

Potential Downstream Target Genes of Aberrant ETS Transcription Factors Are Differentially Affected in Ewing's Sarcoma and Prostate Carcinoma

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Abstract

FLI1 and *ERG*, the major ETS transcription factors involved in rearrangements in the Ewing's sarcoma family of tumors (ESFT) and in prostate carcinomas (PCa), respectively, belong to the same subfamily, having 98% sequence identity in the DNA binding domain. We therefore decided to investigate whether the aberrant transcription factors in both malignancies have some common downstream targets. We crossed a publicly available list of all putative EWSR1-FLI1 target genes in ESFT with our microarray expression data on 24 PCa and 6 non-malignant prostate tissues (NPT) and choose four genes among the top-most differentially expressed between PCa with (PCa *ERG*+) and without (PCa *ETS*-) ETS fusion genes (*HIST1H4L*, *KCNN2*, *ECRG4* and *LDOC1*), as well as four well-validated direct targets of the EWSR1-FLI1 chimeric protein in ESFT (*NROB1*, *CAV1*, *IGFBP3* and *TGFBR2*). Using quantitative expression analysis in 16 ESFT and seven alveolar rhabdomyosarcomas (ARMS), we were able to validate the four genes previously described as direct targets of the EWSR1-FLI1 oncoprotein, showing overexpression of *CAV1* and *NROB1* and underexpression of *IGFBP3* and *TGFBR2* in ESFT as compared to ARMS. Although none of these four genes showed significant expression differences between PCa *ERG*+ and PCa *ETS*-, *CAV1*, *IGFBP3* and *TGFBR2* were less expressed in PCa in an independent series of 56 PCa and 15 NPT, as also observed for *ECRG4* and *LDOC1*, suggesting a role in prostate carcinogenesis in general. On the other hand, we demonstrate for the first time that both *HIST1H4L* and *KCNN2* are significantly overexpressed in PCa *ERG*+ and that ERG binds to the promoter of these genes. Conversely, *KCNN2* was found underexpressed in ESFT relative to ARMS, suggesting that the EWSR1-ETS oncoprotein may have the opposite effect of ERG rearrangements in PCa. We conclude that aberrant ETS transcription factors modulate target genes differently in ESFT and PCa.

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Introduction

The involvement of ETS genes in cancer was first demonstrated by the presence of the oncogene *v-ets* as part of the gag-myb-ets transforming fusion protein of an avian retrovirus, E26 [1]. Their importance in human carcinogenesis is supported by the observations that ETS genes are implicated in chromosomal translocations, giving rise to fusion proteins that play an important role in the genesis of several hematological malignancies, soft tissue tumors and carcinomas [2]. The ETS family of transcription factors is one of the largest families of transcription regulators (27 members in the human genome), and plays an important role in diverse biological processes, including cell proliferation, apoptosis,

differentiation, lymphoid and myeloid cell development, angiogenesis and invasiveness [3–4]. It is characterized by an 85 amino acidic, highly conserved, DNA binding domain (known as ETS domain), which displays sequence specific binding to purine-rich DNA sequences containing a 5'-GGAA/T-3' core sequence [5–6].

The Ewing's sarcoma family of tumors (ESFT) serves as a paradigm for the entire class of ETS-related tumors, since more than 99% of the cases harbor translocations involving ETS genes and *EWSR1* [7]. In 85% of the cases, the ESFT harbors a t(11;22)(q24;q12) chromosomal translocation, resulting in a fusion of the amino terminus of the *EWSR1* gene to the carboxyl

terminus (containing the DNA binding domain) of *FLII*. Fusions between *EWSR1* and other ETS genes, namely *ERG* (10%) and *ETV1*, *ETV4*, or *FEV* (<5%), are alternative pathogenetic mechanisms in ESFT [7]. Prostate cancer (PCa) is the most recent ETS-related neoplasia [8], with the *TMPRSS2-ERG* fusion gene being reported in about 50% of the cases [8–11]. Other, less common gene fusions (1–10%), involve additional ETS family members, such as *ETV1*, *ETV4*, *ETV5*, and *FLII* [12–14]. In both ESFT and PCa these ETS chimeric genes function as aberrant transcription factors, having a pivotal role in promoting transformation and oncogenesis. This hypothesis is consistent with experiments showing that *EWSR1-FLII* knockdown is correlated with decreased cell invasion and increased apoptosis [15–16] and with reports showing that overexpression of *ERG* and *ETV1* in benign prostate cells induces a transcriptional program associated with invasion [17–18].

Identifying the target genes of the ETS fusion genes is crucial to understand the oncogenic pathways of the ETS-positive malignancies and some of them may turn out to be more amenable to targeted therapy than the chimeric/truncated transcription factors themselves. Whereas several target genes relevant for ESFT have been uncovered [19–20], the search for the downstream effectors of aberrant ETS transcription factors in PCa is still in its infancy [21–22]. The major ETS genes involved in rearrangements in ESFT and PCa, *FLII* and *ERG*, respectively, belong to the same subfamily, have 98% sequence identity in the DNA binding domain [23–24], and have been found rearranged in both neoplasias [7–8,13]. In order to investigate whether these ETS fusion genes have some common downstream targets, we crossed a publicly available list of all putative *EWSR1-FLII* direct target genes in ESFT (obtained by chromatin immunoprecipitation coupled with DNA microarrays) [20] with our microarray expression data on PCa with and without *ERG* rearrangements [25] and validated the findings in an independent series of PCa and ESFT.

