Oncogenic ETS Factors in Prostate Cancer

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Introduction

Prostate cancer represents the only carcinoma containing a common chromosomal rearrangement, resulting in an oncogenic fusion gene: *TMRPSS2/ERG*. About one-half of prostate tumors have a rearrangement of chromosome 21 that fuses the promoter and 5′ untranslated region (5′ UTR) of *TMPRSS2* to the open reading frame of *ERG* [1]. *ERG* is normally silent in adult prostate epithelial cells, while *TMRPSS2* is androgen-responsive and highly expressed in the prostate. The *TMPRSS2/ERG* fusion gene usually results in prostate cells that express either a full-length, or N-terminally truncated ERG protein, depending on the breakpoint [2]. Transgenic mouse models indicate that *ERG* expression in prostate epithelial cells promotes carcinogenesis, but only in collaboration with a second oncogenic "hit" [3–6]. *ERG* encodes an ETS family transcription factor that has the potential to alter gene expression patterns. In addition to the *TMPRSS2/ERG*

rearrangement, prostate cancers have other recurrent rearrangements that similarly promote the expression of additional ETS factors such as *ETV1* and *ETV4* [2]. These findings indicate that ETS family transcription factors can play key roles in promoting prostate cancer. This chapter will discuss the role of ETS factors in prostate cancer with a focus on recently uncovered molecular mechanisms that could point the way to ETS-targeted therapeutics.

ETS Family Transcription Factors

What Is an ETS Factor?

The ETS family of transcription factors are encoded by 28 genes in the human genome [7]. ETS factors are defined by a conserved ETS DNA binding domain consisting of a winged-helix-turn-helix structure that can bind DNA monomerically. In vitro DNA sequence specificity of ETS domains have been extensively measured, and every ETS factor requires a GGA(A/T) sequence for high-affinity DNA binding. An extended consensus binding sequence of CCGGAAGT is common in the family, with a handful of members displaying slightly different preferences [8]. Due to this overlapping sequence preference it is difficult to predict which family member will bind an ETS binding sequence in vivo, and competition for binding sites between

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family members is likely. For this reason, when a new ETS factor is expressed in a cell, such as when the *TMRPSS2/ERG* rearrangement occurs in prostate cells, it is possible that changes in gene expression could be the result of transcriptional changes driven by the newly expressed ETS factor, and/or changes caused by displacement of an endogenous ETS factor.

A phylogenetic comparison of the ETS domain divides the ETS family into subclasses of up to three members each, with ERG in the ERG subfamily and ETV1 and ETV4 in the PEA3 subfamily (Fig. 1). Within each of these subclasses there tends to be homology across the entire pro-

tein, however, a comparison between any two subclasses shows that the only homology is in the ETS DNA binding domain. This diversity of sequence outside of the DNA binding domain results in diverse molecular mechanisms, with some members acting as transcriptional activators, and others as transcriptional repressors, and with members responding in distinct ways to signaling pathways [7].

ETS transcription factors are extensively co-expressed in all cell types [9]. There are 8–10 ETS factors that are ubiquitously expressed, while others have varying levels of tissue specificity. Most cell types, normal prostate epithelia
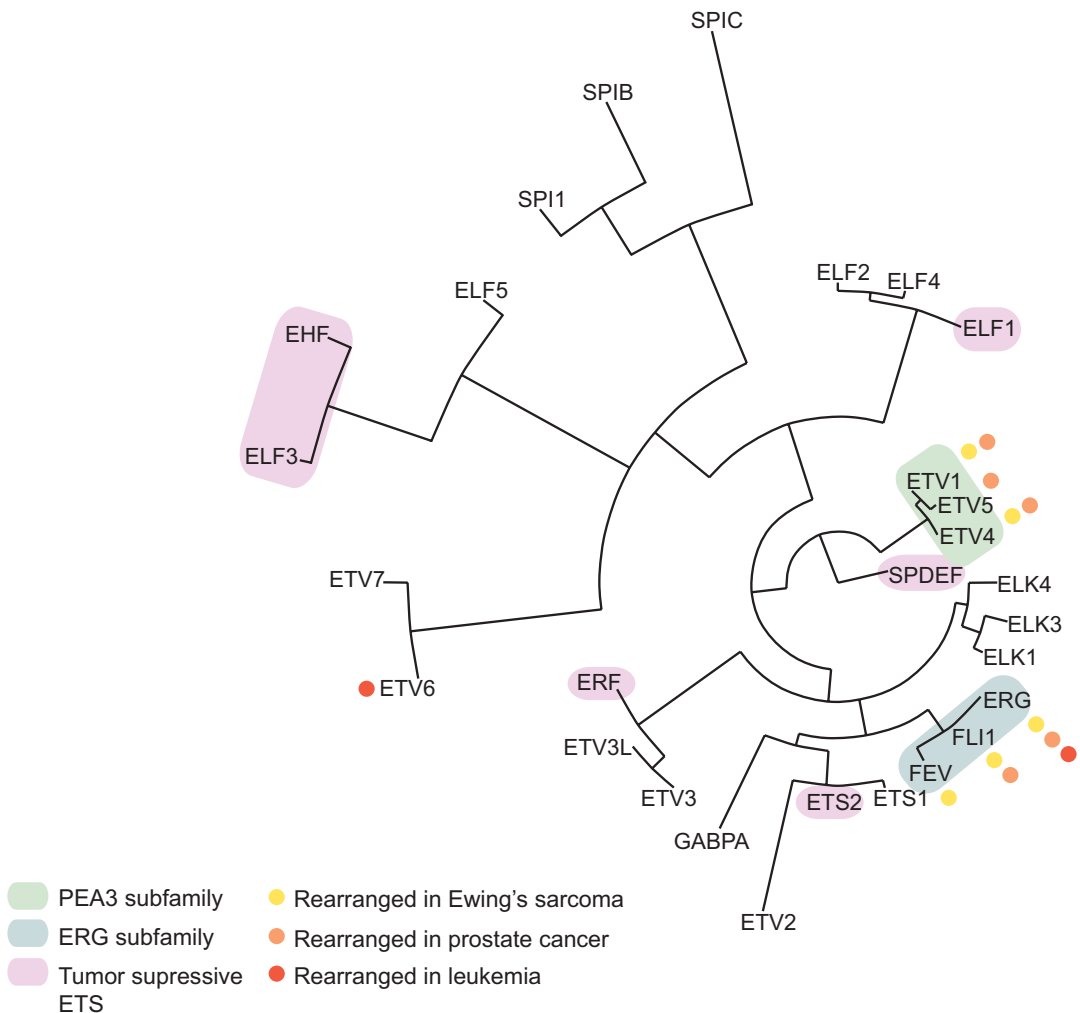

Fig. 1 Circular phylogeny of ETS factors by sequence similarity in the ETS DNA binding domain

included, express 15–20 ETS factors at the mRNA level [9]. SPDEF is the most highly expressed ETS mRNA in normal prostate, and one early name for this gene was prostate-derived ETS factor (PDEF). Likewise, the ETS factors ELF3 and EHF are highly expressed in multiple epithelial cell types including prostate epithelia and were historically named ESE1 and ESE3 for "Epithelial Specific ETS". Consistent with a role in maintaining normal prostate epithelial identity, SPDEF, ELF3 and EHF have all been reported to be potential tumor suppressive genes in the prostate [10–12]. Such tumor suppressive roles will be discussed in more detail later in this chapter.

ETS Factors Aberrantly Expressed in Prostate Cancer

Three members of the ETS transcription factor family, *ERG*, *ETV1*, and *ETV4* are not expressed in normal adult prostate cells [9], but are commonly expressed in prostate cancer cells due to chromosomal rearrangements [2]. Most common is *ERG* overexpression, which is due to *ERG* gene rearrangement in 40–50% of prostate tumors [13, 14]. *ETV1* rearrangement is the second most common and is found in 8–10% of tumors. ETV4 is rearranged in 2–5% of cases. These rearrangements tend to be mutually exclusive, indicating that they have similar roles as oncogenes (Fig. 2). Transgenic expression of *ERG*, *ETV1*, and *ETV4* in mouse prostates indicate that all three are oncogenic, although in all three cases additional "hits" are needed to promote robust tumorigenesis [3–5, 15–17]. These additional hits usually involve activation of oncogenic signaling pathways, and the necessity of these pathways will be discussed later in the chapter. Since ~85% of prostate tumors that harbor ETS rearrangements have the *TMPRSS2/ERG* rearrangement, most research has aimed to understand the molecular consequences of ectopic *ERG* expression in the prostate. Based on the incidence and these experimental findings, it is clear that *ERG*, *ETV1*, and *ETV4* are all prostate cancer oncogenes.

Recent paired-end sequencing of large numbers of prostate tumors has revealed that these tumors can have large numbers of gene rearrangements [14]. Due to this, thousands of genes have been found to be rearranged at low frequency (<2%) in prostate tumors. Inevitably, some of these genes are in the ETS family. But are these rearrangements passenger or driver mutations? In addition to the frequently rearranged *ERG*, *ETV1*, and *ETV4*, the ETS genes *ETV5*, *FLI1* and *ELK4* are rearranged at low frequency in prostate tumors [18–20]. Importantly, *ETV5* and *FLI1* are not expressed in normal prostate cells, however *ELK4* expression in normal prostate is relatively high [9], and it is possible that *ELK4* rearrangements discovered at the RNA level are due to trans-splicing, rather than DNA rearrangement. To date, no transgenic mouse models have been reported that can be used to determine if gene fusion of *FLI1*, *ETV5*, or *ELK4* in the prostate is oncogenic. We and others have used cell line models to test the role of over-expressing various ETS factors. In these studies, *ETV5* has similar roles to *ERG*, *ETV1*, and *ETV4* when expressed in normal prostate RWPE-1 cells [20, 21], suggesting that *ETV5* fusions are oncogenic. In all, we have tested 12 ETS genes, including *FLI1*, in in vitro assays, but only *ERG*, *ETV1*, *ETV4*, and *ETV5* promoted common oncogene-related functions such as cell migration [21, 22]. Intriguingly, these four ETS factors also share a common molecular mechanism, which will be discussed later in the chapter.

