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Exome sequencing of African-American prostate cancer reveals loss-of-function *ERF* mutations

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Abstract

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African-American men have the highest incidence and mortality from prostate cancer. Whether a biological basis exists for this disparity remains unclear. Exome sequencing (n=102) and targeted validation (n = 90) of localized primary hormone-naïve prostate cancer in African-American men identified several gene mutations not previously observed in this context, including recurrent loss-of-function mutations in *ERF*, an ETS transcriptional repressor, in 5% of cases. Analysis of existing prostate cancer cohorts revealed *ERF* deletions in 3% of primary prostate cancers and mutations or deletions in *ERF* in 3–5% of lethal castration-resistant prostate cancers. Knockdown of *ERF* confers increased anchorage-independent growth and generates a gene expression signature associated with oncogenic ETS activation and androgen signaling. Together, these results suggest that *ERF* is a prostate cancer tumor suppressor gene. More generally, our findings support the application of systematic cancer genomic characterization in settings of broader ancestral diversity to enhance discovery and, eventually, therapeutic applications.

INTRODUCTION

Extensive prior work has explored socioeconomic contributions to prostate cancer disparities, however our knowledge of the extent to which molecular and genetic mechanisms may also contribute to prostate cancer disparities has been limited (1–6). The notion that somatic genetic factors may influence tumor biology differentially across distinct ancestral backgrounds is exemplified by high *EGFR* mutation rates in patients of Asian ancestry with non-small cell lung adenocarcinoma (up to 50% of patients), compared to patients of European ancestry (10–15% of cases) (7). Large-scale genomic characterization studies are predominated by tumor samples from patients of European ancestry (8). While of immense value, the limited racial and ethnic diversity of these studies may preclude the detection of genomic events and patterns that are unique or enriched in underrepresented groups. For example, large-scale studies such as The Cancer Genome Atlas (TCGA) have examined the genomic landscape of primary prostate cancer and have been confined mainly to men of European ancestry (81.1%, 270/333) (9–11). In particular, African-American men, who have a 1.4-fold higher incidence and 2.4-fold higher mortality rate from prostate cancer compared to non-Hispanic whites, have been underrepresented in most systematic studies of prostate cancer performed to date (10–12).

We hypothesized that differences in mutational events in African-American prostate cancers may in part underlie these disparities in outcomes. We also reasoned that the power to discover novel cancer genes might increase through inclusion of diverse ancestral backgrounds in large-scale cancer genome studies. To test these hypotheses, we performed whole exome sequencing on a discovery set of 102 localized primary prostate tumors and matched normal controls from a cohort of African-American men and performed targeted sequencing on an extension set of 90 primary prostate tumors.

RESULTS

We focused on intermediate and high-risk prostate cancers corresponding to Gleason grades 7 and higher, or pathologic stage pT2a – pT3c (Supplemental Table 1 and 2). Exome sequencing identified 3059 somatic mutations, corresponding to a median of 7 silent and 23

non-silent mutations per tumor (range: 0–19 silent; 4–47 nonsilent). The median mutation rate for this cohort was ~0.83 mutations/Mb (range: 0.11–1.75), similar to mutation rates in exome sequencing cohorts of primary prostate cancer drawn predominantly from men of European ancestry (10,11).

Overall, the majority of tumors from African-American (AA) men do not harbor recurrent mutations in known cancer driver genes. Instead, most AA prostate cancers harbor somatic copy number alterations (SCNA) that are characteristic of those seen in other published cohorts (Figure 1 and Supplemental Figure 1A) (11,13). However, the overall frequencies of SCNAs appear to be lower in this AA prostate cancer (AAPC) cohort. Comparing the frequency of SCNAs in primary prostate cancer, we found that in 19 loci that undergo recurrent copy number changes, 16 loci were altered at a lower frequency in the AAPC cohort compared to the TCGA cohort (Supplemental Figure 1B, Fisher's exact test; $p < 0.05$). Adjusting for a lower threshold for SCNA in the AAPC cohort (see Supplemental Methods), we found that 10 of the 19 loci were still significantly different in frequencies between the two cohorts (Supplemental Figure 2). Given that the TCGA data set comprises higher frequencies of Gleason 8 and higher tumors in comparison to the AAPC cohort, these copy number differences were less pronounced when stratified by Gleason score yet persisted at certain loci (Supplemental Table 3 and Supplemental Figure 2). For example, overall, *PTEN* deletions were more common in the TCGA cohort (32%) compared to the AAPC cohort (6%), consistent with previous reports, and this difference remained when examining Gleason 7 and Gleason 8 (or higher) tumors (Supplemental Figure 2) (2). We note that focal copy number gains at 17q25.3, a locus containing the gene fatty acid synthase (*FASN*) and a significant amplification by GISTIC analysis in the AAPC cohort, occur in 12.7% of AAPC tumors in contrast to 1% in the TCGA dataset ($p = 0.0001$, Fisher's exact test; Supplemental Figures 1B and 3) (14,15). The mean fraction of the copy number altered genome in the AAPC cohort was lower in comparison to the TCGA cohort (7.94% vs 15.6%; Wilcoxon rank sum test, $p = 0.0025$; Supplemental Figure 4). When we stratified by Gleason grades, the mean fraction of copy number altered genome did not differ significantly between Gleason 7 and lower tumors but did differ between the AAPC and TCGA cohorts in Gleason 8 and higher tumors (12.8% vs 25.7%, Wilcoxon rank sum test, $p = 0.017$; Supplemental Figure 4).

In the AAPC cohort, a focused germline analysis for pathogenic mutations in genes in the DNA repair pathway revealed four patients with germline mutations in *BRCA1* (with a concomitant hemizygous *BRCA1* deletion), *CHEK2* and *ATM* for an overall prevalence of 3.9% (Supplemental Table 4) (16).

In the analysis of somatic mutations, we identified three genes (*SPOP*, *ERF*, *FOXA1*) wherein recurrent base mutations reached statistical significance in the discovery cohort (FDR $q < .1$; Figure 1; Supplemental Table 5) (17). Of the significantly mutated genes, *SPOP* and *FOXA1* have previously been identified as drivers in primary prostate cancer; however, *ERF* has not been implicated in this setting (10,11). *ERF* is a member of the ETS transcription factor family and therefore was of interest given the prominent role that ETS transcription factor rearrangements play in prostate cancer (18). Of the five nonsynonymous *ERF* mutations present in our discovery cohort, three were loss-of-function events (R183*,

K91fs, R218*) and another, which occurred within the ETS DNA-binding domain (Y89C), was predicted to be a damaging event by Polyphen-2 analysis (19). In order to determine whether *ERF* mutations led to decreased ERF expression, we tested whether *ERF* mutants in prostate tumors from the AAPC cohort showed loss of ERF mRNA expression. We used RNA *in situ* hybridization (ISH) to show that *ERF* mutants from the AAPC cohort were associated with a significant loss of ERF mRNA expression (Figure 2A–C and Supplemental Figure 5).