Materials and Methods

Ethics Statement

This study was approved by the institutional review board (Comissão de Ética para a Saúde). Written informed consent was obtained for all participants.

Selection of Candidate ETS Target Genes

To select the ETS candidate target genes, we started from the list of 874 genes shown by Gangwal and colleagues [20] to be bound by *EWSR1-FLII*, the main ETS fusion protein involved in ESFT tumorigenesis. To accomplish this task, they used a combined approach that included chromatin immunoprecipitation and microarray technology. Based on that list, we then used our whole genome expression data on PCa and non-malignant prostatic tissues (NPT) [25], to find out how many of those genes were relevant in prostate carcinogenesis. The genome-wide RNA expression analysis included 6 NPT and 24 PCa: 16 with *ERG* fusion genes (PCa *ERG+*) and 8 without ETS rearrangements (PCa *ETS-*) as determined by FISH and reverse-transcription-PCR (RT-PCR) [9,25]. Then the following selection criteria were applied: a) the gene expression had to be at least 2-fold higher or 1.5-fold lower in PCa harboring *ERG* fusion genes compared to those negative for ETS rearrangements; b) the expression ratio between ETS negative carcinomas and NPT had to be similar (between 0.9 and 1.1).

Four well validated direct targets of the *EWSR1-FLII* chimeric protein in ESFT were selected based on a literature survey. These

included the two upregulated genes *CAVI* [26] and *NROB1* [27] and the two downregulated genes *IGFBP3* [16] and *TGFBR2* [28].

Prostate Cancer and Non-malignant Tissue Specimens

Fifty-six PCa samples were selected from a pool of 200 patients with clinically localized prostate adenocarcinoma consecutively diagnosed and treated with radical prostatectomy at the Portuguese Oncology Institute – Porto (IPO-Porto), Portugal [13]. These samples were chosen in order to represent different molecular subtypes of prostate cancer, as previously classified, and included: 24 samples with *ERG* rearrangements (PCa *ERG+*), 12 with other ETS rearrangements (PCa *oETS+*, which include rearrangements with ETS members of the PEA3 subfamily – *ETV1*, *ETV4* and *ETV5* [24]) and 20 without ETS rearrangements (PCa *ETS-*). For control purposes, 15 NPT were collected from cystoprostatectomy specimens of bladder cancer patients who did not harbor simultaneous prostate carcinoma.

Ewing's Sarcoma and Alveolar Rhabdomyosarcoma Samples

Sixteen samples of ESFT were used. RT-PCR was performed to detect the respective fusion transcripts [29] as part of routine molecular diagnosis at the Department of Genetics of IPO-Porto. Fourteen out of sixteen (88%) samples presented the *EWSR1-FLII* fusion transcript and the remaining two (12%) had the *EWSR1-ERG* chimeric protein. Because the cell of origin of ESFT is not known, we used as control seven alveolar rhabdomyosarcomas (ARMS), which are also small blue round cell tumors but do not express ETS chimeric proteins; instead, they are characterized by the specific translocation t(2;13)(q35;q14) or its variant t(1;13)(p36;q14) giving rise to the fusion genes *PAX3-FKHR* or *PAX7-FKHR*, respectively [30]. Using RT-PCR as part of routine molecular diagnosis in our department [31], the *PAX3-FKHR* was detected in four (57%) samples and the remaining three (43%) had the *PAX7-FKHR* fusion transcript. RNA samples from the 16 ESFT and the seven ARMS were used for the target gene analyses.

Prostate Cell Lines

LNCaP cells were acquired from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and 22Rv1 cells were kindly provided by Dr David Sidransky from the Johns Hopkins University School of Medicine. Both cell lines were cultured under the recommended conditions, being karyotyped by G-banding for validation purposes and tested for Mycoplasma spp. Contamination (PCR Mycoplasma Detection Set; Clontech Laboratories, Saint-Germain-en-Laye, France).

RNA Extraction and cDNA Synthesis

Total cellular RNA was extracted from the prostate tissue samples using the TRIzol® reagent combined with the PureLink™ RNA Mini Kit purification columns (Invitrogen by Life Technologies, Carlsbad, CA), as previously described [25]. Subsequently, 200 ng of RNA were converted into cDNA using the TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. For total RNA extraction from cell lines, the TRIzol® reagent was used, following the manufacturer's recommendations. cDNA was obtained from 500 ng of RNA using random hexamer primers and the H-minus RevertAid cDNA synthesis kit (Fermentas, Ontario, Canada), according to the manufacturer's instructions.

DNA Extraction and Bisulfite Treatment

To assess whether decreased gene expression was associated with DNA methylation, DNA was extracted from prostate tissue samples and from cell lines by the phenol-chloroform method [32], and subsequently subjected to sodium bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA), according to the manufacturer's protocol. CpGenome™ Universal Methylated DNA (Millipore, Billerica, MA) and CpGenome™ Universal Unmethylated DNA (Millipore) were also bisulfite-modified to serve as positive and negative controls, respectively.

Cell Line Treatment with 5-aza-2'-deoxycytidine (DAC)

To evaluate whether promoter methylation of *CAVI*, *IGFBP3* and *ECRG4* was associated with decreased transcript expression in PCa, we treated LNCaP and 22Rv1 prostate cancer cell lines (the first harboring an *ETV1* rearrangement and the second without known ETS rearrangements) with 1 μ M of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (DAC; Sigma-Aldrich), as previously described [33]. After 72 hours of treatment, DNA and RNA were extracted as described above.