ETS Gene Fusions in Prostate Cancer

5′ Fusion Partners and Fusion Products

ETS gene fusions have been assayed in tumor samples by various techniques including reverse transcription polymerase chain reaction (RT-PCR), fluorescence *in situ* hybridization (FISH), and rapid amplification of cDNA ends (RACE). The frequency of each fusion type varies in the literature depending on cohort selection and detection technique, and these incongruities have been
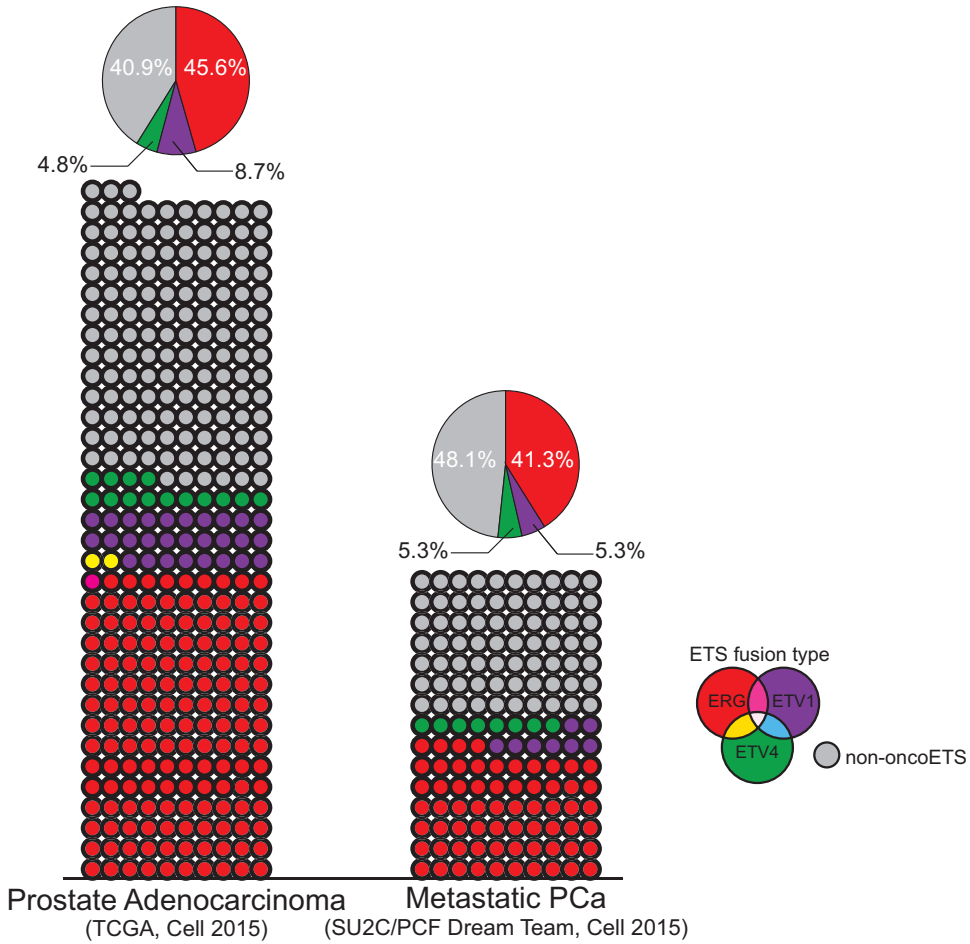
Fig. 2 Distribution of oncogenic ETS fusions across prostate cancer data sets. Three hundred and thirty-three primary prostate adenocarcinoma samples from the TCGA is depicted on the left. Hundred and fifty meta-static prostate cancer samples from the SU2C/PCF Dream Team is depicted on the right. Pie charts representing percentage of each data set with each feature type is displayed above the corresponding bar. Each circle represents a patient sample

reviewed elsewhere [23, 24]. However, recurrent ETS rearrangements exclusively involve fusion of the 3′ end of ETS genes, which include the ETS DNA binding domain. The 5′ partners most often donate promoters that drive high expression in prostate cells, allowing the aberrant expression of oncogenic ETS at high levels. The most common 5′ fusion partners are androgen responsive, yet androgen insensitive and androgen repressed fusion partners have been characterized [23]. *TMPRSS2*, an androgen responsive gene, is the most frequent 5′ partner [1]. While *TMPRSS2/ERG* accounts for the majority of ETS rearrange-

ments, TMPRSS2 is also found rearranged with *ETV1*, *ETV4*, and *ETV5* at lower frequencies [20, 25, 26]. Rarer 5′ fusion partners of *ERG* are *SLC45A3*, *NDRG1*, *and HERPUD1* [23, 24, 27]. *ETV1* 5′ fusion partners include *TMPRSS2*, *SLC45A3*, *HERV-K*, *HERVK17*, *C15ORF21*, *HNRPA2B1*, *OR51E2*, *EST14*, *FLJ35294*, *FOXP1*, and *ACSLS* [1, 2, 24, 28]; and reported *ETV4* 5′ fusion partners are *TMPRSS2*, *SLC45A3*, *CANT1*, *KLK2*, *DDX5*, *HERVK17*, and *UBTF* [28–30]. While 5′ fusion partners are diverse, the congruency of 3′ fusion partners indicates an oncogenic ETS requirement for these rearrangement-driven

prostate cancers. Rare cases in which patients with ERG rearrangements also have ETV1 or ETV4 fusions ([13, 19, 31]; Fig. 2) have been reported and sometimes arise from discrete foci; However, multiple ETS rearrangements have been reported in the same focus [32]. Regardless, these observations exemplify the heterogeneous nature of prostate tumors and tendency towards these genetic alterations.

Aside from diversity at the 5′ end, additional ETS rearrangement diversity can arise from breakpoint site variation. Many *TMPRSS2/ERG* fusions have been reported, and contain either the first, second, or third exon of *TMPRSS2* joined to exon 2, 3, 4, 5, or 6 of *ERG* [31, 33]. Briefly, the most common breakpoint in prostate cancer results in the fusion of the 5′ UTR exon 1 of TMPRSS2 to exon 3 of ERG, resulting in an N-terminally truncated ERG protein (ERGΔ32; named from NCBI ERG isoform 1). Other common fusion products are ERGΔ92 (exon 1 of *TMPRSS2* fused to exon 4 of ERG) and fusions that result in expression of full-length ERG (fusions prior to ERG exon 3). In more rare cases part of the open reading frame of *TMPRSS2* is fused to different breakpoints in *ERG* resulting in some *TMPRSS2* coded amino acids fused to the N-terminus of ERG. At the RNA level, alternative splicing of TMPRSS2/ERG can occur [31]. This potentiates the expression of multiple fusion protein products in the same tumor; however, the oncogenic contribution of different TMPRSS2/ERG variants is not fully understood. Transcripts encoding smaller ERG truncations can be found co-expressed in samples with larger fusion proteins [31]. These smaller truncations are likely alternatively spliced products of *TMPRSS2/ERG*, and it is unknown if they contribute to ERG-mediated oncogenesis.

Demographics of Patients with ETS-Positive Prostate Cancer

Occurrence of the *TMRPSS2/ERG* fusion in prostate cancer differs significantly among different demographic groups. Despite the higher incidence and mortality of prostate cancer in men of African descent [34], this group has a lower frequency of *TMPRSS2/ERG* rearrangement. In a recent meta-analysis, 49% of prostate tumors from men of European decent expressed ERG, while only 27% of tumors from men of Asian descent, and 25% of tumors from men of African descent expressed ERG [35]. ERG-positivity in North Indian patients resembles that of the incidence for Caucasians [36]. It is unknown if these discrepancies are due to common alternative mechanisms that drive prostate tumors in men of African and Asian descent, or if it is due to a lower likelihood of ERG gene rearrangement or repressed ERG function in these populations. The TMPRSS2/ERG fusion is more predominant in early onset prostate cancer, affecting men less than 50 years old [37, 38].

Molecular Stratification of ETS-Positive Prostate Cancers

Classification of prostate cancer into molecular subtypes is an important step in the development and use of precision medicines. ETS-positive prostate cancer has emerged as the largest molecular subtype, but the mutational landscape of ERG-positive (and ERG-negative) prostate cancers are still being investigated. Much of what we know about the molecular stratification of prostate cancer comes from the in depth molecular analysis of 333 primary prostate cancers by The Cancer Genome Atlas Research Network [13]. We will discuss prominent co-occurring and mutually exclusive genetic alterations with ETS fusions (profiling ERG expression in Fig. 3), but further reading should be directed to the above reference. The concurrence between the TMPRSS2/ERG rearrangement and PTEN silencing has been well characterized [36, 39]. The correlation is significant in prostatectomy samples and primary tumors but not in castration resistant prostate cancer (CRPC), where PTEN loss in ERG-positive cases typifies aggressive disease [40]. Other prostate cancer molecular subtypes, such as SPINK1 positive, SPOP mutant, and CHD1 mutant are mutually exclusive with ETS-rearrangements regardless of stage
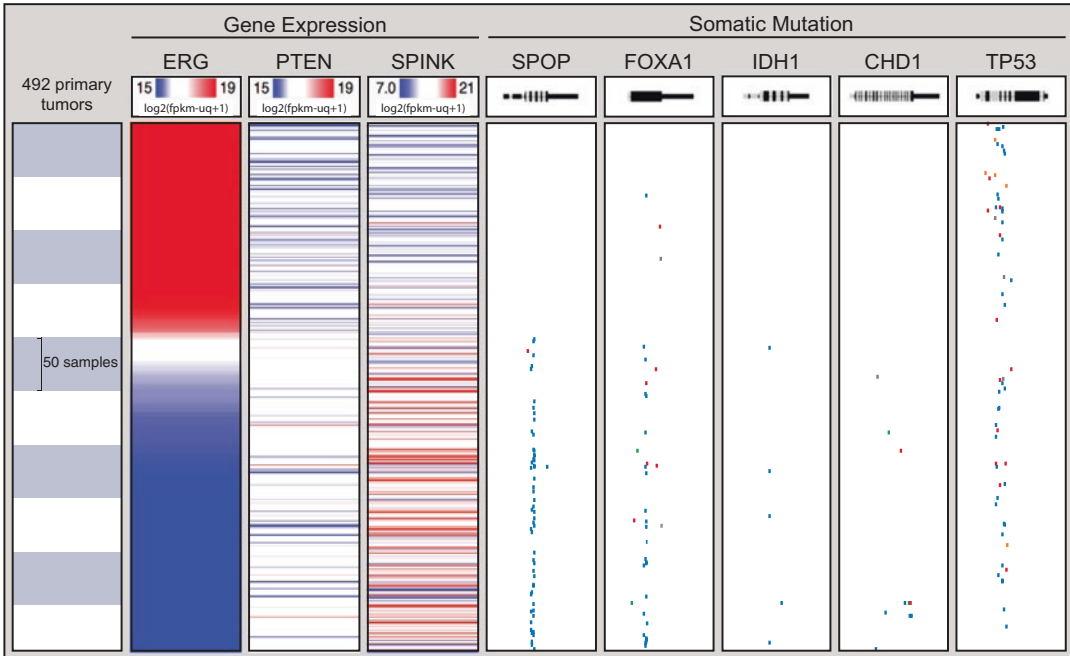
Fig. 3 ERG-centric molecular stratification of primary prostate cancers. The TCGA prostate adenocarcinoma (PRAD) data set was used to rank common molecular features by ERG gene expression and visualized by the UCSC Xena browser [200]. Gene expression profiles for ERG, PTEN, and SPINK are depicted as heat maps. Somatic mutations for the SPOP, FOXA1, IDH1, CHD1, and TP53 genes are shown in relation to gene structure and represented as pixels. Pixel color indicates mutation type, characterized by Xena browser. Samples with no data are excluded