Using GISTIC analysis to examine significant somatic copy number alterations, we also noted that a focal deletion occurred at chr19q13.2 harboring a number of genes including *ERF* and a known tumor suppressor, *CIC*, capicua transcriptional repressor (Supplemental Table 6) (14). This peak represented three hemizygous copy number losses (~3%) of *ERF* in our exome discovery cohort (Figure 2D). These three patients had tumors with higher risk features: Gleason 8, pT3b; Gleason 8, pT3b with PSA of 42.3; and Gleason 7, pT2c with PSA of 12.8; two of these patients had biochemical recurrences. We verified copy number loss of *ERF* in an AAPC tumor using fluorescence *in situ* hybridization (FISH) (Figure 2E–F). To extend the finding of *ERF* copy number loss, we assessed three prostate cancer cohorts for *ERF* deletion by FISH analysis. Only 1 of 105 cases of localized prostate cancer in African-Americans in a previously published cohort demonstrated hemizygous *ERF* deletion (2). None of 33 cases of localized prostate cancer in the predominantly white Early Detection Research Network (EDRN) cohort showed deletion in *ERF*. This evaluation included all tumor nodules in the same prostate gland, when multiple foci were present. We also interrogated 82 cases of advanced CRPC, four of which harbored *ERF* hemizygous deletions (~5%) (20). These cases were part of the Precision Medicine Clinical Trial at Weill Cornell and an updated analysis of the whole exome sequencing data from this cohort shows 8 of 175 cases (~5%) harbor genomic alterations of *ERF*, including 6 cases with deletion (3 hemizygous and 3 homozygous), one case with hemizygous deletion and concomitant H31P missense mutation, and one additional case with a G299 frameshift deletion (Supplemental Table 7) (21).

To determine whether *ERF* might represent a recurrently mutated gene in primary AAPC, we expanded the data set by including an additional 90 prostate cancer samples (AAPC extension cohort; Supplemental Table 8) and performed targeted hybrid capture sequencing for 41 known or putative prostate cancer genes (Supplemental Table 9). Tumor-normal pairs from this additional AA prostate cancer cohort were sequenced at high coverage (mean target coverage: tumor 335×, normal 348×; Supplemental Methods). In the extension cohort, we identified five additional nonsynonymous *ERF* mutations, three of which were predicted loss-of-function frameshift mutations (Figure 3A and Supplemental Table 10). In total, the prevalence of *ERF* mutations in the discovery and extension AA primary prostate cancer cohorts was 5.2% (10/192). We validated by Fluidigm array 7 of 7 of the *ERF* mutations that we were able to evaluate (Supplemental Figure 6). Thus, *ERF* is recurrently mutated in primary prostate cancer in AA men.

While *ERF* was found to be a significantly mutated gene in the AAPC cohort, it did not reach statistical significance in the TCGA cohort (n=333) (Figure 3B) (11). However, taking into account deletions as well as mutations in *ERF*, the frequency of somatic alterations in

ERF are comparable between the AAPC cohort and other primary prostate cancer cohorts (5% vs 3%), suggesting that loss-of-function by mutation or deletion may be mechanisms to dysregulate *ERF*. Overall, combining publically available exome or whole genome sequencing datasets from primary prostate cancer revealed that *ERF* was altered in ~3% of primary prostate cancer cases by either mutation (0.76%; 5/661) or by homozygous deletion (2.2%; 15/661; Supplemental Figure 7 and 8A; Supplemental Table 11) (9–11). Therefore, *ERF* is recurrently mutated or deleted in primary prostate cancer.

Although *ERF* was not previously recognized as a recurrently mutated gene in primary prostate cancer, lethal castration-resistant prostate cancer (CRPC) cohorts showed missense or loss-of-function *ERF* mutations in ~3% of CRPC tumors (Supplemental Figure 7 and 8B–C) (22,23). An updated analysis of genomic data from a cohort of CRPC cases shows that *ERF* is recurrently mutated at a frequency of ~3% (8/269) and undergoes copy number loss (hemizygous or homozygous) at a frequency of ~17.5% (47/269) (Supplemental Figure 8C) (22). We also examined prostate cancer cell line data from the CCLE and COSMIC databases and found that *ERF* is mutated in one out of six prostate cancer cell lines (DU-145; p.A132S) (24,25).

We next asked whether alterations in *ERF* might be associated with more aggressive disease. *ERF* copy number loss was associated with a number of aggressive pathologic features including higher Gleason grade (Supplemental Figure 9; $p=0.0035$), higher pathologic T stage ($p=0.00696$), and residual tumor ($p=.0435$) (www.firebrowse.org). Of the five patients with *ERF* mutations (5/492) in the TCGA dataset, 4 had Gleason 8 or higher tumors (3 with Gleason 9) and two of these patients experienced biochemical recurrences. Among the 8 patients with *ERF* mutations or deletions in the AAPC exome cohort, three experienced biochemical recurrences (vs. 16 of 73 *ERF* wt) and 4 of 8 were pT3 (vs. 31 of 94 *ERF* wt). Moreover, a germline analysis of *ERF* in the AAPC cohort revealed a novel *ERF* coding variant (S295I) in a patient with Gleason 9 prostate cancer that experienced a biochemical recurrence (Supplemental Table 12). Overall, these data raise the possibility that *ERF* mutations and deletions may be linked to more aggressive forms of prostate cancer.

ERF was first characterized as an ETS and Ras tumor suppressor protein with a transcriptional repressor function (26,27). In addition to prostate cancer, mutations in *ERF* occur in other tumor types at similar frequencies: stomach adenocarcinoma (~4%), colorectal adenocarcinoma (~4%), and Ewing Sarcoma (~3%), which is also notably driven by a common ETS rearrangement, *EWS-FLI* (28–30). We asked whether *ERF* might function as a tumor suppressor in prostate cancer cells. Using lentiviral shRNAs, we knocked down *ERF* in the PC-3 prostate cancer cell line and demonstrated a significant increase in anchorage-independent growth of prostate cancer cells (Figure 3C; Supplemental Figure 10). *ERF* knockdown also increased invasion of PC-3 cells and increased mouse tumor xenograft growth (Supplemental Figure 11A–C). Overexpression of *ERF* reduced colony growth while a mutant *ERF* harboring a mutation (Y89C) identified in the AAPC cohort, diminished this effect (Supplemental Figure 12A–C). Furthermore, *ERF* knockdown in another prostate cancer cell line (LNCaP) and immortalized prostate epithelial cell line (RWPE-1) showed increased growth proliferation but no significant increase in invasion (Supplemental Figure 13A–H). Overexpression of wild-type *ERF* in the DU-145 cell line

harboring a mutation in *ERF* had no effect on proliferation in a focus formation assay but led to a reduction in colony growth in a low-attachment assay and decreased invasion (Supplemental Figure 14A–D). These results were consistent with a possible tumor suppressor role for *ERF* in prostate cancer.

