Original Article

Sumoylation of transcription factor ETV1 modulates its oncogenic potential in prostate cancer

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Abstract: The transcription factor ETS variant 1 (ETV1) is capable of promoting prostate tumorigenesis. We demonstrate that ETV1 can be posttranslationally modified by covalent attachment of small ubiquitin-like modifier 1 (SUMO1) onto four different lysine residues. In human embryonic kidney 293T cells, mutation of these sumoylation sites stimulated the transactivation potential of ETV1 at the matrix metalloproteinase 1 (MMP1), but not Yes-associated protein 1 gene promoter, while ETV1 protein stability and intracellular localization remained unchanged. In stark contrast, sumoylation-deficient ETV1 was repressed in its ability to stimulate the MMP1 promoter and to cooperate with a histone demethylase, JmjC domain-containing 2A (JMJD2A), in LNCaP prostate cancer cells. Mutation of sumoylation sites enhanced the ability of ETV1 to interact with the histone deacetylase (HDAC) 1, but had basically no impact on complex formation with HDAC3 or JMJD2A. Further, compared to non-sumoylated ETV1, its sumoylated forms were less able to bind to the transcription factor, SMAD family member 4. Lastly, in contrast to wild-type ETV1, sumoylation-deficient ETV1 repressed LNCaP cell growth. Altogether, these data suggest that sumoylation modulates ETV1 function in a cell type-specific manner, possibly by altering the spectrum of transcriptional cofactors being recruited. Notably, SUMO pathway components SUMO1, ubiquitin-like modifier activating enzyme 2 and ubiquitin conjugating enzyme 9 were upregulated in prostate tumors, implying that enhanced sumoylation indeed promotes ETV1’s oncogenic activity during prostate cancer formation.

Keywords: ETV1, posttranslational modification, prostate cancer, SUMO, transcription

Introduction

ETS variant 1 (ETV1), formerly also designated as ETS-related 81 (ER81), is a member of a transcription factor family that comprises 28 proteins in humans and is characterized by the ETS DNA-binding domain, which interacts with GGAA-containing target sequences [1, 2]. Knockout of ETV1 in mice led to defective connections between sensory and motor neurons, which caused motor discoordination and eventually death approximately one month after birth [3]. Further, ETV1 is required for rapid conduction in the heart, and overexpression of ETV1 induced atrial arrhythmias in mice and has been observed in respective human patients [4-6]. ETV1 has also been implicated as a promoter of oncogenesis. Most significantly, the ETV1 gene was found to be translocated in 5-10\% of all human prostate carcinomas, which results in overexpression of full-length ETV1, N-terminal deletions, or fusion proteins that retain most of the ETV1 amino acids, including the ETS domain [7, 8]. Mimicking this overexpression of ETV1 in transgenic mouse models led to the development of prostatic intraepithelial neoplasia, which is a precursor of prostate adenocarcinoma [9, 10]. In addition, ETV1 overexpression synergized with homozygous deletion of the PTEN tumor suppressor in the induction of adenocarcinomas in the mouse prostate [11]. ETV1 overexpression was also associated with higher Gleason score and increased disease recurrence after prostatectomy, suggesting that ETV1 especially marks aggressive prostate tumors [10-12].

Similar to prostate cancer, ETV1 has been found to be translocated to the EWS gene in a subset of Ewing tumors. This generates an EWS-ETV1 fusion protein that is endowed with the potent N-terminal activation domain of EWS.
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and the DNA-binding domain of ETV1 [13, 14]. A consequence is the constitutive activation of ETV1 target genes and potentially of other genes regulated by GGAA microsatellites that may facilitate neoplastic transformation [15]. Moreover, amplification of the ETV1 gene was reported to occur in up to 40% of all melanomas and ETV1 synergized with the V600E mutant of BRAF, which is a major driver of melanomagenesis, in the transformation of melanocytes [16]. Overexpression of ETV1 was also noted in colorectal tumors and its downregulation compromised the growth of colon cancer cells [17, 18].