Quantitative RT-PCR (qRT-PCR)

In order to determine the relative expression levels of selected genes, qRT-PCR was performed. Primers and probes for the selected genes and the endogenous control (glucuronidase beta, *GUSB*) were acquired as pre-developed TaqMan® Gene Expression Assays from Applied Biosystems (by LifeTechnologies, Foster City, CA) (Supplementary Table S1). *GUSB* gene was used for normalization of the expression levels of the selected genes. All samples were run in triplicate and multiple negative controls were included in each plate. Relative expression values were obtained by the comparative Ct method [34].

Methylation-specific PCR (MSP) and Quantitative MSP (qMSP)

To confirm the presence of a CpG island in the promoter region of the genes of interest, their RefSeqs were obtained from the USCS Genome Browser Database (<http://genome.ucsc.edu/>), including the 2 Kb sequence upstream of the first exon, and these were subsequently analyzed *in silico* using CpG Island Searcher software, according to the algorithm described by Takai and Jones (2002) [35]. The primers' sequences for *CAVI*, *IGFBP3*, and *LDOC1* have been published elsewhere [36–38] and the primers' sequences for *TGFBR2* and *ECRG4* are shown in Supplementary Table S1, all being acquired from Metabion (Martinsried, Germany). MSP assays were carried on prostate samples using 2 μ L of template modified-DNA in a 20 μ L PCR reaction containing 0.2 mM of dNTPs mix (Fermentas, Ontario, Canada), 0.25 μ M of each primer and 0.5 U of DyNAzyme™ II Hot Start (Finnzymes) in 1x DyNAzyme™ II Hot Start Reaction Buffer (Finnzymes, Vantaa, Finland). PCR was then performed according to the DyNAzyme™ II Hot Start manufacturer's conditions. Considering the limited amount of bisulfite-treated DNA available for the MSP analysis, samples were selected according to the lowest expression for each gene (14 for *ECRG4*, 10 for *CAVI*, eight for *IGFBP3* and *LDOC1* and seven for *TGFBR2*) (Supplementary Table S2).

For qMSP on DAC-treated cell lines, 2 μ L of bisulfite modified-DNA were amplified with 0.25 μ M of each primer in 1x Power SYBR® Green PCR Master Mix (Applied Biosystems). β -Actin (*ACTB*, Supplementary Table S1) was used as an internal

reference gene to normalize for DNA input and the qMSP reaction was performed as previously described [33].

Chromatin Immunoprecipitation (ChIP) and Quantitative PCR (qPCR)

We used VCaP cells and the rabbit anti-ERG monoclonal antibody (Epitomics, Burlingame, CA) to detect ERG binding to the promoter of *HIST1H4L* and *KCN2*, as previously described [25]. Briefly, 2×10^6 cells were used for each immunoprecipitation with the EZ-Magna ChIP™ G kit (Millipore), following manufacturer's instructions [39]. To select for putative ETS binding sequences in the promoter regions, a bioinformatic survey of the 10 kb sequence upstream of the translation start site was conducted using ConSite [40]. Three promoter regions of *HIST1H4L* (−454, −728 and −2266), each containing two putative ETS binding sequences, and three promoter regions of *KCN2* (−1442, −1833 and −4083), the first two containing one putative ETS binding sequence and the last containing three, were selected for qPCR analysis of the ERG-immunoprecipitated chromatin. Primers were designed using the Primer3 online software and acquired from Metabion. Primers for a negative control region were also included to correct for unspecific binding (Supplementary Table S1) [41]. qPCR was performed using Power SYBR® Green (Applied Biosystems), according to the manufacturer's recommendations. Serial dilutions of the input fraction were used to calculate primers' efficiency. Results are shown as a fold enrichment of ERG bound chromatin relative to IgG and corrected to the negative control region [42].

Statistical Analysis

Differences in relative expression values of each gene in different groups were assessed by the Kruskal-Wallis non-parametric test, followed by pair-wise comparisons using the Mann-Whitney non-parametric test. The Chi-square test was used to assess the statistical significance of the differences in the frequency of methylation between NPT and PCa samples and a t-test was applied to qPCR and qMSP data. A *p*-value below 0.05 was considered statistically significant. The statistical analyses were performed using the Statistical Package for Social Sciences software, version 15.0 (SPSS Inc., Chicago, IL).

Results

Microarray Expression Data and Candidate Target Gene Selection

After crosschecking the list of *EWSR1-FLI1* target genes in ESFT [20] with our microarray expression data on PCa and NPT, and applying the aforementioned selection criteria, seven potential ETS target genes emerged. Two genes were overexpressed in PCa with ERG fusion genes, namely *HIST1H4L* and *KCN2*, and were chosen for validation. Five genes were underexpressed in PCa with ERG fusion genes, namely *ABCD1*, *ECRG4*, *KCNMA1*, *LDOC1* and *SLC7A4*. *ECRG4* and *LDOC1* were selected for further analysis based on their putative function as tumor suppressor genes in other cancer types [43–44].

The expression of the selected target genes in Ewing's sarcoma (*CAVI*, *NR0B1*, *IGFBP3* and *TGFBR2*), together with the expression of *HIST1H4L*, *KCN2*, *ECRG4* and *LDOC1*, was then validated in an independent series of PCa with and without ETS gene fusions, as well as in a series of ESFT and ARMS.