Given its known role as a repressor of the ETS transcription factor family, we hypothesized that loss of *ERF* might activate a transcriptional program that resembles the output of ETS transcriptional activators such as ERG or ETV1 (31,32). To test this hypothesis, we performed lentiviral shRNA knockdown of ERF in an immortalized prostate epithelial cell line (LHS-AR) and two prostate cancer cell lines (VCaP and LNCaP) that each harbors oncogenic ERG (VCaP) or ETV1 (LNCaP) rearrangements (Supplemental Figure 10) (33). We then generated transcriptome data (RNA-seq) to derive a gene signature of ERF knockdown from the top 100 genes upregulated, according to the difference of means, when ERF is knocked down in comparison to control (See Methods). The ERF knockdown (KD)_UP signature from the LHS-AR cells was correlated with ETV1 and ERG signatures across the Cancer Cell Line Encyclopedia (CCLE) and was correlated with ETV1's target expression across the TCGA and CRPC tumor data sets (Figure 4A; Supplemental Figure 15). We then generated a combined ERF KD_UP signature from VCaP and LNCaP cell lines, hypothesizing that in the context of ETS activation that ERF loss might augment an ETS oncogenic transcriptional program. We found that this signature correlated with ERG pathway expression across TCGA and CRPC tumor data sets (Figure 4B). Supporting the idea that *ERF* loss or dysfunction may function similarly to ERG activation, *ERF* deletions and mutations were mutually exclusive of *ERG* rearrangements in the AAPC exome cohort (Figure 1). Furthermore, mutations and homozygous deletions of *ERF* were mutually exclusive of *ERG* fusion events in the published TCGA data set (Supplemental figure 16; Fisher's exact test: $p < .05$). In addition, overexpression of wild-type ERF in DU-145, an *ERF* mutant cell line, diminished the ERF KD signatures and ERG signature (Supplemental Figure 17A).

We next asked whether the ERF KD signature could be associated with a more aggressive phenotype in prostate cancer. We tested whether the ERF KD gene signature was also correlated with features of aggressive prostate cancer in the TCGA dataset and found that higher Gleason scores correlated with the ERF KD gene signature (Supplemental Figure 17B).

In an unbiased pathway analysis of cell lines from the CCLE, we found an androgen signaling (AR) signature (NELSON_RESPONSE_TO_ANDROGEN_UP) as the top correlated signature with respect to the ERF KD signature_UP (Supplemental Figure 18) (34). We projected this ERF KD signature into the RNA sequencing datasets generated from tumor samples in cohorts of primary prostate cancer (TCGA) and castration-resistant prostate cancer (CRPC) (11,22). We found that AR signatures were correlated with the ERF KD signature_UP in the TCGA and CRPC datasets (Figure 4C) (35). These data suggest that AR signatures are correlated with the transcriptional program of *ERF* knockdown and that loss of *ERF* is associated with a transcriptional program that can mimic ETS activation and may impinge on androgen signaling. To test the hypothesis that *ERF* loss may promote androgen signaling, we used the CRISPR/Cas9 system to target the *ERF* coding sequence in

prostate cancer cells (36). We showed that loss of *ERF* is associated with an increase in androgen-dependent growth (Figure 4D).

We also investigated other genes that were recurrently mutated but did not reach statistical significance in the AAPC discovery cohort. Y-box binding protein 1 (*YBX1*) (K81T, R244*, N76S), was mutated three times and has been implicated as an oncogene in prostate cancer but had not been previously identified as recurrently mutated in any prostate cancer cohorts (37). We identified missense mutations in the steroid hydroxylase *CYP11B1*, as well as other cytochrome P450 family members that occurred in a total of ~14% of samples in the discovery cohort (Supplemental Table 13).

Among known cancer genes, we observed that *PIK3CA*, which is recurrently mutated at a frequency of ~3% (20/667) in primary prostate cancers was not mutated in the AAPC discovery or extension cohorts (Fisher's Exact Test, $p=0.0115$; Figure 1) (10,11,30). Previous analyses also suggest that *PTEN* is less commonly deleted in cohorts of men of African ancestry (2,38). Our data suggest that alteration of the PI3K signaling pathway either through *PTEN* deletion or *PIK3CA* mutation is a less common event in AAPC. Interestingly, we identified missense mutations in *FOXA1* (F254V and H247L) in the AAPC cohort that occurred at the same residues only in men of African ancestry in the TCGA dataset (F254V and H247Y), raising the possibility of somatic mutations in prostate cancer that may be associated with ancestry (39).

Finally, to test the addition of tumors from African-American men to a large cohort of primary prostate cancer largely from men of European ancestry, we performed a combined analysis of the AAPC discovery ($n=102$) and TCGA ($n=457$) cohorts which nominated several new significantly mutated genes including *SMARCA1* and *ZFH3* (Supplemental Figure 19) (17).

DISCUSSION

Prostate cancer sequencing studies have been comprised primarily of men of European ancestry and there have been few large studies focused on men of African ancestry. Here we use exome sequencing to identify genomic features of primary prostate cancer in AA men. We report the identification of recurrent mutations in *ERF*, a novel prostate cancer gene that had not been previously appreciated. While analysis of this cohort identified *ERF* as a significantly mutated gene in prostate cancer, we note that *ERF* was mutated and located within a focal deletion in the TCGA cohort, composed primarily of men of European ancestry. Focal deletions at chr19q13.2 encompass *ERF* and *CIC*, a tumor suppressor that has been shown to regulate the ETS factors *ETV1*, *ETV4*, and *ETV5* (40). Our exome sequencing study implicates *ERF* as a potential target of these deletions. Still we cannot exclude the possibility that deletion of both *ERF* and *CIC* contribute to prostate cancer. Therefore, our data in conjunction with TCGA data suggest that *ERF* can be altered through mutation or deletion.