(Fig. 3) [19, 39–44]. Interestingly, in some primary prostate cancers, ETV1 and ETV4 have been found overexpressed but *not* rearranged. Although the mechanism is undefined, these tumors are mutually exclusive with those harboring ETS rearrangements [13]. Tumor heterogeneity is an important factor in calling molecular subtypes; for example, mutational co-occurrence increases from single cell to single foci to whole tumor. The same tumor can be called ETS-positive or ETS-negative, depending on where the biopsy was taken [31]. Correct calling of the molecular subtype of a tumor underlies a critical obstacle in linking molecular subtypes to clinicopathological indicators and treatment course.

Clinicopathological Value of Oncogenic ETS

Because the TMPRSS2/ERG fusion gene is unique to prostate cancer cells and because ETS rearrangements result in much higher expression of oncogenic ETS in malignant tissue compared to adjacent normal tissue [1, 45, 46], the presence of the TMRPSS2/ERG fusion and/or expression of oncogenic ETS have emerged as clinical biomarkers of a cancerous prostate. Yet the connection between various clinicopathological indicators and ETS-positivity has not been fully determined. Cohort selection, evaluation, and mutational background differences potentially underlie the controversial prognostic value of oncogenic ETS. In a cohort of patients with localized prostate cancer undergoing "watchful waiting" therapy, a significant correlation was found with TMPRSS2/ERG-positive tumors and lethality [47]. Other groups have reported similar relationships between ERG positivity and active surveillance progression [48–50], suggesting the value of TMPRSS2/ERG as a prognosticator for risk of disease progression. However, in post-prostatectomy patients, ERG protein expression was found to correlate positively with longer pro-

gression free survival [40]. Similarly, ERG-positivity was found to be associated with longer recurrence free survival and longer overall survival in localized and castration resistant prostate cancer [51]. In contrast, no connection was found between ERG and prognosis in a separate radical prostatectomy cohort [52]. Additionally, patients with *TMPRSS2/ERG* and loss of PTEN were found to have shorter survival than ERG-/PTEN-patients [53]. ERG expression levels, rather than mere presence, correlates with aggression and disease stage and could be used for a more accurate prognosis [54]. It should be noted that because most ERG gene rearrangements result in androgen-driven ERG expression, the level of ERG in these prostate tumors may be indicative of high activity of the androgen receptor (AR), and it may be difficult to parse out whether phenotypes are due to high ERG or high AR activity. Clearly, a multivariate approach is necessary for understanding the risk associated with oncogenic ETS-positivity.

Recently, efforts have been made to develop screening assays to detect TMPRSS2/ERG by transcription-mediated amplification in the urine and serum to allow for early detection without biopsy. In this application TMPRSS2/ERG represents an ideal biomarker as it is an RNA that is completely tumor specific, and therefore the detection of even small amounts can be considered positive. The Mi-Prostate Score (MiPS) is a commercially available logistic regression model that uses detection of urine TMPRSS2/ERG as well as urine prostate cancer antigen 3 (PCA) normalized to PSA to predict prostate cancer and high-grade prostate cancer on biopsy [55]. MiPS has been shown to reduce biopsies by 51% [56]. PCA3 and TMPRSS2/ERG urine levels are significantly associated with high grade disease [53].

Since methods for detecting ETS fusions are abundant, relatively inexpensive, and precise, another hope is that variants of ETS fusions might contain more prognostic value. Several splice variants of TMPRSS2/ERG have been reported in clinical samples. Retention of both exons 10 and 11 of *ERG* is correlated with advanced disease [57]. Additionally, expression of *TMPRSS2/ERG*

fusions resulting from distinct breakpoints may have prognostic value. The type VI fusion, in which the *TMPRSS2* start codon in exon 2 is in frame with exon 4 of *ERG*, is associated with aggressive disease measured by early PSA recurrences and seminal vesicle invasion [33].

Generation of TMPRSS2/ERG Fusions

How do gene fusions occur in prostate epithelial cells? The prevalence of chromosomal rearrangements in leukemias and lymphomas could be due to the misapplication of the cellular machinery used to rearrange the immunoglobulin and T cell receptor loci. However, the discovery of common chromosomal rearrangements in prostate cancer was a surprise, as there is no such obvious mechanism to explain their occurrence. It is not clear why prostate cancer appears to be unique among carcinomas in this molecular cause. Prostate cancer is similar to many types of sarcoma, where oncogenic fusion genes are also common. Interestingly, the *TMPRSS2/ERG* fusion can be generated in rare cells in cell culture. Two prerequisites have been found necessary for the formation of the *TMPRSS2/ERG* fusion: (1) induced proximity of breakpoint regions and (2) DNA damage directed to breakpoint sites and subsequent aberrant repair. AR and topoisomerase II beta (TOP2B) bind to the TMPRSS2 and ERG breakpoints [58, 59], bringing these distal regions into close proximity in response to androgen stimulation in androgen responsive cells [58, 60]. TOP2B recruitment facilitates double stranded DNA breaks at the TMPRSS2 and ERG breakpoints, which upon recombination produce TMPRSS2/ERG [59]. Additionally, treatment with ionizing radiation [60] or TNFα [61] allows for *de novo* formation of TMPRSS2/ERG in androgen stimulated cells. Injecting a prostate cancer cell line into LPS treated air pouches of immunocompetent mice allowed formation of *TMPRSS2/ERG* [61]. Recently, the oncoprotein BRD4 has been shown to promote formation of TMPRSS2/ERG by facilitating DNA double stranded break repair via non-homologous end joining [62].

ETS Factors as Oncogenes and Tumor Suppressors

The Physiological Role of Oncogenic ETS

The normal function of oncogenic ETS in endogenous tissue should give clues to their functions when aberrantly expressed in prostatic tissue. ERG, ETV1 and ETV4 are tissue specific transcriptions factors and thus induce tissue specific gene programs important for development and maintenance. Oncogenic ETS factors belong to two subfamilies of ETS factors: the PEA3 and ERG subfamilies (Fig. 1). The PEA3 subfamily consists of ETV1, ETV4, and ETV5; and the ERG subfamily contains ERG, FLI1 and FEV. While homology between members of the same subfamily is high, members of different subfamilies only have homology in the ETS DNA binding domain, therefore ERG has little sequence similarity with ETV1 and ETV4. ETS factors are found throughout metozoan lineages. While some ETS factors are ubiquitous housekeepers, numerous developmental studies in mice and zebrafish show distinct spatio-temporal expression patterns of ERG, ETV1, and ETV4 orthologs. The tissue specific expression patterns of ERG, ETV1, and ETV4 suggest that these ETS factors control distinct gene programs that give rise to specialized organ function. ERG is predominantly expressed in hematopoietic stem cells and endothelial cells [63], regulating programs important for stem-cell self-renewal in hematopoietic cells and angiogenesis and migration in endothelial cells [64–67]. The ETVs, on the other hand, are mostly expressed in embryonic epithelial tissues including the developing lung, kidney, salivary gland, and mammary gland and promote branching morphogenesis during murine embryogenesis [68]. While the ETVs seem to function redundantly as fibroblast growth factor (FGF) signaling effectors [69–72], slightly divergent spatio-temporal expression throughout development and adulthood suggests tissue-specific functions [73, 74]. A common feature between ERG and the PEA3 factors may be functions related to the maintenance of tissue specific stem cells. Mice with ERG mutations have defects in hematopoietic stem cell function [75]. Similarly, mice with ETV5 knockouts are male-sterile due to loss of self-renewal in spermatogonial stem cells [76]. Interestingly, overexpression of ERG in mouse hematopoietic cells induces leukemia [77–79], suggesting that proper regulation of ERG levels is crucial for maintaining a normal cellular state.

Oncogenic ETS in Prostate Cancer Pathogenesis and Progression

Since the discovery of recurrent ETS rearrangements, a casual role for the oncogenic ETS in prostate carcinogenesis and disease progression has been under question. Oncogenic ETS gene rearrangements occur early, or even prior to disease onset, with evidence suggesting that ETS rearrangements drive prostatic neoplasia [31, 46]. Expression of oncogenic ETS factors alone in mouse models is not sufficient to drive formation of prostate tumors [6, 80]. Rather, a second hit, for example *PTEN* inactivation or *TP53* loss, is required for development of the disease [3, 4, 6, 16, 17, 81]. However, one mouse study reported that prostate specific expression of ERG at levels comparable to ERG-positive human prostate cancers allow 50% of mice over 2 years of age to develop tumors [82], suggesting an age-related component to ERG-mediated carcinogenesis.

Interestingly, the TMPRSS2/ERG fusion can drive expression from the wild-type ERG allele [83], creating a feed-forward loop. This allows continual, androgen-independent expression of ERG in CRPC, suggesting a requirement or oncogenic addiction to ERG. In fact, expression of oncogenic ETS is found in advanced metastatic disease, with ERG expression levels in CRPC comparable to expression in primary tumors [40, 84], with one study citing an increase in ERG expression from PIN to metastatic disease [51]; however, it is still unclear whether this expression actively contributes to an aggressive phenotype.