CAV1 Relative Expression

CAV1 was significantly overexpressed in ESFT when compared to ARMS, showing a median 4.9 fold increase (Figure 1A). On the other hand, *CAV1* was significantly underexpressed in PCa ETS+ when compared to PCa ETS-, presenting a median 1.5 fold decrease (not shown). Although there was no significant difference in *CAV1* expression between PCa *ERG*+ and PCa ETS-, *CAV1* expression in PCa oETS+ was significantly lower when compared to PCa ETS-, with a median 5.5 fold decrease (Figure 2A). *CAV1* expression was significantly lower (3.3 fold decrease) in PCa in general when compared to NPT (Figure 1B).

NROB1 Relative Expression

NROB1 relative expression was significantly higher in ESFT when compared to ARMS, showing a median 8.3 fold increase (Figure 1A). On the contrary, *NROB1* was poorly expressed in PCa and NPT (Figure 1B) and there were no significant differences in relative expression between these groups or among different molecular subgroups of PCa (not shown).

IGFBP3 Relative Expression

IGFBP3 expression was significantly decreased in ESFT when compared to ARMS, exhibiting a median 7.7 fold decrease (Figure 1A). On the other hand, *IGFBP3* relative expression did not show significant differences in different molecular subgroups of PCa, except between PCa *ERG*+ and PCa oETS+ (the last group presenting a median 2 fold decrease expression level; Figure 2B). Globally, *IGFBP3* was significantly underexpressed in PCa when compared to NPT, presenting a median 2.7 fold decrease (Figure 1B).

TGFBR2 Relative Expression

TGFBR2 was significantly underexpressed in ESFT when compared to ARMS, showing a median 3.7 fold decrease (Figure 1A). In contrast, *TGFBR2* expression did not show significant differences among the three molecular subgroups of PCa (not shown). However, when considering PCa as a sole entity we observed that *TGFBR2* expression was significantly lower when compared to NPT (Figure 1B).

HIST1H4L Relative Expression

HIST1H4L relative expression was not significantly different between ESFT and ARMS (Figure 3A). On the other hand, although the expression of *HIST1H4L* was not significantly different between PCa in general and NPT (Figure 3B), PCa *ERG*+ presented higher *HIST1H4L* expression levels when compared to PCa oETS+ (median 3.0 fold increase), PCa ETS- (median 1.9 fold increase) and NPT (median 2.1 fold increase) (Figure 4A).

KCNN2 Relative Expression

KCNN2 was poorly expressed in ESFT and ARMS, but it was significantly underexpressed in the former when compared to the later, showing a median 9.4 fold decrease (Figure 3A). On the other hand, although the expression of *KCNN2* was not significantly different between PCa in general and NPT (Figure 3B), the relative expression of *KCNN2* in PCa ETS+ was significantly higher when compared to PCa ETS- ($p=0.011$), showing a median 1.7 fold increase (not shown). This significant overexpression was found when comparing PCa *ERG*+ with either PCa oETS+ or PCa ETS-, with a median 3.7 and 3.0 fold increase, respectively, but not between PCa oETS+ and PCa ETS- (Figure 4B). *KCNN2* was also significantly overexpressed in PCa *ERG*+ when compared to NPT, showing a median 1.7 fold increase, but was significantly underexpressed in PCa oETS+ and PCa ETS- when compared to NPT, displaying a median 2.2 and 1.8 fold decrease, respectively (Figure 4B).

ECRG4 Relative Expression

ECRG4 relative expression was not significantly different between ESFT and ARMS (Figure 3A). Similarly, there were no significant differences in *ECRG4* relative expression among the different molecular subgroups of PCa (not shown). However, *ECRG4* expression was significantly decreased (2.7 fold) in PCa when compared to NPT (Figure 3B).

LDOC1 Relative Expression

There was no significant difference in *LDOC1* expression between ESFT and ARMS (Figure 3A). Likewise, *LDOC1*