Dysregulation of ETS transcription factors plays a major role in prostate carcinogenesis and several studies have reported a lower prevalence of *TMPRSS2-ERG* rearrangements in

prostate tumors of African-American men (2,41). Here we present evidence of a novel mechanism of affecting ETS transcriptional output through an ETS transcriptional repressor. Furthermore, alteration of *ERF* may also be associated with more aggressive prostate cancers.

We observed a number of recurrent SCNAs that differed between the primary AAPC and TCGA cohorts. The limitations of this comparison with TCGA include differences in technologies (exome sequencing vs. SNP arrays) and stringencies for copy number detection. Still, our results, in conjunction with other studies that have examined specific copy number alterations, suggest that overall the somatic copy number alteration landscape may be distinct in primary prostate cancer in African-American men. In addition, we find that alterations of the PI3K signaling pathway through deletion of *PTEN* or mutation of *PIK3CA* are uncommon in primary AAPC, suggesting that distinct patterns of genomic alterations may occur in this cohort with implications for precision medicine.

Our results suggest that increasing the ancestral diversity of study populations for cancer genomic characterization may help increase the discovery potential of these studies, which we believe to date have not included sufficiently large numbers of men from African ancestry. Given the relatively lower mutation rate of prostate cancer, larger cohorts of AA prostate cancer patients may be required to identify recurrently mutated genes that may contribute to prostate carcinogenesis or to aggressive prostate cancer features in this population. These studies will inform whether alterations in these genes may be enriched in certain ancestral groups. Recent studies have implicated prostate tumor location, differential gene expression, and somatic genomic events such as *LSAMP* deletions in prostate cancers in AA men (2–4,42–44). Our study suggests that there are still unexplained reasons for the aggressive nature of prostate cancer in AA men that is only partially explained by the genomic studies to date. Additional studies focused on metastatic CRPC samples from AA men and development of methodologies to integrate analyses of somatic and germline data may improve our understanding of the nature of aggressive prostate cancer in these patients (23,45–47). Our results suggest that inclusion of sufficient numbers of patients of African ancestry in cancer genomic studies may enable the discovery of new cancer genes and inform the inclusion of diverse populations towards precision cancer medicine (48).

METHODS

Cohort description and pathology evaluation

The discovery cohort comprised specimens from Weill Cornell Medicine (WCM) and Karmanos Cancer Center (Supplemental Table 2). The extension cohort comprised samples from Karmanos Cancer Center, Johns Hopkins University/Prostate Cancer Biorepository Network (PCBN), and Roswell Park Cancer Institute (Supplemental Table 8). Archival pathology specimens were obtained retrospectively from four different institutions under Institutional Review Board protocols: WCM (IRB #1007011157), Roswell Park Cancer Institute (IRB #BDR-035413), Johns Hopkins University (IRB # NA_00048544), and Karmanos Cancer Center (IRB # 044812MP4E). H&E stained slides were reviewed by study pathologists at WCM (JMM, BDR, FK, MAR), RPCI (GA), JHU (AM) and KCC. Annotated slides containing tumor and benign tissue were used for somatic and germline

DNA. Clinical and pathologic data are summarized in Supplemental Tables 2 and 8. Data on *ERG* rearrangement, *PTEN* deletion, *SPOP* mutation and *SPINK1* expression was available for the WCM cohort, as previously published (2).

***ERF* fluorescence *in situ* hybridization (FISH)**

To assess *ERF* deletion in tissues, we developed a dual-color FISH assay consisting of a locus specific probe (W12-2967N22) plus reference probe spanning a stable region of the chromosome (RP11-46I12). All clones were tested on normal metaphase spreads of CGH target slides as previously described (9,21). *ERF* deletion was defined as the presence of 0 or 1 copies on average per nucleus compared to two reference signals. At least 100 nuclei were evaluated per tissue section using a fluorescence microscope (Olympus BX51; Olympus Optical, Tokyo, Japan).

***ERF* RNA *in situ* hybridization (RNAish)**—This single-color chromogenic detection assay uses pairs of specially designed oligonucleotide probes that, through sequence-specific hybridization, recognize both the specific target *ERF* RNA sequence and the signal amplification system (see *ERF* Oligonucleotide list, Supplemental Table 14) (Affymetrix, Inc., Santa Clara, CA). Based on unique coordinates on chromosome 19, unique target probe oligonucleotides were designed (see *ERF* Map sequence, Supplemental Figure 20). The latter is designed to hybridize in tandem to the target RNA. Cross-hybridization to other sequences is minimized by screening against the entire human RNA sequence database. The signal amplification system consists of the preamplifier, amplifier, and enzyme-conjugated label probe, which assemble into a tree-like complex through sequential hybridization. Signal amplification occurs at target sites bound by probe pairs only. Nonspecific off-target binding by single probes does not result in signal amplification. All steps of *ERF* RNAish staining of the slides were performed manually (49). Briefly, formalin-fixed, paraffin-embedded (FFPE) unstained tissue sections (5 μ m) are mounted on positively charged microscopic glass slides and deparaffinized in xylene and dehydrated through a series of alcohols. The dehydrated sections are then treated and sequential hybridization of probe and amplifiers are performed according to Affymetrix protocols. The rehydrated sections are treated with 3% hydrogen peroxide at room temperature for 10 minutes to block endogenous peroxidase. Sections are then boiled in 1 \times citric buffer (10 nmol/L Nacitrate, pH 6.0) for 15 minutes and incubated with protease (2.5 mg/mL; Sigma Aldrich, St. Louis, MO) at 40°C for 30 minutes. The slides are hybridized sequentially with target probes (20 nmol/L) in hybridization buffer A (6 \times saline sodium citrate [SSC] buffer [1 \times SSC is 0.15 mol/L NaCl and 0.015 mol/L Na-citrate], 25% formamide, 0.2% lithium dodecyl sulfate [LDS], and blocking reagents) at 40°C for 2 hours, signal preamplifier in hybridization buffer B (20% formamide, 5 \times SSC, 0.3% LDS, 10% dextran sulfate, and blocking reagents) at 40°C for 30 minutes, amplifier in hybridization buffer B at 40°C for 30 minutes, and horseradish peroxidase- or alkaline phosphatase-labeled probes in hybridization buffer C (5 \times SSC, 0.3% LDS, and blocking reagents) at 40°C for 15 minutes. Hybridization signals are detected under brightfield microscope as red colorimetric staining followed by counterstaining with hematoxylin. Signals are granular and discrete red dots corresponding to individual RNA targets.

Quantitative ERF RNAish analysis

RNAscope® SpotStudio™ Software from Definiens, Inc. (Cambridge, MA) was utilized for image analysis of ERF RNA *in situ* expression at a single cell resolution.