Cell line models have been used to address the causal role of oncogenic ETS in various disease stages. Introducing oncogenic ETS expression

vectors into normal immortalized prostate cells results in increased oncogenic potential [21, 22]. This suggests normal prostate cells are susceptible to transformation when oncogenic ETS become expressed and recapitulates early-stage or indolent disease. Prostate cancer cell lines with ETS gene rearrangements include VCaP and NCI-H660 which have *TMPRSS2/ERG* [85]. Both LNCaP and MDA-PCa-2B cells harbor an ETV1 gene rearrangement [2]. PC3 and 22Rv1 both express high levels of ETV4 protein, but no rearrangement of this gene is apparent [86]. Knockdown of ERG in VCaP cells results in a loss of oncogenic functions [82, 87–90]; similar effects are observed when knocking down ETV1 and ETV4 in LNCaP and PC3 cell lines, respectively [15, 91, 92]. VCaP mouse xenograft studies suggest a requirement of ERG for tumor formation [88, 93]. Because the VCaP, LNCaP, and PC3 cell line models are all derived from advanced metastatic disease, these findings suggest that continued oncogenic ETS expression is important for this phenotype.

In an effort to reconcile molecular subtypes with cellular phenotypes, many groups are determining if certain cell types in the prostate are more susceptible to ETS rearrangements, and whether ETS rearrangements alter lineage outcomes during disease development. Multiple reports have found that ERG functions to block differentiation [94–96], reminiscent of ERG function in hematopoietic stem cells. Similarly, ERG repressed genes are involved in luminal and neuroendocrine differentiation in prostate-specific TMPRSS2/ERG transgenic mice. However recent work from multiple groups indicate that ERG expression actually promotes luminal cell fates in prostate cells. For example, ERG-positive patient specimens are classified as luminal [97]. Additionally, in a PTEN negative/TP53 mutant background, mice expressing transgenic ERG in the prostate grew tumors with a luminal epithelial phenotype [98]. These discrepancies suggest that ERG expression may function to define different cellular identities based on the mutational background, begging the need for additional mouse models that accurately represent co-occurring mutations in human prostate cancer.

Recurrent ETS Fusions in Other Cancers

Aside from prostate cancer, recurrent ETS fusions are found in the Ewing's family of tumors, and in leukemias (Fig. 1) [99–101]. However, these cases differ from prostate gene fusions in that Ewing's and leukemia gene fusions encode fusion proteins with emergent properties that depend on both fusion partners. In prostate cancer, ETS gene fusions usually only express full length or truncated ETS protein. Chromosomal translocations in Ewing's sarcoma create a fusion protein that includes the C-terminus and DNA binding domain of an ETS transcription factor and the N-terminus of another protein. The hallmark fusion, *EWS-FLI1*, occurs in ~85% of cases [102]. Rarer 3′ ETS fusion partners with EWS include: ERG, ETV1, ETV4, and FEV [100, 101, 103–106]. Additionally, ERG and FEV can be fused with the 5′ partner FUS, but these cases of Ewing's family tumors are rare [102]. In acute myeloid leukemia (AML), *ERG* can be fused to the 5′ partner *FUS* [107, 108]. Notably, EWS and FUS are paralogs, and therefore all of these fusions are likely to encode proteins with similar molecular properties. This similarity suggests a shared selective pressure that drives the formation of these events across distinct cell types. The ETS gene *ETV6* (TEL) is also commonly fused in lymphoid and myeloid leukemia [109], but the resulting fusion proteins do not include the ETS DNA binding domain, and therefore are likely to act through distinct mechanisms.

Tumor Suppressive ETS Factors

Of the six ETS genes with the highest mRNA expression in normal prostate (*SPDEF, EHF, ETS2, ELF3, ELF1,* and *ERF*), each exhibits tumor suppressive functions in prostate cells [10–12, 110–112]. In many cases these functions are due to binding site competition with oncogenic ETS. Although this likely plays a role, lower expression levels of these ETS genes do not always correlate with oncogenic ETS expression,

so other mechanisms appear to be at work as well. ERF is mutated in a small portion of ETS tumors [113], and this mutation is mutually exclusive with oncogenic ETS rearrangements. ERF binds many of the same sites as oncogenic ETS factors, but acts as a repressor [110]; and loss of ERF results in gene signatures similar to expression of oncogenic ETS [113]. The role of ETS2 as a prostate tumor suppressor is particularly interesting, as the *ETS2* gene lies between *TMPRSS2* and *ERG* on chromosome 21, and one copy is lost in the interstitial deletion that is the most common cause of the *TMPRSS2/ERG* rearrangement. A mouse model indicates that this interstitial deletion results in more aggressive disease than expression of *ERG* without this deletion, and appears to result from the loss of *ETS2* [112]. Interestingly, *ETS2* can act as a tumor suppressor in other cell types, and the presence of *ETS2* on chromosome 21 has been attributed for the lower incidence of some tumor types in people with down syndrome [114]. *SPDEF*, *EHF* and *ELF3* all appear to promote epithelial differentiation, and deletion of these factors is implicated in epithelial to mesenchymal transition and carcinogenesis [10–12]. *ELF1* is frequently co-deleted with the tumor suppressor *RB1* in advanced prostate cancer. *RB1* deletions that co-occur with *TP53* loss of function are thought to promote resistance to hormonal therapies in castration resistant prostate cancer [115, 116], and the concomitant loss of *ELF1* may contribute to this resistant phenotype [111].

Molecular Mechanisms of Oncogenic Function

ERG, ETV1, and ETV4 all are able to activate similar transcriptional programs, which result in phenotypes related to oncogenesis including cell migration, invasion, and de-differentiation [22]. Understanding the mechanism by which oncogenic ETS initiate these gene expression programs is essential for guiding therapeutic efforts. Here we will detail what is known about DNA binding, chromatin accessibility, protein interacting partners, and post-translational modifications

that underlie mechanisms of oncogenic ETS in the prostate epithelium.

DNA Binding

All members of the ETS transcription factor family contain a highly conserved 84–90 amino acid ETS domain, which is sufficient to bind DNA as a monomer [117]. Structurally, the ETS DNA binding domain exists as a winged helix-turn-helix (wHTH) domain consisting of three alpha helices and four beta-strands [7]. To date 47 high resolution structures of the ETS domain have been solved by X-Ray crystallography or solution NMR and have been published and deposited into the Protein Data Bank (PDB), including every ETS factor rearranged in prostate tumors [118–130]. Many of these structures have been solved both in the presence and absence of DNA [121], as well as with an additional DNA binding partner [124, 128]. Taken together, these studies provide insight as to how the ETS domain interacts with DNA and how specific regions of the ETS domain or other binding partners alter this DNA binding specificity.

The core recognition motif of the ETS domain is a four base consensus 5′-GGA(A/T)-3′ in which two invariant arginine residues make contacts with the guanine bases [7]. Although no direct contacts are made with bases outside the GGAA core, the ETS domain binds DNA in a region spanning 12–15 base pairs, and binding is mediated by positioning of the phosphodiester backbone, water mediated hydrogen bonding, hydrophobic, and electrostatic interactions [7]. Although subtle differences exist in the primary amino acid sequences of various ETS factors, these differences do not dramatically alter the DNA-protein interface. The ETS transcription factor family can be grouped into four classes based on minor differences in their DNA sequence binding preference in vitro [8]. The first class contains half of the ETS factors including the PEA3, TCF, ETS, ERF, and ERG subfamilies and has highest affinity for 5′-ACCGGAAGT-3′ [8]. This class of ETS factors contains all of the oncogenic ETS factors. The second class includes

all members of the TEL, ESE, and ELF subfamilies and differs from the first class only by a cytosine base rather than an adenine base at the beginning 5′ nucleotide. The third class contains members of the SPI subfamily and prefers adenine rich sequences 5′ to the core GGA sequence. The fourth class exhibits preferential binding for thymine at the final position in the GGA(A/T) core sequence and is comprised only of the ETS factor SPDEF. It is not clear to what degree these class differences influence binding site selectivity in vivo.

Autoinhibition of DNA binding plays an important regulatory role for many ETS factors and could be exploited as a therapeutic target. Autoinhibition is the process where regions of the protein outside of the DNA binding domain inhibit DNA binding, often in a regulated manner. All of the oncogenic ETS factors are subject to autoinhibition [121, 125]. Autoinhibition of DNA binding occurs by a similar mechanism in multiple members of the ETS family. In this mechanism, regions both N and C terminal of the ETS domain form an autoinhibitory module which is sustained by a hydrophobic pocket of amino acids [121, 124, 125, 131]. Full length ERG protein binds to a target DNA sequence in vitro with a K_D = 120 nM, whereas the ETS domain of ERG without its inhibitory modules binds to DNA with a K_D = 37 nM, indicating that the inhibitory modules on ERG play a modest (~3-fold) role in regulating DNA binding [125]. While autoinhibition of ERG plays a modest role, members of the PEA3 subfamily exhibit robust (~10–30-fold) autoinhibition when comparing minimal DNA binding domains to full length proteins [121]. The N- and C-terminal inhibitory regions of the ETS domain in the PEA3 family act independently to inhibit DNA binding, but can also act cooperatively to inhibit DNA binding at higher than additive levels. In the PEA3 family, the C-terminal inhibitory region forms an alpha helix, which packs against the ETS domain and distorts Helix 3 (H3), the helix responsible for direct DNA base contacts. The N-terminal region is predominantly disordered and also inhibits DNA binding through interactions with H3 [121]. Autoinhibition can be relived or enhanced through post-translational modifications and through interactions with other proteins [124, 129, 132]. The prototype for this mechanism comes from the ETS factor ETS1, where phosphorylation of multiple residues in the N terminal inhibitory domain by CAMKII reinforces autoinhibition of DNA binding by ~50-fold [132]. In terms of the oncogenic ETS factors, acetylation of members of the PEA3 subfamily in an N-terminal inhibitory domain relieves autoinhibition and increases transcriptional activation [121, 133]. ETV1 autoinhibition has also been demonstrated to be relieved by protein-protein contacts with USF1 [134].

