERG fusions make distinct contributions to prostate tumorigenesis

Although both Tmprrs2-ERG and Tmprss2-ETV1 cooperate with loss of a single copy of Pten in leading to localized PIN lesions, similar to those demonstrated previously in mice overexpressing ERG or ETV1 under the Probasin or viral promoters (Carver et al. 2009; King et al. 2009; Zong et al. 2009) or in mice expressing ERG from an extended human TMPRSS2 promoter (Casey et al. 2012), our mechanistic and animal model studies suggest they do so differently (Fig. 7F). Ectopic ERG expression likely represses the differentiation program of prostate cells (e.g., represses AR and AR targets and up-regulates EzH2 and its targets, as suggested previously) (Yu et al. 2010). Interestingly, it has been reported that Pten loss leads to a castration-like phenotype by suppressing androgen-responsive gene expression through modulation of AR transcriptional activity. Moreover, conditional deletion of AR further promotes proliferation of prostate cells with Pten loss (Mulholland et al. 2011). We speculate that TMPRSS2-ERG may act in a similar fashion by downregulating AR and, consequently, promoting cell proliferation. Indeed, the ERG expression pattern in our murine models (Figs. 1C, 2B,D) suggests that ERG may be required primarily at early stages of the disease but may be not as functionally relevant at late stages. This may explain our observation that ERG cooperates with Pten haploinsufficiency (i.e., under a more sensitized Pten+/− background), whereas its contribution under the total Pten loss background appears far less. The high levels of ERG expression are often observed in localized fusion-positive human prostate cancers may be a secondary consequence of high activity of AR signaling in such tumors in general (i.e., similar to what we observed in Pb-AR;T-ERG prostate) (Fig. 5E–G) rather than a critical requirement of ERG overexpression at this stage. In contrast, ectopic ETV1 expression appears to enhance androgen signaling and reprogram the metabolism of prostate cells, processes critical for both early and advanced stages of the disease. Activation of the PI3K/AKT pathway drives anabolic metabolism and tumorigenesis (Ward and Thompson 2012). We propose that TMPRSS2-ETV1 cooperates with Pten loss by further enhancing metabolic reprogramming, in particular, by favoring steroid biosynthesis, a pathway critical for invasive adenocarcinoma cells. The cooperation between ETV1 and Pten loss is also consistent with the recent finding that combined loss of Pten and COP1, a ubiquitin ligase that negatively regulates ETV1 levels, leads to more invasive prostate adenocarcinomas (Vitari et al. 2011).

In aged PbCre;T-3Mb-Erg,Pten+/− males, we also observed invasive prostate cancer (Fig. 2E). Interestingly, in such invasive cancers, we observed a mosaic pattern of Erg expression (Fig. 2E). Several possibilities can explain this observation. Erg-expressing prostate cells may send signals to Erg-negative cells so that high levels of Erg expression are not needed in all cells of the invasive cancer. Alternatively, Erg may not be critical for the development of advanced cancer, and another genetic or epigenetic change may contribute to advanced disease. One potential genetic change is haploinsufficiency of one or more deleted genes in the interstitial region. Interestingly, ETS2, a gene residing within the interstitial region, was recently proposed to be a tumor suppressor contributing to aggressive prostate cancer cases carrying TMPRSS2-ERG fusions with deletion (Grasso et al. 2012). Whether haploinsufficiency of the deleted genes (e.g., ETS2) contributes to the advanced cancer phenotype awaits further investigation.
Distinct roles of ETV1 and ERG in prostate tumorigenesis have implications for prostate cancer therapy

Our analysis of gene expression and patient outcome data sets underscores the relevance of distinct features of ETV1-regulated pathways to invasive adenocarcinoma progression. ETV1-defined, but not ERG-defined, gene sets are associated with high Gleason score and metastasis (Fig. 7E, Supplemental Fig. S18C). Our observation that ERG expression does not correlate with the worse outcome is consistent with a recent meta-analysis describing no association of ERG with Gleason score, clinical outcome, or recurrence of the disease including 62 cohorts (Pettersson et al. 2012). Of note, ERG mRNA and protein level analysis (Markert et al. 2011; Pettersson et al. 2012) showed that TMPRSS2-ERG fusion status does not always correlate with the TMPRSS2-ERG transcriptional signature or ERG protein level in prostate cancer patients. Accordingly, most recent clinical studies have supported high ERG expression levels as a favorable prognosis biomarker (Bismar et al. 2012; Kimura et al. 2012, Suh et al. 2012). Consistent with our findings, however, ETV1 expression at the transcript level has been associated with a greater Gleason score and recurrence of the disease [Attard et al. 2008b, Shin et al. 2009]. Unfortunately, thus far, it has not been possible to study clinical relevance of ETV1 at the protein level due to the lack of satisfactory antibodies. Moreover, ETV1, rather than ERG, is among AR ChIP targets defined recently from primary CRPC patient samples (Sharma et al. 2013). Last, there is also a high overlap between ETV1-associated, castration-associated, and recurrent prostate tumor signatures (Supplemental Fig. S18E). Although TMPRSS2-ETV1 fusions are only found in ~1%-2% of all prostate cancer cases, prostate tumors with elevated ETV1 expression [5%-10% of all cases] are enriched in advanced disease [Fig. 7A,B], suggesting that the ETV1-driven oncogenic program may be selected for during prostate cancer progression.