Exome sequencing and analysis—For our WES discovery cohort we collected treatment-naïve radical prostatectomy specimens collected from two primary sites: New York City (Weill Cornell Medicine) and Detroit (Karmanos Cancer Institute/Wayne State University). All patients were self-reported African-American. All tissue DNA was extracted from formalin-fixed paraffin embedded (FFPE) tissues. We sequenced 128 matched tumor-normal pairs and after quality control for contamination and low tumor purity (Supplemental Table 15), we analyzed 102 matched pairs (See Supplemental Methods). The baits for exon capture targeted 98.2% of genes in the Consensus CDS (CCDS) database. A mean target coverage depth of 100x per sample was achieved, with 80% of targets covered at a depth of 20x.

Somatic alterations, Filters, and Germline analysis—We used Mutect and the Indelocator (<http://www.broadinstitute.org/cancer/cga/indelocator>) for calling single nucleotide changes and insertion/deletions (Supplemental Methods). We used an FFPE filter to remove mutations likely from FFPE artifacts. We also used the GATK HaplotypeCaller to find germline variants within a specific gene.

Germline Variant Interpretation—The analysis of germline variants focused on variants identified among 20 genes that are associated with autosomal dominant cancer-predisposition syndromes. These genes were chosen for their crucial role in the maintenance of DNA integrity. Pathogenicity of germline variants was determined according to the most recent guidelines published jointly by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP). Germline variants were evaluated against published literature and publicly available databases such as ClinVar and variant-specific databases. In addition, population-based frequency databases including 1000 genomes and the Exome Aggregation Consortium (ExAC) were examined. Only pathogenic and likely pathogenic variants with high or moderate penetrance were reported in this study. Low penetrance variants were excluded.

DNA sequencing validation—For validation, we used the Fluidigm Access Array microfluidic device. PCR products were barcoded, pooled and subjected to Illumina sequencing on a MiSeq instrument.

Cell Culture/Lentiviral transduction—LNCaP and PC3 cells were cultured in RPMI (Gibco) with 10% Fetal bovine serum. VCAP cells were cultured in Dulbecco's Modified Essential Medium with 10% fetal bovine serum. Cells stably expressing shRNAs were generated by seeding cells at 3×10^5 cells per well of a 6-well followed by transduction with lentivirus expressing given shRNAs. shRNAs in pLKO vectors were obtained from the Genetic Perturbation Platform at the Broad Institute: shERF.1 - shRNA TRCN0000349615: CCTGGTGTCTTCCGAGTCTAT; shERF.2- shRNA TRCN0000273970: CCACACCCAAAGCGTCTACAA. shRNA TRCN0000013908:

CCTGTCTCTGTGGGTTTCTAA; shRNA TRCN0000013911: GAGGTGACTGACATCAGTGAT; Selection was performed subsequently with RPMI media with 10% FBS containing puromycin (2ug/ml). Cell lines were kindly provided by Dr. William C. Hahn in 2014–16 and were authenticated annually by DDC Medical or by the Molecular Diagnostics Laboratory at the Dana-Farber Cancer Institute using short tandem repeat (STR) profiling. The LHS-AR cell line was provided by Dr. William C. Hahn (33).

Western Blot Analysis—Cell lysates were prepared by lysing cells in 1% NP-40 with protease inhibitors (Roche) and phosphatase inhibitors (Calbiochem). Lysates were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using the iBlot system (Life Technologies). Immunoblotting was performed using LI-COR reagents (Odyssey Blocking Buffer and IRDye 800CW and IRDye 680RD secondary antibodies) according to the manufacturer’s instructions (LI-COR Biosciences). Fluorescence detection was performed using an Odyssey CLx Infrared Imaging System, and quantitation was performed using Image Studio software (LI-COR) Anti-ERF antibody was purchased from Abcam (ab61108). Antibody for vinculin was purchased from Sigma (#V9131).

qRT-PCR—Total RNA was isolated using RNAeasy (Qiagen). cDNA was prepared using ~1ug of RNA and the Superscript III kit (Life technologies). ERF transcript levels were quantified using SYBR green (Applied Biosystems) and measured using Quantstudio 6. The following primers were used for ERF:

ERF_1_FP 5’ GCA AGC CCC AGA TGA ATT ACG 3’

ERF_1_RP 5’ CCC CTT GGT CTT GTG CAG AA 3’

RNA sequencing—Total RNA was isolated using RNAeasy (Qiagen) and then processed with the NEBNext PolyA mRNA Magnetic Isolation Module (NEB, E7490) and then further processed with the NEBNext Ultra Directional RNA Library Prep kit (NEB, E7420S). Libraries for RNA sequencing were then sequenced on a NextSeq (Illumina).

ERF signature

To generate the ERF signatures we analyzed the RPKM RNASeq profiles of ERF shRNA knockouts in LNCaP and VCAP cell lines (see ERF shRNA experiments above). We independently rank genes according to the difference of means between the shERF-infected LNCaP and control samples. The same procedure was performed for the VCAP cell line. To obtain a consensus shERF signature we compute the overlap between the top 400 differentially expressed (up- and down-regulated) genes in LNCaP and VCAP cell lines. This overlap resulted in 65 up-regulated genes and 61 down-regulated genes. These two gene sets are being provided as part of the supplementary information and will also be made publicly available as part of the C6 sub-collection of the Molecular Signatures Database (MSigDB) in a future release (Supplemental Table 16) (50).

Soft Agar Assays—PC3 cells were transduced with lentivirus generated with pLKO-puro-shRNA plasmids. Twenty-four hours post-infection, cells were selected with 2 μ g/ml puromycin. After 48 hours of selection, cells were split for qRT-PCR assays and for passaging in normal serum-containing media for soft agar assays. 1×10^4 cells were suspended in 1ml of 0.33% select agar in RPMI/FBS and plated on a bottom layer of 0.5% select agar in 6-well plates. Each cell line was analyzed in triplicate. Colonies were photographed after 11 days and quantified using CellProfiler.

CRISPR/Cas9 experiments—sgRNA guide sequences targeting the ERF coding region were cloned into the plentiCRISPR vector (pxpr001).(36) sg4 ERF: CAC CGG GGT ACA TCG GGC TCA GCG T sg5 ERF CAC CGG ATC CCC GCG CCC GAC CAC C; control guide sg5 GFP: GAAGTTCGAGGGCGACACCC. Lentivirus was produced in 293T cells and then LNCaP cells were lentivirally transduced followed by puromycin (2 μ g/ml) selection. Western blot analysis was used to confirm *ERF* knockdown. LNCaP cells were seeded at a density of 1×10^4 cells in triplicate in 12-well plates in RPMI media supplemented with 10% charcoal-stripped serum (CSS) (Gibco #12676). The following day cells were treated with R1881 synthetic androgen in RPMI/CSS media and media was changed every 3–4 days. After 14 days cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet solution. Cells were photographed using a Leica microscope and imaging software. Quantification of crystal violet uptake for each sample was performed by de-staining cells with 10% acetic acid and measurement of absorbance at 595 nm using a SpectraMax 190 instrument.