Gene Regulation

Genome-wide mapping techniques such as chromatin immunoprecipitation sequencing (ChIP-seq) have been conducted on all of the oncogenic ETS factors in prostate cancer cell lines [6, 21, 22, 95], in mouse models of prostate cancer [89], and in normal prostate cells engineered to express exogenous oncogenic ETS [22]. To a lesser extent, oncogenic ETS factor DNA binding has been interrogated in other malignancies [135] and in the context of their normal physiological expression [136]. These ChIP-Seq experiments support and expand on basic biochemical studies of ETS factor DNA binding in vitro and provide critical insights into how ETS factors regulate their target genes in cells.

Studies of oncogenic ETS factor DNA binding coupled with whole transcriptome RNA-Sequencing (RNA-Seq) have further enhanced our understanding of the ETS regulome in prostate cancer and how it contributes to oncogenesis. These studies have also further interrogated ETS factor DNA binding and gene regulation in contexts relevant to prostate cancer such as PTEN deletions [6], with and without androgen treatment [95], and with knock-down/deletion of tumor suppressive ETS [95]. Taken together, these studies have provided insight into how ETS factor binding across the cistrome contributes to gene regulation in prostate cancer. This next section will summarize the aforementioned studies

and will highlight recent key findings as to how oncogenic ETS factors regulate gene expression.

Members of the ETS family of transcription factors are extensively co-expressed in every cell type, and prostate epithelial cells are no exception [9]. As summarized in previous sections, the highly conserved ETS domain differs little in its sequence preference in vitro and accordingly, there is extensive competition between ETS factors to bind target genes in prostate cells [22]. Taking this information into account, measuring the contribution of a single ETS transcription factor's role in gene regulation has proved challenging. However, mis-expression of a single oncogenic ETS factor in the prostate results in dramatic changes in gene transcription. This information alongside studies of tumor suppressive ETS factor deletions in prostate cancer demonstrate the delicate balance of the ETS regulome.

Early studies mapping ETS family transcription factors discovered two distinct types of ETS binding site in the genome, "redundant" sites that can be bound by any ETS factor, and "specific" sites that tend to favor binding by one or a subset of ETS factors [7, 137]. Redundant ETS binding tends to occur at consensus ETS binding sequences common in the proximal promoters of CpG island-containing housekeeping genes. Specific binding tends to occur in tissue-specific enhancers and coincides with weaker match to consensus ETS binding sequences. Specificity for enhancer binding within the ETS family has been attributed to specific cooperative binding interactions with neighboring transcription factors such as ETS1 binding with RUNX1 or ELK1 binding with SRF [137, 138]. Like other ETS factors, oncogenic ETS factors expressed in prostate cells bind both housekeeping promoters and tissue-specific enhancers [22]. It is possible that oncogenic ETS could play a role in altering housekeeping gene expression, however this has not been described and it may be that redundancy of ETS function at these sites masks any role for this binding. Instead it has been proposed that it is the binding to tissue-specific enhancers that mediates oncogenic ETS function. As this specific binding is thought to be

influenced by neighboring transcription factors, we will discuss several proposed interactions between oncogenic ETS and other transcription factors that may mediate oncogenic gene expression programs.

ETS/AP1

One class of enhancer bound by oncogenic ETS factors in prostate cancer cells contains an ETS binding sequence that is followed by a sequence recognized by the AP-1 class of transcription factors. AP-1 consists of a dimer of JUN and FOS family transcription factors. At an ETS/AP-1 sequence, a single ETS protein could bind next to a JUN homodimer or a JUN/FOS heterodimer [139]. In vitro biochemical experiments recently demonstrated that oncogenic ETS factors can bind with AP-1 cooperatively, where the affinity of the ETS factor increases when AP-1 is present. In contrast, several tumor-suppressive ETS factors showed anti-cooperative binding in the presence of AP-1 [140]. Amino acid substitutions in the ERG ETS domain interfere with the interaction with JUN and FOS, and the interaction mutants of ERG lose the ability to transcriptionally cooperate with AP-1 in luciferase assays [141]. Furthermore, ChIP-Seq data indicates that ERG exhibits preferential binding at ETS/AP1 sites in prostate cells compared to tumor suppressive ETS factor EHF [140]. ETS/AP1 enhancer elements regulated by oncogenic ETS factors in prostate cancer are found near genes involved in cell migration, cell morphogenesis, and cell development and include genes such as *PLAU*, *VIM*, and *ETS1* [22].

Both Ras/ERK signaling and differential binding of the JUN transcription factor family play important roles in regulation at ETS/AP1 enhancers [139]. ETS factors present in normal prostate epithelial cells, such as ETS1, can activate gene expression through binding ETS/AP-1 sequences, but this activation requires high levels of Ras/ERK signaling [142]. High Ras/ERK signaling can be caused by the activating KRAS mutations common in many types of carcinoma. However, prostate tumors rarely have activating KRAS

mutations. One unique characteristic of the oncogenic ETS factors commonly found in prostate tumors is that they can activate ETS/AP1 enhancers in the presence of low levels of Ras/ERK activation [22]. Thus, one model suggests that oncogenic ETS factors essentially replace the role of activated KRAS in prostate cancer. Ras/ERK signaling is also involved in regulating Jun family transcription factors at ETS/AP1 enhancers. In the absence of Ras/ERK activation, c-Jun acts as a transcriptional activator and JunD as a transcriptional repressor of ETS/AP1 target genes, however, the converse is true in the presence of high Ras/ERK activation [139]. Thus, it has been proposed that c-Jun is the JUN family member likely to function with oncogenic ETS in prostate tumors.

GGAA Microsatellites

Within the preferred ETS binding sequence, the central GGA(A/T) nucleotides are the most important for high-affinity binding. Ewing's sarcoma is caused by a fusion oncogene that encodes a protein with an ETS DNA binding domain. This fusion protein most commonly consists of the N-terminus of the EWS protein fused to the ETS factor FLI1 [21]. Studies in Ewing's sarcoma indicate that this EWS/FLI1 fusion can bind an unusual regulatory sequence consisting of repeats of the sequence GGAA, which can extend over several hundred base pairs [143]. These GGAA microsatellite repeats regulate several oncogenes critical for tumorigenesis in Ewing's Sarcoma, including *NR0B1*, *NKX2-2*, *AND CCDN1* [144, 145]. The nature of these repeat elements suggests that EWS/FLI1 oligomerizes on DNA to form an activating transcriptional hub. Whether other ETS transcription factors also exhibit the ability to oligomerize and activate transcription in cells through GGAA repeats remains to be shown. However, the recent finding that the prostate cancer oncogenic ETS factors function with the EWS protein in transcription suggests common mechanisms between Ewing's sarcoma and prostate cancer [21]. In fact, preliminary studies have demonstrated that oncogenic ETS factors bind to GGAA microsatellite regions in prostate cells and that oncogenic ETS factors can activate a luciferase reporter regulated by a GGAA repeat element to a similar degree as EWS/FLI1 [21]. Thus, activation of genes regulated by GGAA repeats might be a common mechanism by which ETS factors promote both prostate cancer and Ewing's sarcoma.

Androgen Receptor

The function of oncogenic ETS factors in the prostate intersects in several ways with transcriptional regulation by the androgen receptor (AR). The most common oncogenic rearrangements, such as TMPRSS2/ERG put the oncogenic ETS factor under transcriptional control of AR. Further, oncogenic ETS proteins alter AR-dependent gene expression programs. Several studies report a physical interaction between oncogenic ETS, including ERG, with AR [95, 146]. ERG interacts with AR in normal endothelial cells, suggesting this interaction could play normal functions [89]. Importantly, non-oncogenic, and tumor-suppressive ETS factors have also been shown to bind AR, so the importance of the ETS/AR interaction in oncogenesis is still unclear [147]. Genomic interrogation shows co-enrichment of ERG and AR at many gene regulatory sequences [95]. Unlike ETS and AP-1, it is not clear whether the ERG and AR binding sequences at these enhancers or promoters are near each other, or that ERG and AR can influence each other's binding.

Contrasting work has been published as to whether ERG activates or attenuates AR transcriptional activity [6, 16]. In fact, one hypothesis is that this function is context dependent, and that ERG can promote oncogenic functions of AR, while inhibiting tumor suppressive AR functions. ERG attenuates AR transcription and prostate lineage specificity in VCaP cells, and epigenetic regulators EZH2 and HDAC1/2 have been implicated in this repression [148]. Conversely, ERG acts as an activator of androgen-independent genes that are responsible for prostate cancer cell invasion and growth [22, 89, 95, 149]. In the context of

PTEN loss, ERG can restore AR target gene expression and increases AR binding across the cistrome independent of AR protein levels and circulating testosterone [6, 16]. These results suggest that ERG can promote survival of cells that are dependent on AR transcriptional activity. Taken together these studies demonstrate that ERG regulation of AR transcription is context dependent and that PTEN status is a major determinant as to how ERG impacts transcription.

ETV1 is also intimately involved in AR transcriptional activity and is involved androgen metabolism in prostate cells [16]. ETV1 cooperates with AR signaling in LNCaP cells and in PTEN deficient mouse models of prostate cancer [16, 150]. ETV1 directly binds and upregulates genes associated with steroid hormone biosynthesis and androgen metabolism, which results in increased testosterone production and subsequent increased AR transcriptional activity [16]. To a lesser extent, ETV4 has been implicated in AR signaling, since survival and metastatic potential of mouse prostate cells is reduced upon ETV4 knockdown [15]. Interestingly, estrogen receptor (ER) signaling has been implicated in growth and migration of cells harboring TMPRSS2-ETV5 fusions suggesting that ETV5 is involved in steroid hormone signaling as well in prostate cancer [151].

Transcriptional Activation and Repression

Deciphering whether the role of a transcription factor in gene regulation is activating or repressive has proven to be a challenging task for multiple reasons: (1) transcription factors often regulate other transcriptional regulators, which themselves effect gene expression, (2) enhancer elements are often Kb-Mb in distance away from the genes that they regulate and may not always regulate the nearest gene, and (3) multiple transcription factors and enhancers regulate the transcription of any given gene. Families of transcription factors with similar consensus sequences and binding sites in vivo further complicate this problem as lower gene expression observed when a new factor is introduced may

simply be due to less activation function than a factor that was displaced. Despite all of these complications, progress has been made to decipher the molecular mechanisms and contexts by which oncogenic ETS factors directly activate or repress transcription.