In summary, our data suggest that ETV1 is a novel marker of aggressive prostate cancer, and the oncogenic program it drives may be an important therapeutic target for treating advanced prostate cancer. Metabolic enzymes (such as HSD17B7) that are regulated by ETV1 may be explored as therapeutic targets. Moreover, ETS factors have been described to modulate the Ras/MAPK pathway (Hollenhorst et al. 2011). Interestingly, ETV1 overexpression, but not that of ERG, is associated with Ras/MAPK activity in a range of tumors, including ETV1-dependent melanoma and gastrointestinal stromal tumor, where ETV1 is a master regulator of lineage (Chi et al. 2010, Jane-Valbuena et al. 2010). These observations raise the possibility that MAPK inhibitors may be explored to target ETV1-overexpressing tumors. In conclusion, our study suggests that tumors characterized by an ETV1 expression signature through either translocation or other mechanisms represent a distinct biological entity associated with aggressive prostate cancer. Future research should focus on exploring novel therapeutic approaches for this entity.

ETV1 promotes aggressive prostate cancer phenotype

Materials and methods

Mouse lines

Tmprss2-ETS conditional knock-in mice were generated by standard gene targeting. Pb-Cre (Pb-CreD) transgenic mice were acquired from the Mouse Models of Human Cancers Consortium (MMHCC) repository. Pten conditional knockout mice [Ptenfl/fl] and Pb-AR [Fvb-Tg[Pbsn-Ar*E231G]] transgenic mice were acquired from JAX. Ptenfl/fl mice were generated by crossing Ptenfl/fl mice to Gata1-Cre mice. All studies were approved by the Institutional Animal Care and Use Committee (IACUC).

Pathology, immunostaining, and flow cytometry

Standard protocols were followed.

Cell lines

Cell lines were obtained from American Type Culture Collection and cultured accordingly. ERG or ETV1 overexpression and silencing experiments were performed by standard protocols.

RT–PCR

Real-time RT–PCR was performed according to standard protocols. All primer sequences are listed in Supplemental Table S2.

Gene expression microarray analysis

RWPE-1 stable cell clones [R.ERG, R.ETV1, and R.CTL] were grown under normal conditions. VCaP and LNCaP cells, 24 h after ERG or ETV1 RNAi, respectively, were grown in hormone-depleted conditions for 2 d, and then in the presence or absence of 10 nM DHT for 16 h. Mouse primary prostate cells were FACSorted and processed according to standard procedures. Affymetrix HG133 plus 2.0 or Mouse Genome 430 2.0 expression arrays were used for human or mouse samples, respectively. Gene Pattern software (Reich et al. 2006) was used for data normalization, extraction of expression values, and generation of GTC files for GSEA (Subramanian et al. 2005). A bidimensional comparison plot was used to compare differentially expressed genes (P < 0.05 by two-tailed t-test) in RWPE-1 cells upon overexpression of either ETV1 or ERG.

ChIP and ChIP-on-chip

BioChIP–chip for ETV1 was performed as described (Kim et al. 2008), and conventional ChIP–chip reaction for ERG was as described (Kim et al. 2004). Affymetrix Human Promoter 1.0R array hybridization was performed at the Dana-Farber Cancer Institute Microarray Core Facility. Peak identification was calculated by MAT score [Johnson et al. 2006]. For ChIP-PCR experiments, conventional ChIP reactions were performed. Antibodies used were as follows: anti-AR (N20X), anti-ERG (C17X) and anti-rabbit IgG from Santa Cruz Biotechnology, and anti-ETV1 kindly provided by Dr. Litovchick. The online DAVID functional annotation tool [Huang et al. 2009] and the IPA tool [Ingenuity Systems, Inc.] were used to determine the enrichment for all “FAT” GO terms and canonical pathways/networks in each gene set.

Patient tumor data analysis

Gene sets associated with indolent and aggressive prostate cancer were extracted from the Swedish, MSKCC, Sharma, and
Glinsky cohorts (Glinsky 2004; Setlur et al. 2008; Taylor et al. 2010; Sharma et al. 2013) and analyzed for their mutual overlap between tumor cohort-derived signatures (differentially expressed genes: fold change, >1.5; false discovery rate [FDR], <0.05) and ERG- and ETV1-associated gene sets obtained in our studies. The overlap between gene sets was represented by a connectivity network, where the width of the connector edge was $-\log_{10}(P\text{-value})$. The $P$-value was derived from a hyper-geometric distribution by using Fisher exact test to analyze the significance of the mutual overlap. Cytoscape software version 2.8 (Cline et al. 2007) was used for the visualization of gene sets overlapping the network.

**Statistics**

All statistics were based on a Student’s $t$-test, unless otherwise indicated. Dot plots and histograms show data means, and error bars are standard error of the mean (SEM). All statistics were performed using the data analysis package within Microsoft Excel or the analysis tool within GraphPad Prism 5.0. Kaplan-Meier survival analysis was performed using GraphPad Prism 5.0.

**Steroid metabolism measurement**

Steroids from RWPE-1 and androgen-deprived LNCaP cells were extracted following Lemmen et al. (2002) and quantified by LC/MS at the Harvard FAS Center for Systems Biology.

**Testosterone measurement**

The intraprostatic testosterone levels were measured by a mouse testosterone ELISA kit (Calbiotech) based on the manufacturer’s instructions. Briefly, mouse prostates were microdissected in cold PBS and lysed in RIPA buffer. Testosterone levels were calculated as the total amount per gram of total protein.

**Accession number**

The Gene Expression Omnibus accession number is GSE39388.

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**References**


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