Animal Experiments—All animal experiments were approved by the Dana-Farber Cancer Institute Institutional Animal Care and Use Committee and were performed in accordance with institutional and national guidelines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

Systematic genomic sequencing of prostate cancer in African-American men revealed new insights into prostate cancer, including the identification of *ERF*, a new prostate cancer gene, somatic copy number alteration differences, and uncommon *PIK3CA* and *PTEN* alterations. This study highlights the importance of inclusion of underrepresented minorities in cancer sequencing studies.

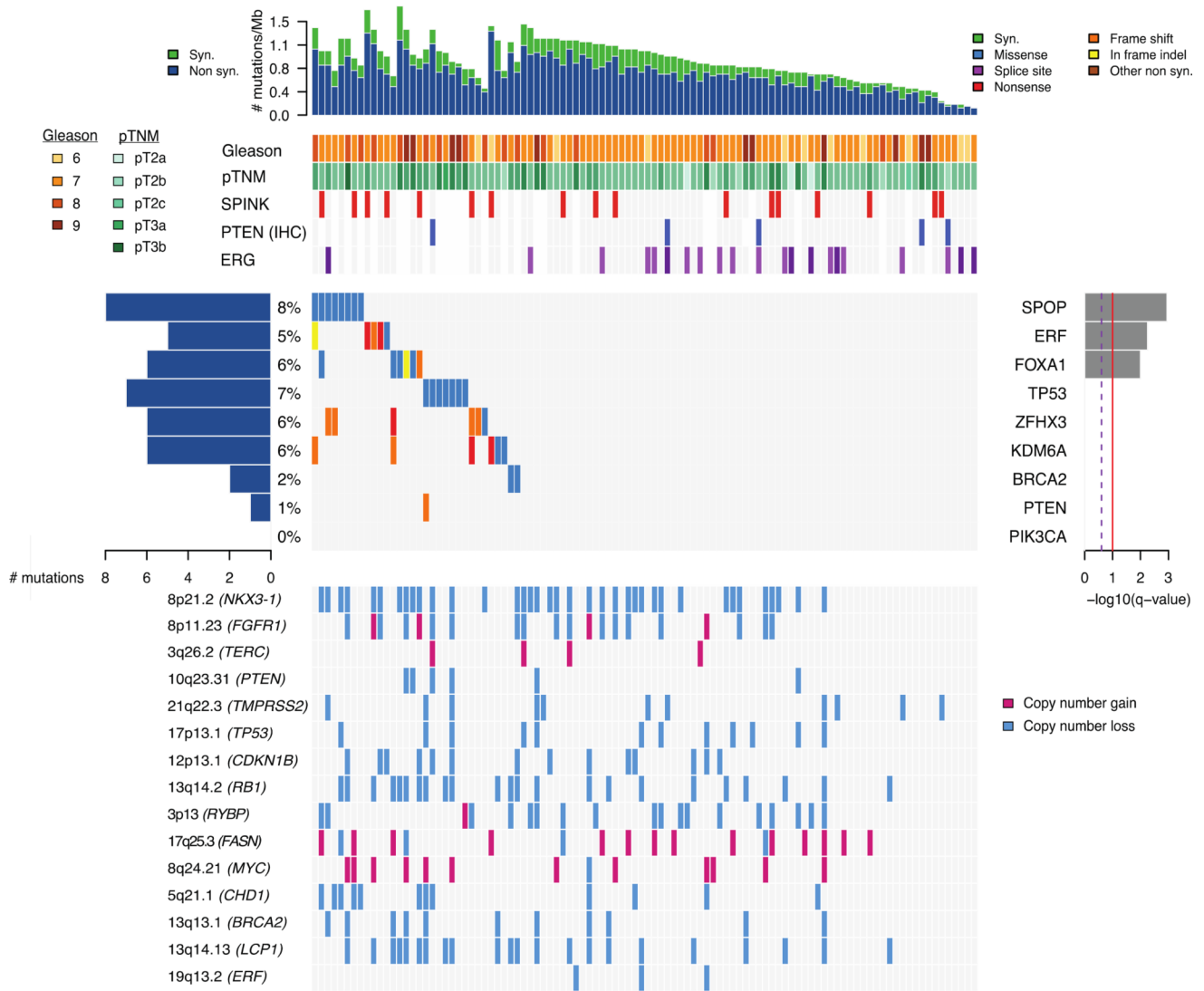


Figure 1. Exome mutation plot of 102 primary prostate cancers from African-American men Significantly mutated genes (*SPOP*, *ERF*, *FOXA1*) determined by MutSigCV ($q < 0.1$) and other selected prostate cancer genes are shown with mutation frequencies and types of mutations. Mean mutation rate per sample is shown in the top panel. Pathologic features of the prostate tumors including Gleason score, pathologic stage, and ERG by FISH, PTEN deletion by immunohistochemistry, and SPINK1 overexpression are shown in the second panel for available samples. Somatic copy number events across the samples at recurrently affected loci with noted prostate cancer genes in parentheses are shown in the bottom panel.