An activation function for a transcription factor can be predicted when there are direct interactions with known transcriptional co-activators such as CBP and p300. CBP/p300 are homologous acetyltransferases that directly acetylate histones and transcription factors at both promoters and enhancers [152]. All of the oncogenic ETS are acetylated by, and interact with, p300, however the mechanism of acetylation and interaction varies [153]. The N-terminus of ERG is acetylated by p300 at a KGGK motif, which results in recruitment of the transcriptional co-activator BRD4 that binds these acetylated lysines through its bromodomain [152, 154]. Initially, the ERG-BRD4 interaction was described in acute myeloid leukemia (AML), a cancer with ERG-dependencies [152], but now has also been shown in prostate cells. The interaction between ERG and BRD4 is of particular interest because of the potential therapeutic value of BRD4 inhibitors such as JQ1 and iBET, and because these inhibitors can decrease the ERG/BRD4 interaction [154]. ERG KGGK acetylmimetics can also increase invasion of normal prostate cell lines [154]. However, a highly prevalent ERG fusion product, ERG Δ92, does not contain the KGGK motif. Therefore, the relevance of the ERG-BRD4 interaction in prostate cancer, and whether it differs based on the fusion location is still unclear. Genome wide co-occupancy between oncogenic ETS factors and p300 in cells has not been investigated extensively in the context of prostate cancer and could provide further insights into specific sites of oncogenic ETS regulation.

While CBP/p300 is important for oncogenic ETS function, it also binds to and is important for the function of many non-oncogenic ETS factors. What then, is the mechanism that allows ERG, ETV1, and ETV4 to be oncogenic, when normal prostate cells express at least 15 other ETS factors? We recently reported that ERG, ETV1,

ETV4, and ETV5, and no other ETS factor interact with the Ewing Sarcoma Breakpoint Region 1 (EWS) protein [21]. In this context EWS acts as a co-activator, and the EWS interaction is essential for ETS-mediated cell migration, anchorage-independent growth, clonogenic survival, and tumor formation. EWS can also interact with ETV1 in developing limb buds of mouse and chick embryos, suggesting that EWS cooperates with oncogenic ETS in normal tissue [155]. The exact mechanism by which EWS regulates ERG transcriptional activity is not well understood. EWS has been implicated in a variety of co-activator and co-transcriptional activities including splicing, RNA Polymerase II CTD phosphorylation, and phase separation, all of which indicate that it is a multifunctional regulator of gene expression [21, 156]. Importantly, EWS is fused to the ETS DNA binding domain of various ETS factors in Ewing's Sarcoma, which suggests that there is a common oncogenic EWS/ETS function in both prostate cancer and Ewing's sarcoma [21].

A potential co-activator specific for the PEA3 subfamily is the mediator subunit MED25. ETV1, ETV4, and ETV5 interact with MED25 through a conserved N-terminal transactivation domain [157]. The ETV4 ETS domain also interacts with MED25, and both the transactivation domain and the ETS domain contact MED25 at multiple interfaces. MED25 has high affinity ($K_d = 50$ nM) for ETV1 and ETV4 but does not interact with ERG. The MED25 interaction with ETV4 relieves auto inhibition and promotes DNA binding. Genome-wide analysis of MED25 and ETV4 binding showed co-enrichment at ETS-motif containing enhancers [158]. While the specific interaction between the ETVs and MED25 suggests a functional link, studies have not been performed to explore the role on prostate cancer phenotypes.

In addition to transcriptional activators, ERG has been demonstrated to interact with epigenetic transcriptional repressors EZH2, HDAC1, and HDAC2 [148]. These repressors appear to be involved in ERG interplay with AR as addition of DHT alters binding of these repressors across AR and ERG binding sites and attenuates transcription of specific ERG and AR co-bound genes. Transcription of the *VCL* gene in prostate cells is activated by AR and attenuated by ERG, however, the addition of an EZH2 enzymatic inhibitor relieves this attenuation [148]. FOXO1, a forkhead transcription factor frequently inactivated in prostate cancers, interacts directly with the N-terminus of ERG [89]. FOXO1 binding functions to inhibit ERG-mediated transcriptional activation. Unlike the ERG-AR interaction, FOXO1, which is also normally expressed in endothelial cells, does not interact with ERG in that cell type. This suggests that ERG function in prostate cells is specifically inhibited by FOXO1 whereas, in endogenous tissue, ERG function is unrestrained by this mechanism [89].

There is a dichotomy as to how ERG can act as a transcriptional activator in certain contexts and as a transcriptional repressor in others. It is still not clear if both activating and repression functions are critical for the oncogenic properties of ERG. It is possible that ERG acts as an activator at some regulatory elements and a repressor at others due to differing interactions with other proteins at these sites, or via displacement of endogenous ETS factors at a subset of sites. It is also possible that there is a dynamic switch between activating and repressing functions mediated by signaling pathways. ERG regulation by signaling will be summarized later in the chapter.

Oncogenic ETS and Chromatin

Expression of ERG in prostate cells can drastically influence chromosome topology and organization [159]. Upon ERG expression, novel intra- and inter-chromosomal interactions form with ERG binding sites enriched at these novel interacting loci. Dynamic nuclear co-localization of specific genes into transcriptional hubs contribute to gene activation or repression. To support this concept, the majority of ERG-regulated genes (65%) exhibit cis-interactions in cells and are enriched for genes involved in cell adhesion, skeletal system development, and migration [159]. However, the exact mechanism by which

ERG drives changes in chromosome structure and topology is not understood.

ERG interacts with various chromatin modifying enzymes including p300, HDAC1, HDAC2, EZH2, KDM4A, PRMT5, and SETDB1 [148, 152, 160, 161]. These interactions suggest that ERG modifies the local chromatin at its binding sites to alter gene transcription. ERG binding could facilitate an assortment of histone modifications as its interacting partners have the ability to methylate, demethylate, acetylate, and deacetylate various sites on histone tails. The influence of the PEA3 family on chromatin is less understood. ETV1/4/5 interact with P300, and ETV1 interacts with KAT2B [133, 153]. The enzymatic subunit of PRC2, EZH2, is upregulated in prostate cancers and is associated with aggressive disease [162]. Several groups have shown direct ERG binding and activation of the EZH2 locus [95, 163]. High levels of EZH2 results in down regulation of EZH2 targets through epigenetic silencing and dedifferentiation of prostate cancer cells [95]. ERG physically interacts with EZH2 in a manner that is regulated by post-translation modification. For example, phosphorylation of ERG Ser-96 by ERK disrupts EZH2 binding and allows ERG to function as a transcriptional activator [164]. However, modulation of ERG activity by the EZH2 interaction is not fully understood.

Signaling Pathways and Oncogenic ETS

Post translational modifications can alter the transcriptional output of transcription factors through multiple different mechanisms including effects on the rates of degradation, nuclear/cytoplasmic shuffling, altering interaction partners, and altering DNA binding. Multiple signaling pathways have been implicated in the activation of the oncogenic ETS transcription factors through a variety of post-translational modifications (Fig. 4). This section will summarize the various signaling pathways and post-translational modifications that regulate the transcriptional activities of oncogenic ETS factors in the prostate.

The Ras/MAPK pathway appears to play a role in activating the transcriptional activity of all of the prostate cancer oncogenic ETS factors. In vitro evidence demonstrates that all of the oncogenic ETS factors can be phosphorylated by multiple downstream kinases in the pathway (ERK2/JNK1/P38a) [165]. ERK2 can phosphorylate all of the oncogenic ETS factors in vitro and is critical for their transcriptional activation in vivo [166–168]. ERG can be phosphorylated by ERK2 at S96, S215, and S283 in various cell types. Phosphorylation at S283 in leukemic cells enhances stem cell features and cell proliferation and is associated with increased ERG binding at specific loci [169]. Phosphorylation of ERG at S96 and S215 occurs in prostate cells, and S96 phosphorylation is critical for ERG to activate transcription and promote cell migration [164]. Phosphorylation of S215 induces a conformational change in ERG necessary for subsequent S96 phosphorylation. ERG S96 phosphorylation decreases affinity for EZH2 and the polycomb repressive complex 2 (PRC2). This results in loss of the recruitment of EZH2 across the ERG cistrome and promotes activation of ERG target genes [164]. If Ras/MAPK signaling is important for ERG function, why do prostate tumors rarely have the Ras/MAPK pathway activating mutations that are common in other carcinomas? We have published findings suggesting that activating mutations in KRAS lead to phosphorylation of the ubiquitously expressed ETS protein ETS1, and subsequent activation of a gene expression program that promotes cell migration, invasion, and epithelial to mesenchymal transition (EMT) [142]. However, ERK2 has a higher affinity for ERG than for ETS1, and thus ERG requires lower levels of Ras/ERK signaling to be phosphorylated [165] and to activate the same cell migration/EMT gene expression program [22]. We hypothesize that these low levels of Ras/MAPK can be attributed to growth factors in the tumor microenvironment, rather than mutation. Less is understood about the mechanisms by which Ras/MAPK signaling effects the function of PEA3 subfamily members in prostate cells. The transactivation potential of ETV5 is greatly increased when Ras/MAPK signaling is activated above basal levels, and this increase in activation potential is
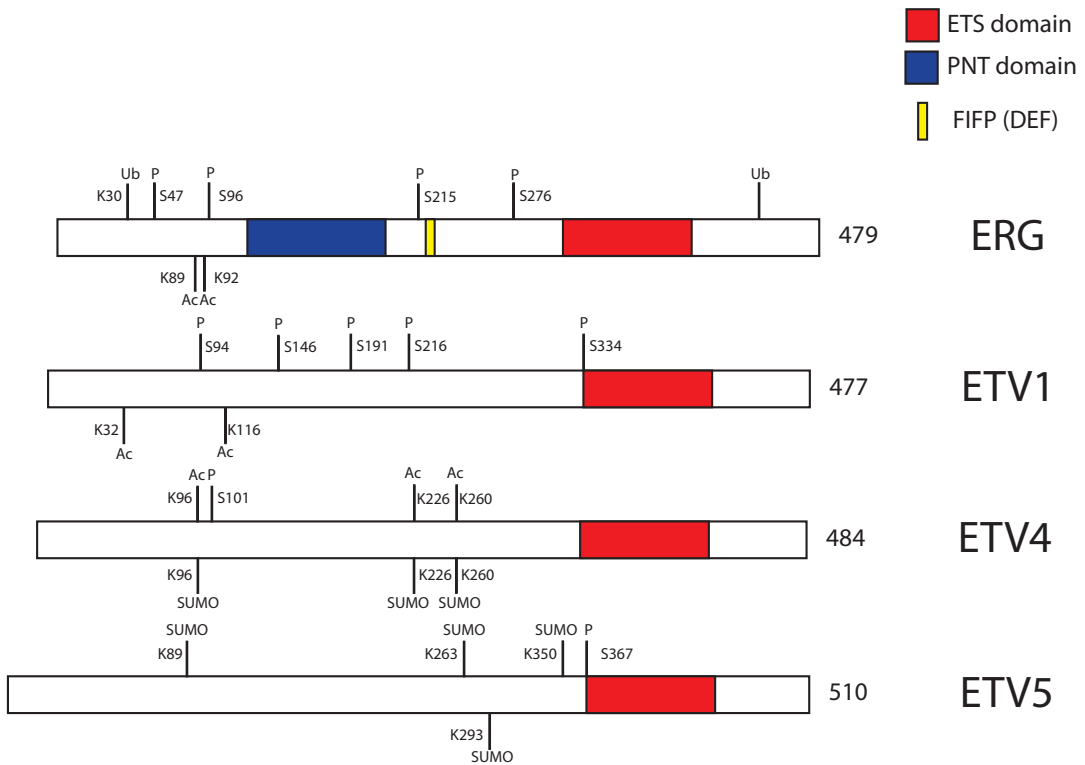
Fig. 4 Post-Translational Modifications of Oncogenic ETS Proteins. ERG (NP_001230358.1), ETV1 (NP-004947.2), ETV4 (NP_001073143.1), and ETV5 (NP_004445.1) proteins depicted with major structural domains: ETS DNA binding domain (Red), Pointed or PNT domain (Blue), and ERK docking sequence FIFP or DEF domain (Yellow). Post-translational modifications indicated by lines corresponding to respective amino acids on the protein. Acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and sumoylation (SUMO) modifications have been curated from literature describing these modifications in cells. C-terminal ubiquitination site on ERG has not been assigned to a specific residue