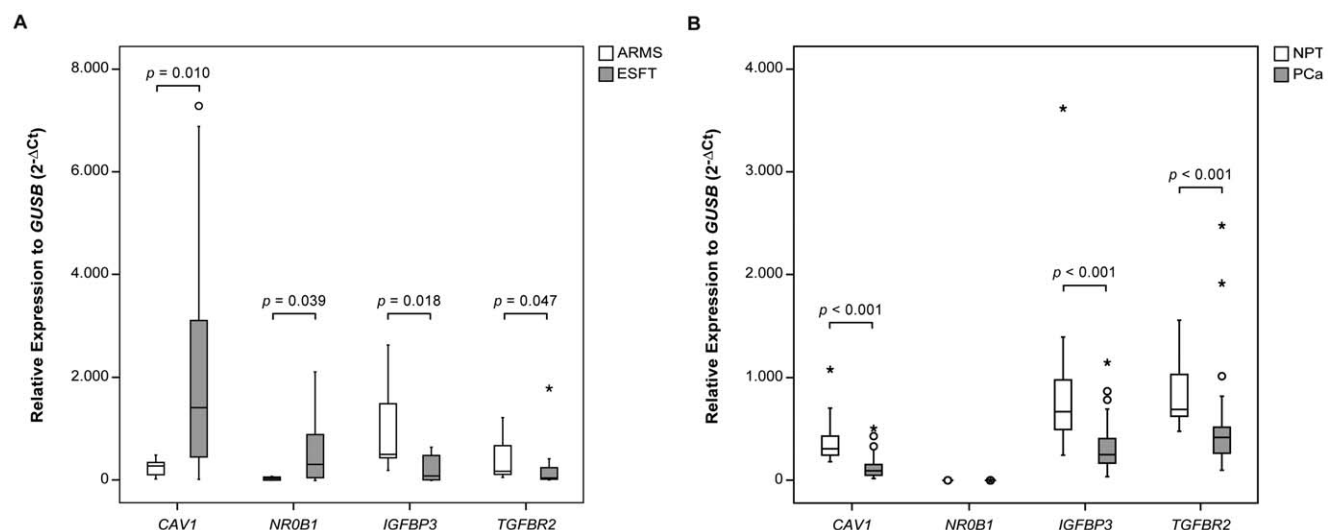


Figure 1. Box-plot representation of the qRT-PCR data for the four genes described as EWSR1-FLI1 targets (*CAV1*, *NROB1*, *IGFBP3* and *TGFBR2*). A) ESFT versus ARMS samples; B) PCa versus NPT samples. A p value is shown whenever the differences in each two group comparison reach significance ($p < 0.05$).

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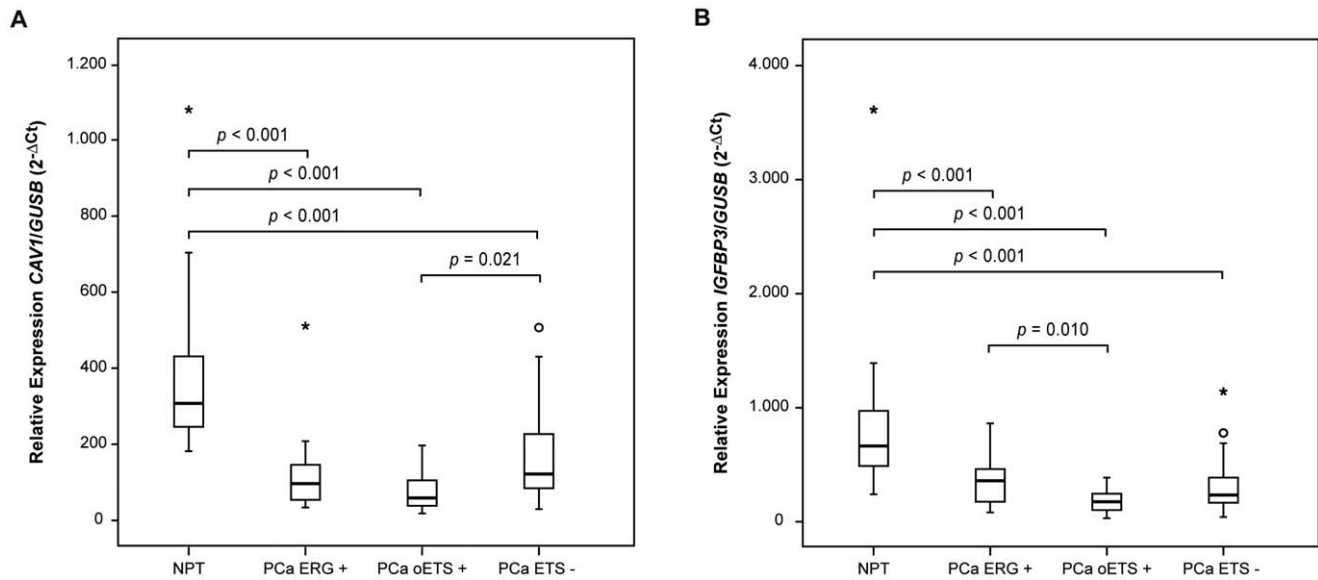


Figure 2. Box-plot distribution of *CAV1* and *IGFBP3* expression in PCa sample subgroups. A) *CAV1* expression; B) *IGFBP3* expression. A p value is shown whenever the differences in each two group comparison reach significance ($p < 0.05$).
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expression did not present significant differences among the different molecular subgroups of PCa (not shown). Nonetheless, *LDOC1* was underexpressed (1.8 fold decrease) in PCa in general when compared to NPT (Figure 3B).

Promoter Hypermethylation and Downregulation of *CAV1*, *IGFBP3* and *ECRG4* in PCa

The promoter methylation status of *CAV1*, *IGFBP3*, *TGFBR2*, *ECRG4* and *LDOC1* was evaluated in prostate tissue samples (Supplementary Table S2). Although we were not able to detect differences among PCa subgroups, overall, higher promoter methylation frequencies of *CAV1*, *IGFBP3* and *ECRG4* were found in PCa compared to NPT ($p = 0.010$ for *CAV1*, $p < 0.001$ for

IGFBP3 and $p = 0.008$ for *ECRG4*). No methylation was detected at the *TGFBR2* and *LDOC1* promoters in prostate tumor samples. DAC-treatment of the *ETV1* rearrangement-positive cell line LNCaP resulted in decreased methylation of *CAV1* promoter and *de novo* *CAV1* expression, although the difference did not reach statistical significance ($p = 0.07$; Supplementary Figure S1). A slight increase in *IGFBP3* expression was also observed in LNCaP cells after DAC treatment, although not statistically significant ($p = 0.15$; data not shown). The ETS-negative cell line 22Rv1 showed basal expression of *CAV1* and *IGFBP3*, which did not change after DAC treatment. *ECRG4* was not expressed in both cell lines and DAC treatment was not sufficient to induce *de novo* *ECRG4* expression (data not shown).