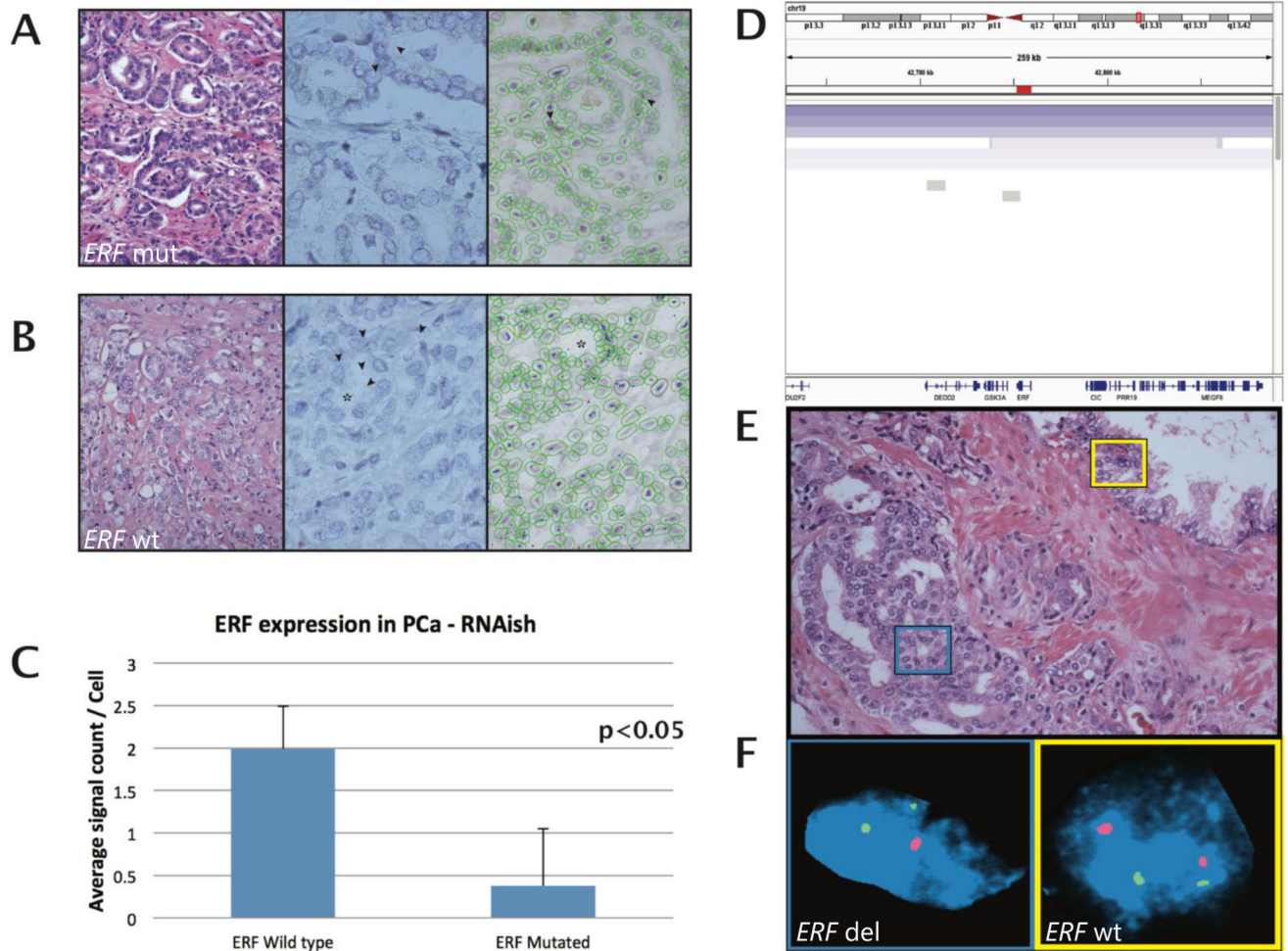


Figure 2. *ERF* RNA expression is significantly lower in *ERF* mutated prostate cancer

A) Histology of prostatic adenocarcinoma, Gleason score 3+4=7 (case STID21598), (left). This representative *ERF* mutated tumor demonstrates only few *ERF* RNA signals as highlighted by arrowheads (center). RNA signals are seen as scattered yellow dots during image fragmentation for automated analysis (right). B) Histology of prostatic adenocarcinoma, Gleason score 4+3=7 (case STID21603), (left). In contrast to *ERF* mutated cases, this representative *ERF* wt prostate cancer demonstrates numerous signals by RNA *in situ* hybridization (asterisk, center). These are highlighted as yellow dots for automated image analysis (right). C) Automated image analysis of three *ERF* mutant cases vs. six *ERF* wt prostate cancer cases demonstrates a significant difference ($p < 0.05$) in RNA expression by this *in situ* assay. D) IGV screenshot of deletions spanning the *ERF* locus in the AAPC exome cohort E) Validation of *ERF*-deleted prostate cancer by fluorescence *in situ* hybridization (FISH). Top panel shows histology of prostatic adenocarcinoma, Gleason score 4+3=7 (case STID12329). F) In the bottom panel, a FISH assay shows hemizygous *ERF* deletion in the tumor (left image; blue square in panel F), as demonstrated by only one target (red) signal and two centromeric (green) reference signals. In contrast, *ERF* wild type status (right image; yellow square in panel F) is seen in nuclei of adjacent benign glands. (Original magnification: H&E at 40 \times ; FISH images at 100 \times under oil immersion).