dependent on the N-terminal activation domain [167]. Activation of the Ras/MAPK pathway has been implicated in increasing the metastatic potential of prostate cells, which express ETV4, and is important for ETV4 target gene activation in esophageal adenocarcinoma [170]. ETV1 transcriptional activity is also activated by ERK phosphorylation, and this phosphorylation occurs in the activation domain [166]. The exact mechanisms by which Ras/ERK signaling allows for PEA3 family activation remains to be investigated.

The PI3K/AKT signaling pathway is clearly important for ERG-mediated pathogenesis. ERG expression in prostate cells is not sufficient for transformation, but ERG can drive tumor growth when coupled with mutations that activate PI3K/AKT signaling [3–5]. In prostate tumors this PI3K/AKT activation is often associated with loss of the PI3K/AKT pathway inhibitor PTEN, and in fact, PTEN deletion is more common in ERG-positive tumors. While ERG and AKT could function in parallel pathways, one study suggests that AKT activation is necessary for ERG to activate target genes and cell migration in cell line models [86]. Interestingly, while this function required AKT, it did not require mTORC complexes, which are downstream of AKT. It has not been reported that AKT can directly phosphorylate the oncogenic ETS proteins, and so far, any molecular mechanism of synergy between ERG and AKT signaling is unclear [86].

Other kinases have been implicated in the activation of oncogenic ETS transcription factors. Protein Kinase A (PKA) activates ETV1 and

ETV5 through phosphorylation at the very N-terminal region of their respective ETS domains [167, 171]. Ribosomal S6 Kinase 1 (RSK1) also positively regulates ETV1 function through phosphorylation [171]. In vitro evidence demonstrates that P38a and JNK1 can phosphorylate all members of the PEA3 subfamily, however, only ETV1 has thus far been demonstrated to be phosphorylated by these kinases in cells [165]. Taken together, these data demonstrate that there is extensive cross talk between multiple signaling pathways and oncogenic ETS transcription factors. Phosphorylation by these different kinases, in general, results in increased activation of transcription by the oncogenic ETS factors.

Acetylation by CBP/p300 is another post-translational modification that effects transcriptional activation by oncogenic ETS transcription factors. ERG acetylation by CBP/p300 occurs at K89 and K92 and results in recruitment of the transcriptional regulator BRD4 [152]. Multiple hematopoietic transcription factors appear to utilize this mechanism to activate target gene transcription [152]. ETV1 and ETV4 are also targets of acetylation by CBP/p300, however, to date ETV5 has not been demonstrated to be acetylated [153]. Acetylation of ETV1 occurs at two residues at the N-terminus of the protein, which stabilizes the protein and increases its transcriptional activity [133]. Regulation of ETV4 by acetylation occurs through a dynamic mechanism, which involves cross talk of multiple post-translational modifications including phosphorylation, acetylation, sumoylation, and ubiquitination [172].

While phosphorylation and acetylation appear to regulate transcriptional activation of the oncogenic ETS proteins, both ubiquitination and sumoylation can repress their transcriptional activity. Although the exact SUMO-transferase has not been identified, ETV4 and ETV5 are sumoylated at multiple residues [172, 173]. The lysine residues sumolyated on ETV4 are also the same residues that are acetylated by CBP/P300, meaning that only one modification or the other can exist on the protein at any one time. Sumoylation of ETV4

opposes the acetylation-mediated stabilization of the protein and ultimately results in protein degradation and decreased transcriptional activation. Sumoylation of ETV5 also contributes to repression of target genes, however, this appears to occur by a mechanism other than altering protein stability [173]. The E3 ubiquitin ligase adaptor SPOP promotes ubiquitination of ERG at both the N- and C-terminal ends, however, the N-terminal ubiquitination seems to have a larger effect on protein stability [174, 175]. The E3 ubiquitin ligase adapter COP1 is involved in transcriptional activation and stabilization of the PEA3 subfamily [176]. ETV5 appears to have an N-terminal and C-terminal binding site for the complex, similar to ERG [177]. Different components of the ubiquitin ligase complex are also involved in regulation of ETV5 stability, as the presence of DET1 is necessary for its degradation.

ETS transcription factors are also able to modulate signaling pathways through the activation or repression of their downstream target genes. Ectopic expression of ERG in a prostate epithelial cell line can increase phospho-ERK and phospho-AKT levels indicating activation of both pathways [86]. Transcription of PTEN is repressed by ERG in prostate cancer cells [178]. However, in a mouse model ERG expression did not correlate with downregulation of the PTEN gene [3], indicating that regulation of these pathways is highly context dependent. The role of the PEA3 family in modulation of the Ras/ERK and PI3K/AKT pathways has not been extensively investigated in the prostate. ERG regulates Wnt/β-catenin/LEF signaling in both the context of vascularization and in prostate cancer [66, 179]. ERG is required for blood vessel development and sprouting angiogenesis in zebrafish, and this process is regulated through activation of Wnt/β-catenin signaling [66]. In the prostate, ERG binds to and increases transcription of various Wnt proteins and ultimately results in more active β-catenin. ERGs activation of Wnt/β-catenin signaling results in epithelial to mesenchymal transition of prostate cells and increases survival and invasive properties of prostate cancer cells [179].

Targeting Oncogenic ETS Factors

There are two major challenges to designing therapies that target oncogenic ETS factors. First, transcription factors such as ETS family members, do not have easily targetable binding pockets and have been described as "undruggable". However recent advances have been made in targeting transcriptional function by either developing small molecules that disrupt protein-protein interactions, or by indirectly targeting enzymes that work in transcriptional complexes. Second, since the oncogenic ETS factors belong to a large family of proteins, a challenge in therapeutic design apart from efficacy is specificity. To reduce off-target effects, oncogenic ETS targeting drugs should not cross react with non-oncogenic ETS and also should not affect the physiological function of these factors in normal tissue. For instance, inhibiting ERG requires that tumor suppressive ETS such as EHF remain active in the prostate and that ERG function is unaffected in endothelial cells. This section will describe efforts to target oncogenic ETS factors such as ERG (diagrammed in Fig. 5).

Understanding the differences between oncogenic ETS and tumor suppressive ETS may be key in developing targeted therapies. Therefore, identifying targetable regions of ETS factors that are specific for oncogenic mechanisms is crucial for future prostate cancer therapies. Two recent studies have used knowledge of the molecular mechanisms detailed above to construct a tumor suppressive ETS function more like oncogenic ETS. First, fusing the activation domain of an oncogenic ETS interacting partner, EWS, to the tumor suppressive ETS SPDEF and EHF results in the gain of oncogenic function [21]. This suggests that the oncogenic properties of ETS factors contribute to regions outside the ETS domain that allow for specific protein-protein interactions. Another key protein-protein interacting partner specific to the oncogenic ETS is the AP-1 transcription factor. Oncogenic ETS are able to activate ETS-AP1 sites by cooperatively binding with the AP-1 transcription factor. Tumor suppressive ETS instead bind anti-cooperatively with AP-1. Mutating positively charged residues

in the AP-1 interface of EHF to the corresponding residues in ERG disrupt this anti-cooperative binding [140]. This suggests that these residues contribute specifically to oncogenic ETS function.

ERG Targeting Peptide

An ERG inhibitory peptide was recently identified that forms high affinity interactions with the ETS DNA binding domain of ERG [180]. This peptide inhibits interactions between ERG and important protein binding partners AR and the catalytic subunit of DNA-PK (DNA-PKcs) in a dose-dependent fashion. Treatment of ERG expressing cells with the peptide caused a significant decrease in ERG-mediated cell migration by interfering with the DNA binding ability of ERG and triggering ERG degradation. Importantly, this peptide had little effect on normal ERG functions in endothelial cells, indicating that normal and oncogenic ERG functions are separable. Treatment of LNCaP, a cell line that overexpresses ETV1, with this peptide showed a similar effect. The mechanism that might allow this peptide to specifically target only oncogenic functions, or whether it targets only oncogenic ETS factors, remain unclear.