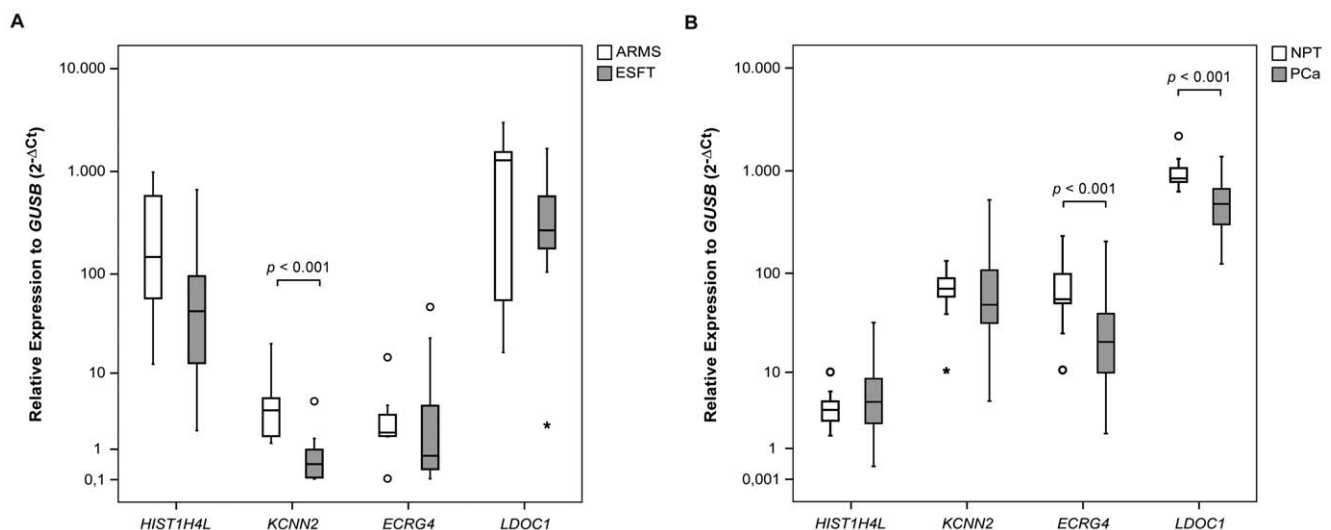


Figure 3. Box-plot representation of the qRT-PCR data for the four genes described as EWSR1-FLI1 targets and associated with PCa samples harboring *ERG* rearrangements (*HIST1H4L*, *KCNN2*, *ECRG4* and *LDOC1*). A) ESFT versus ARMS samples; B) PCa samples versus NPT samples. A p value is shown whenever the differences in each two group comparison reach significance ($p < 0.05$).
doi:10.1371/journal.pone.0049819.g003