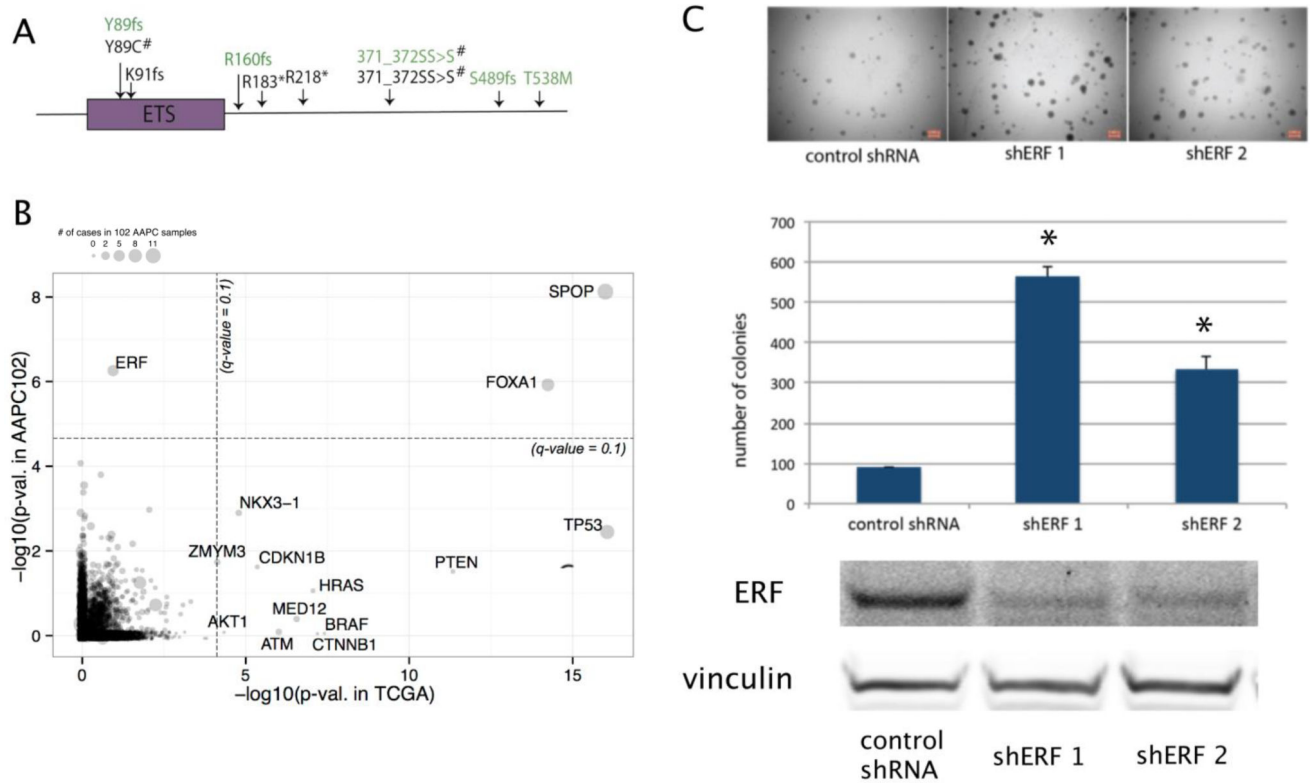


Figure 3. *ERF* is mutated in the APC cohort and can act as a tumor suppressor

A) Types and locations of mutations in *ERF* found in the discovery (black) and extension (green) APC cohorts. Mutations were validated by Fluidigm Access Array except those indicated by #. B) Mutation significance p-values for genes from the APC cohort (n=102) and TCGA (n=333) were plotted. Dotted lines are drawn at the q-value = 0.1 for significance within each cohort. C) Anchorage-independent growth assays of *ERF* knockdown in PC-3 prostate cancer cell lines were performed in triplicate. Colonies were quantified with CellProfiler, * p<.05, student's T test. Data shown is representative of two independent experiments. Western blot results are depicted for knockdown of *ERF* with two short hairpins and control hairpin.

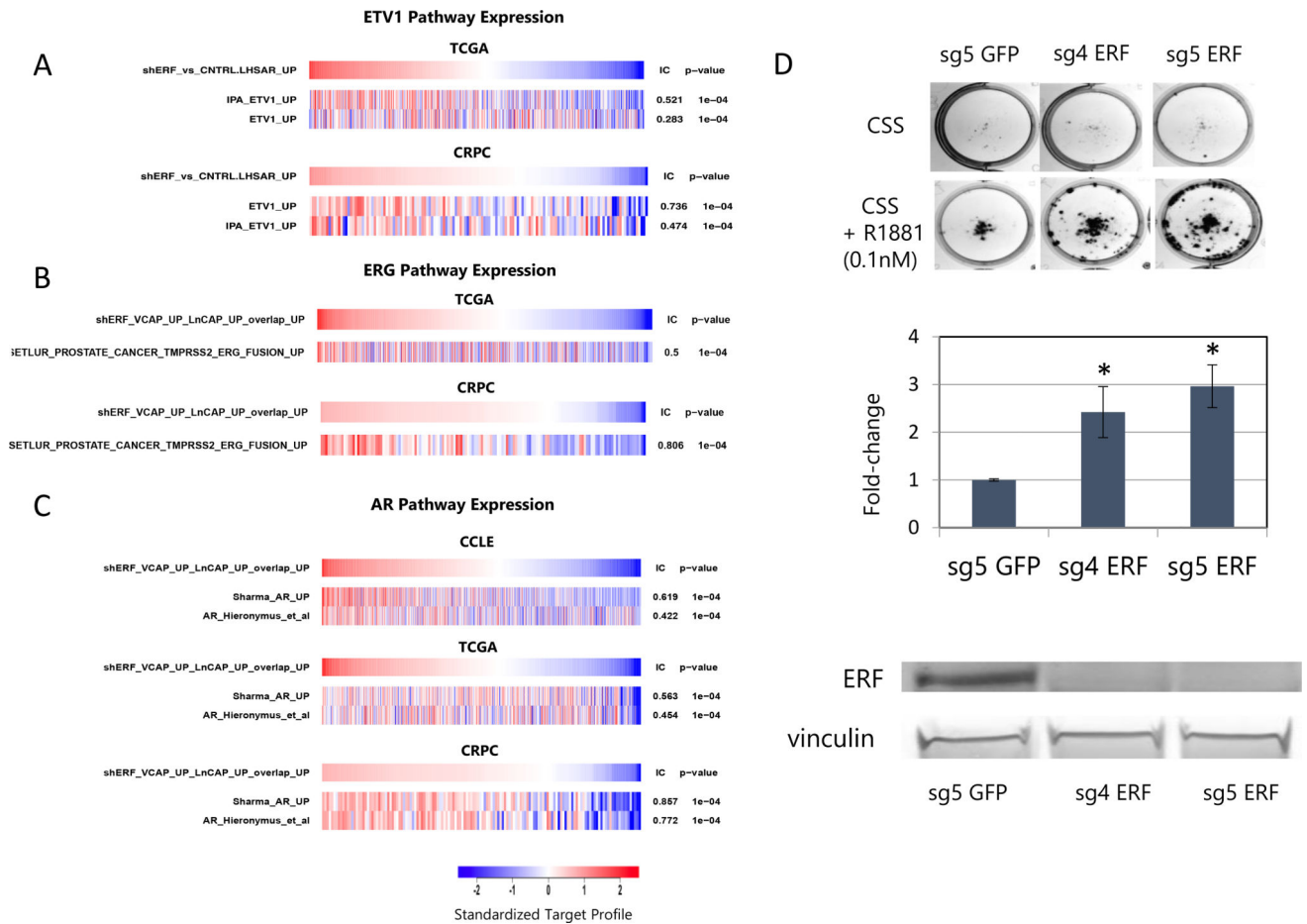


Figure 4. The association of *ERF* knockdown signatures with the single-sample GSEA enrichment profiles of ETS and AR Gene sets

A) An *ERF* knockdown signature profile in the prostate epithelial cell line LHS-AR associates with the enrichment profiles of ETV1 gene sets in the TCGA and CRPC expression datasets B) A composite *ERF* knockdown signature profile, i.e. the enrichment profile of the overlap between *ERF* knockdown signatures in the VCAP and LNCaP cell lines, associates with the enrichment profile of an *ERG* gene set across the TCGA and CRPC expression datasets C) The same composite *ERF* knockdown signature association with enrichment profile of *AR* gene sets across the CCLE, TCGA, and CRPC datasets. D) Focus formation assay of LNCaP-sgERF cell lines in the context of charcoal-stripped serum (CSS) and androgen treatment (0.1nM R1881). Crystal violet staining was quantitated and compared to control. Mean and standard error of three replicates is shown. * $p < 0.05$ by Student's t-Test, fold-change sg4 ERF vs sg5 GFP, sg5 ERF vs sg5 GFP. Data shown are representative of two independent experiments.