Small Molecule Inhibitors

A cell-based immuno-assay screen was used to identify ERGi-USU as a small molecule that altered ERG protein levels [181]. ERGi-USU was able to selectively inhibit ERG protein expression in the ERG-expressing prostate cancer cell line VCaP, but not ERG expressed in non-prostate cancers or in endothelial cells. ERGi-USU treatment inhibited VCaP xenograft growth in nude mice and was found to function by directly binding to and inhibiting RIOK2, a kinase important for ERG protein stability. One group targeted ERG-DNA complexes with the di-(phenyl-thiophene-amidine) compound DB1255 [182]. DB1255 interacts with ETS binding motifs in DNA and blocks ERG binding, impeding activation of reporter genes. An *in silico* approach was used to identify small mol-
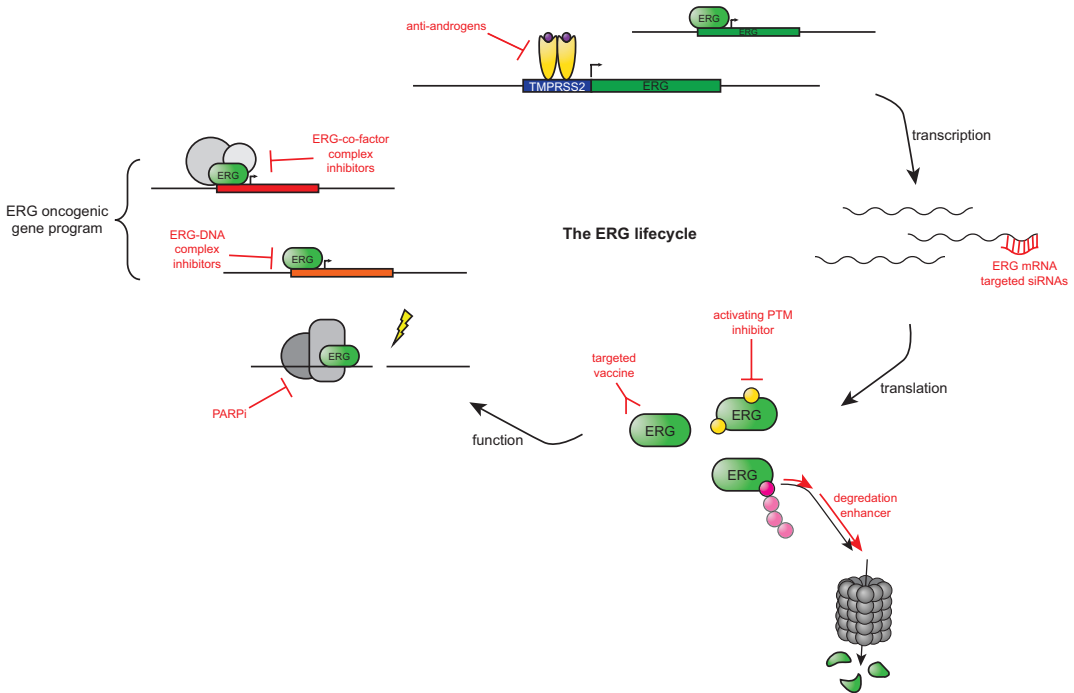
Fig. 5 A cartoon depiction of the ERG lifecycle. Diagramed is ERG expression from gene to protein and ERG function. Potential nodes of inhibition are depicted in red. Expression of TMPRSS2/ERG is driven by binding of ligand-bound AR (yellow pillars with purple circles) to the TMPRSS2 promoter. Additionally, ERG protein can bind the endogenous ERG promoter and drive ERG transcription. Transcription of the ERG gene produces ERG mRNA, which can be silenced by the RNAi pathway. ERG transcripts are translated into protein. Post translational modification of ERG can be activating (yellow circle), repressing (not shown), or target ERG protein for proteasomal degradation (pink circle). Potential ways of regulating ERG protein include ERG targeted vaccines, inhibition of activating post translational modifications, and promotion of degradation. To activate oncogenic gene programs, ERG binds gene regulatory elements with various co-factors and other transcription factors. Various inhibitors can target this function, such as ERG-DNA complex inhibitors and ERG-co-factor complex inhibitors. General transcription inhibitors may synergize with ERG inhibitors to yield a greater effect. When aberrantly expressed, ERG promotes DNA damage and thus PARP inhibitor treatment of ERG-rearranged cells promotes synthetic lethality

ecules that target the ETS domain of ERG. Compound VPC-18005 inhibited ERG-DNA complex formation and inhibited migration and invasion in cells expressing ERG. Additionally, VPC-18005 decreased metastasis in a zebrafish xenograft model [183]. The small molecule YK-4-279 was developed as an inhibitor of EWS-FLI1 for Ewing's Sarcomas [184]. Given that FLI1 is an ETS transcription factor with high homology to ERG, the effect of YK-4-279 has been tested for other oncogenic ETS factors. Treatment of the prostate cancer cell lines VCaP or LNCaP with YK-4-279 resulted in the inhibition of ERG or ETV1 transcriptional activity, respectively, as well as ETS-mediated cell invasion. However, YK-4-279 treatment of PC3 cells, which overexpress ETV4, had no effect on invasion [185]. In mouse xenograft tumor models YK-4-279 has been effective against prostate tumors expressing both ERG [186] and ETV1 [187]. BRD32048 was identified as a small molecule inhibitor of ETV1 via a microarray screen [188]. Treatment with BRD32048 caused a significant dose-responsive decrease in invasion of an ETV1 translocation-positive prostate cancer cell line but had no effect on prostate cancer cells that do not express an ETV1 fusion. BRD32048 functions by direct binding to ETV1, preventing p300 acetylation and promoting degradation.

PARP1 Inhibitors

ERG interacts with the DNA damage repair protein PARP1 and DNA-PKcs, a kinase in the PI3K pathway [189]. This complex is required for cell invasion, intravasation, and metastasis. Importantly, inhibiting PARP1 significantly decreased tumor growth in ETS-positive xenografts but had no effect on ETS-negative xenografts. It was hypothesized that aberrant ERG expression causes DNA damage, allowing for PARP inhibition to promote synthetic lethality specifically in ETS-positive cells. While this study suggested that ERG acts independently of XRCC4-mediated non-homologous end joining (NHEJ), another study found that ERG blocks XRCC1-mediated NHEJ to promote radiosensitization to PARP inhibition [190]. Treatment of ERG expressing prostate cancer cells with the PARP inhibitor olaparib significantly reduced clonogenic growth. Olaparib treatment increased the DNA damage marker gamma-H2AX in ERG-positive cells but not ERG-negative cells. Expression of ERG in a xenograft model was able to confer radiation resistance that was subject to reversal by the PARP inhibitor ABT-888 [191]. The PARP inhibitor rucaparib was shown to synergize with radiation in the ETS-positive cell lines VCaP and LNCaP [190]. Although more research is needed to pinpoint the mechanism, the PARP1-ERG axis provides an attractive prostate cancer target.

ERG Targeting Vaccine

The development of immune checkpoint inhibitors has renewed interest in the use of immunotherapy for many cancer types. However, prostate cancer tends to be resistant to such therapies. Since ERG expression is specific to prostate tumor cells, as compared to normal prostate, one effort has been to use this observation to promote an immune response. ERG peptides are processed and presented on the surface of prostate cancer cells that express the HLA-A2.1 type of MHC class 1 molecules [192]. HLA-A2.1+ cells are the most common human leukocyte antigen

in Caucasians, the demographic that most frequently has ERG rearrangements [193]. Mice expressing human HLA-A2.1 or both prostate-specific ERG and human HLA-A2.1, when immunized with an ERG derived peptide, were found to have significantly more cytotoxic T lymphocytes than the control mice. Additionally, co-culture of T-cells from ERG peptide immunized mice with ERG expressing prostate cancer cells resulted in an increased measures of T-cell activation compared to cells lacking ERG expression [192].

Chemoresistance

ERG-positive patients with CRPC are twice as likely to develop resistance to docetaxel treatment than ERG-negative CRPC patients. Immunoprecipitation experiments revealed that cytoplasmic ERG interacts with tubulin through the PNT domain. Deletion of the ERG PNT domain restores sensitivity to docetaxel treatment, suggesting that the ERG-tubulin interaction functionally contributes to taxane resistance in prostate cancer [194]. Additionally, detection of *TMPRSS2*/ERG in the blood of metastatic CRPC patients is predictive of docetaxel resistance [195]. ERG induces chemoresistance in leukemia cells [196]. These data may help clinicians determine a course of treatment by ERG subtyping.

Degradation of ETS Factors

It is clear that the protein stability of oncogenic ETS proteins is highly regulated, and it is possible that this process could be manipulated for therapeutic benefit. The tumor suppressor SPOP regulates ERG protein stability. Interestingly, SPOP loss-of-function mutations are present in 6–15% of prostate cancers but are mutually exclusive with ETS rearrangements [42, 175, 197, 198]. While wild type ERG protein is amenable to SPOP mediated degradation, most ERG fusion products are resistant through loss of the N-terminal degron [174, 175]. Similarly, the E3 ubiquitin ligase

COP1 targets the PEA3 factors for degradation through an N-terminal degron that is lost in most prostate cancer gene rearrangements [176]. It is possible that these mechanisms contribute to ERG and PEA3 factor upregulation, which is observed in some rearrangement-negative tumors. However, the E3 ubiquitin ligase TRIM25 is able to bind and ubiquitinate both full length and N-terminally truncated ERG, as it targets the C-terminal ERG degron [199]. The deubiquitase enzyme USP9X binds to and deubiquitinates ERG [93]. A small molecule inhibitor of USP9X, WP1130, destabilizes ERG protein and causes an increase in tumor growth in mice xenografted with ERG-expressing prostate cancer cells [93], suggesting that targeting ERG protein stability may be an effective prostate cancer treatment.

Conclusion

Recurrent gene rearrangements that involve ETS transcription factors typify the largest molecular subgroup of prostate cancer. Information on the molecular details of ETS factor function are essential to the movement towards precision therapies for prostate cancer patients. Genomic and biochemical studies have uncovered much about how ETS factors bind to and regulate chromatin to alter gene expression. However, the key to the development of effective and specific ETS therapies may be through teasing apart differences between oncogenic and tumor suppressive ETS function and understanding differences in physiological versus pathological roles.

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