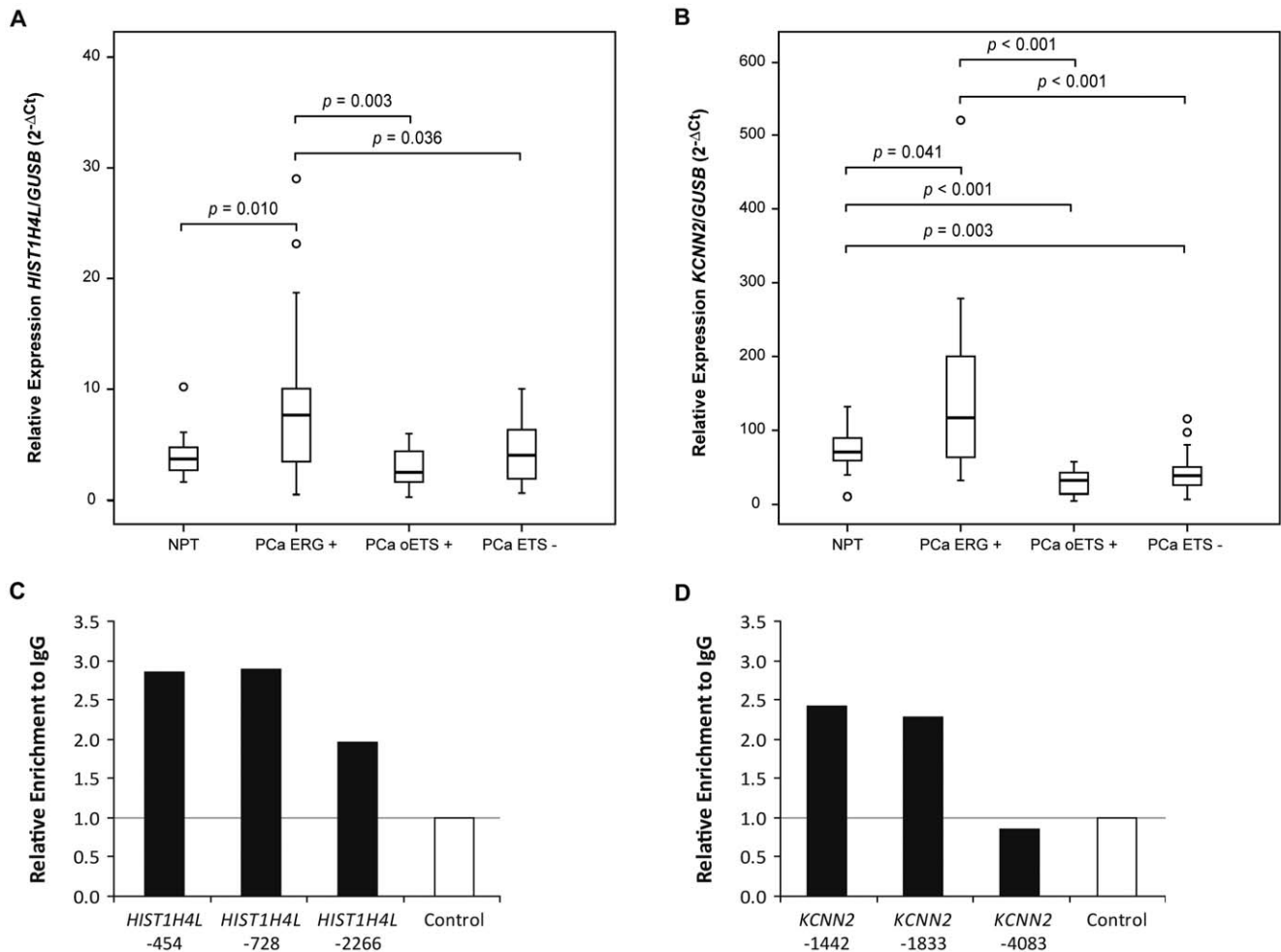


Figure 4. Analyses of *HIST1H4L* and *KCNN2* expression and their regulation by ERG in PCa samples harboring *ERG* rearrangements. **A)** and **B)** Box-plot distribution of *HIST1H4L* and *KCNN2* expression in PCa sample subgroups, respectively. A *p* value is shown whenever the differences in each two group comparison reach significance ($p < 0.05$). **C)** and **D)** qPCR of ERG-immunoprecipitated chromatin from VCaP cells showing ERG binding to three regions of the *HIST1H4L* promoter and to two regions of the *KCNN2* promoter, respectively. doi:10.1371/journal.pone.0049819.g004

ERG Binds to *HIST1H4L* and *KCNN2* Promoter Regions

Using ChIP of VCaP cells, we were able to detect ERG binding to the three regions tested for the *HIST1H4L* promoter (-454, -728 and -2266) and to two regions of the *KCNN2* promoter (-1442 and -1833) (Figures 4C and 4D).

Discussion

The ETS family of transcription factors is one of the largest involved in the regulation of a variety of different genes that play key roles in proliferation, apoptosis, differentiation, hematopoiesis, metastasis, tissue remodeling, angiogenesis and transformation [3–4]. Identification of the target genes for normal and oncogenic ETS genes may lead to a better understanding of the mechanisms underlying malignant transformation. All ETS family members bind to 5'-GGAA/T-3' DNA sequences and ETS target genes may be identified upon the presence of a functional binding site in their regulatory regions [5–6]. The crucial role of ETS chimeric proteins in the development of ESFT is well documented [45], and although it has been shown that EWSR1-FLI1 and EWSR1-ERG bind ETS sequences *in vitro* with similar specificities and affinities as the wild-type transcription factors [46–47], it has recently been

shown that EWSR1-ETS chimeric proteins induce chromatin structure alterations that lead to transcription dysregulation [48]. Contrary to ESFT, in PCa the most common ETS fusion member is *ERG* (about 90% of the fusion positive cases), and only 1–10% of the cases have fusion genes involving other ETS members, namely, *ETV1*, *ETV4* and *ETV5* (which cluster in the PEA3 subfamily), and *FLI1* (which clusters with *ERG* in the ERG subfamily) [8,12–14,23]. The ETS domain of the PEA3 subfamily displays 60% homology with the ERG subfamily, but there is no significant homology outside the ETS domain as indicated by the presence of a PNT domain in the ERG subfamily but not in the PEA3 subfamily [6,23]. Recently, we showed that while some genes are specifically and differentially expressed between PCa harboring *ERG* or *ETV1* rearrangements, others are commonly dysregulated between these tumor molecular subtypes and PCa without ETS rearrangements, with *ETV4* and *ETV5* positive tumors clustering together with those with *ETV1* rearrangement [33]. As *FLI1* and *ERG* belong to the same subfamily and share 98% of sequence homology in the DNA binding domain [23–24], we questioned whether in a different cell background they would show dysregulation of the expression of the same genes.

We started by analyzing the expression of four well-validated targets of the EWSR1-FLI1 oncoprotein in ESFT and we used ARMS for control purposes. We validated the dysregulation in the expression of four genes previously described as direct targets of the EWSR1-FLI1 oncoprotein in ESFT, showing overexpression of *CAVI* and *NROB1* and underexpression of *IGFBP3* and *TGFBR2* [16,26–28]. We then evaluated the expression of these genes in PCa with and without ETS rearrangements. None of these genes showed significant expression differences between PCa *ERG+* and PCa *ETS-*, suggesting that *ERG* proteins do not regulate their expression in this tumor type. However, the expression of *CAVI*, *IGFBP3* and *TGFBR2* is decreased in PCa in general, suggesting a role in prostate carcinogenesis. Our data suggest that regulation of *CAVI* expression may be, at least in part, controlled by promoter methylation, which has also been reported by others [49]. Although we found decreased *CAVI* expression especially in PCa oETS+ and the *ETV1*-positive LNCaP cell line showed increased expression of *CAVI* after DAC treatment, the methylation status of the *CAVI* promoter in PCa oETS+ samples was heterogeneous, while in other tumor samples *CAVI* was more consistently methylated (Supplementary Table S2). This suggests that *CAVI* promoter methylation and ETS transcription factors do not cooperate in the regulation of *CAVI* expression in PCa. Although *IGFBP3* also showed a greater underexpression in PCa oETS+ when compared to PCa *ERG+*, there was a non-significant increase in *IGFBP3* expression after DAC treatment of the *ETV1*-positive LNCaP cell line, precluding a consistent relationship between higher *IGFBP3* methylation levels and *ETV1* rearrangements. We also confirmed that *TGFBR2* expression is reduced in PCa [50–51], which is compatible with the tumor suppressor role of *TGFBR2* in PCa cells described by others [52], but promoter methylation does not seem to be involved. On the other hand, we found that *NROB1* was poorly expressed in PCa and in NPT, so our data do not support the previously reported immunoreactivity of DAX1 (protein encoded by the *NROB1* gene) in a significant proportion of PCa [53].

Based on our microarray findings of differential expression of *ECRG4*, *LDOC1*, *HIST1H4L* and *KCNN2* between PCa harboring *ERG* rearrangements and those without ETS fusions, we decided to validate these data in an independent series of tumors. Among the five genes downregulated in PCa *ERG+*, we choose *ECRG4* and *LDOC1* for further study based on their tumor suppressor activity in other cancer models (see below; [43–44]). We also evaluated the expression of these genes in ESFT and ARMS in order to verify if there was any significant difference in their expression that might be attributable to *EWSR1*-ETS rearrangements. We here report for the first time that expression of both *ECRG4* and *LDOC1* is significantly decreased in PCa when compared to NPT. However, this was independent of the ETS status, contrarily to our initial microarray data suggesting a specific underexpression in PCa with *ERG* fusion genes. Consistent with a recent study that has associated CpG island hypermethylation of *ECRG4* with recurrence in prostate carcinoma [54], our MSP analysis showed a significantly higher methylation frequency in PCa comparing with NPT, thus representing a mechanism of gene silencing that might be involved in all molecular subgroups of PCa. In LNCaP and 22Rv1 cell lines, however, DAC treatment was not sufficient to allow *de novo* *ECRG4* expression, thus suggesting that other regulatory mechanisms may act in *ECRG4* underexpression. The mechanism of *LDOC1* downregulation is currently unknown, but because we did not find aberrant promoter methylation at this locus, other epigenetic or genetic alterations are probably causally involved. Finally, although the chimeric EWSR1-FLI1 protein has been found to bind the promoter of both *LDOC1* and *ECRG4*

in vitro [20], we here show that their expression is not significantly different between ESFT and ARMS, thus suggesting that either the expression of these genes is not regulated by that chimeric protein in ESFT or that a different regulatory mechanism in ARMS is regulating the expression of *LDOC1* and *ECRG4* to similar levels.

Our microarray findings of differential expression of *HIST1H4L* and *KCNN2* in different molecular subsets of PCa were confirmed by qRT-PCR in an independent series. *HIST1H4L* is a gene that encodes a histone, which is a basic nuclear protein responsible for the nucleosome structure of the chromosomal fiber in eukaryotes. We here show for the first time that *HIST1H4L* expression is specifically and significantly increased in PCa harboring *ERG* fusion genes, both when compared to other PCa molecular subtypes and with NPT. These findings indicate that *HIST1H4L* is a potential target of *ERG* fusion genes, as also illustrated by our demonstration of direct binding of *ERG* to the *HIST1H4L* promoter, but the mechanism whereby it is involved in prostate carcinogenesis is still unknown. *KCNN2* codes for a small conductance Ca^{2+} -activated potassium channel involved in the regulation of the neuronal excitability [55], and, to our knowledge, we here show for the first time that this gene is overexpressed in PCa harboring *ERG* rearrangements when compared to the other subtypes of PCa and to NPT. On the other hand, *KCNN2* was underexpressed in both PCa with other ETS rearrangements and in those without ETS rearrangements when compared to NPT. These data suggest that *KCNN2* regulation may be mediated by the aberrant *ERG* transcription factor in a particular subtype of PCa (PCa *ERG+*), as also illustrated by our demonstration of direct binding of *ERG* to the *KCNN2* promoter, and that different ETS can have specific roles, even in the same cellular context. Conversely, we show for the first time that *KCNN2* is significantly underexpressed in ESFT when compared to ARMS. Since it has been previously shown that EWSR1-FLI1 binds to the promoter of *KCNN2* *in vitro* [20], it seems reasonable to assume that this downregulation of *KCNN2* in ESFT might be directly mediated by the chimeric transcription factor. On the other hand, although *HIST1H4L* was also found as a direct target of the EWSR1-FLI1 chimeric protein [20], our data showed that the expression of *HIST1H4L* was not significantly different between the ESFT and ARMS, thus suggesting that either EWSR1-FLI1 does not regulate *HIST1H4L* expression *in vivo* or that other regulatory mechanism in ARMS is regulating *HIST1H4L* to similar expression levels.

In conclusion, using two different models of ETS-related tumors, we show that, despite of the conservation of the DNA binding domain of the ETS family of transcription factors, ETS proteins can modulate common target genes in different manners, as well as achieve specificity by controlling distinct genes.

Supporting Information

Figure S1 Comparative methylation and expression levels of *CAVI* after DAC treatment of LNCaP and 22Rv1 prostate cancer cell lines.

(TIF)

Table S1 Assay ID or sequence of the primers used in this study.

(DOC)

Table S2 MSP analysis data of prostate samples.

(DOC)

Author Contributions

Conceived and designed the experiments: MRT. Performed the experiments: MJC PP JDBS MA VLC. Analyzed the data: MJC PP FRR.

Contributed reagents/materials/analysis tools: NC RIS RAL RH CJ. Wrote the paper: MRT PP.

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