A role of ETV1 in tumorigenesis has also been implicated in the breast. Overexpression of the receptor tyrosine kinase HER2/ERBB2, which is an underlying cause of breast cancer in ~20% of all cases, led to the overexpression of ETV1 in respective transgenic mice, and ETV1 is also highly expressed in several human breast cancer cell lines [19, 20]. Moreover, ETV1 is capable of stimulating the HER2/ERBB2 gene promoter, and a feed-forward mechanism of upregulation for these two proteins has even been proposed [21]. Consistently, ETV1 appears to be co-overexpressed with HER2/ERBB2 in human breast tumors [22, 23]. Further, downregulation of ETV1 caused decreased growth of human breast cancer cells in vitro and in a xenograft model [24]. Another receptor tyrosine kinase, KIT, may also elevate ETV1 activity in gastrointestinal stromal tumors and this appears to facilitate their growth [25, 26].

Biochemical analysis indicates that ETV1 is heavily modified by posttranslational modification. In particular, phosphorylation by MAP kinases, MAPKAP kinases, PKA and ATR occurs on various serine and threonine residues and can affect ETV1's transactivation potential, DNA-binding activity, and/or protein stability [27-33]. Likewise, the interaction with and acetylation by the cofactor p300 affects the function of ETV1 [34-36]. An in vitro screen for proteins that become modified with SUMO1 (small ubiquitin-like modifier 1) identified ETV1 as a potential substrate for sumoylation [37], but the lysine residues being modified and the function of this posttranslational modification have remained unexplored. Hence, we studied in this report the relevance of the covalent attachment of SUMO1 onto ETV1.

Materials and methods

Preparation of protein extracts

Human embryonic kidney 293T cells were grown in 12-wells and transiently transfected by the calcium phosphate coprecipitation method [38], 50 ng 6Myc-ER81/ETV1 construct [34] or empty vector pCS3+-6Myc were cotransfected with 50 or 500 ng HA-SUMO1 or 500 ng Flag3-SUMO1 (these SUMO1 expression vectors encode for amino acids 1-97 of human SUMO1). Total DNA amount was adjusted with pBlueScript KS+ (Stratagene) to 2.2 µg. 36 h after transfection, cells were washed once with phosphate-buffered saline and then incubated on ice with 800 µl of 40 mM HEPES (pH 7.4), 10 mM EDTA, 150 mM NaCl with and without 10 mM N-ethylmaleimide (NEM; diluted from a freshly prepared 200 mM stock solution in H2O). After centrifugation (3 min, 2500 rpm), the cell pellet was lysed for one hour on ice with 100 µl of 10 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 30 mM Na3P2O7, 1% Triton X-100 (pH 7.1) supplemented with 1 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotenin, 1 µg/ml pepstatin A, 0.5 mM Na3VO4 and with or without 10 mM NEM. The lysate was cleared by centrifugation and then subjected to SDS polyacylamide gel electrophoresis followed by anti-Myc western blotting [39]. To test sumoylation of ETV1 mutants, 300 ng HA-SUMO1 or empty vector pEV3S, 100 ng 6Myc-ER81/ETV1 constructs (wild-type and point mutants) and 1800 ng pBlueScript KS+ were employed for the transfection of 293T cells, which were lysed in the presence of 10 mM NEM as above.

Anti-SUMO1 immunoprecipitations

HeLa and SW620 cells grown in 10 cm dishes were washed once with ice-cold phosphate-buffered saline plus 10 mM NEM and then lysed as above in the presence of 10 mM NEM. One 10-cm dish was lysed in a volume of approximately 1.2 ml and subjected to immunoprecipitation with control (Santa Cruz Biotechnology sc-7966) or anti-SUMO1 (Santa Cruz Biotechnology sc-5308) mouse monoclonal antibody essentially as described before [40]. This was followed by SDS polyacrylamide gel electrophoresis and western blotting [41] with rabbit anti-ER81/ETV1 antibody #959 [33].
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**Luciferase assays**

Human 293T cells were grown in poly-L-lysine-coated 12-wells to ~25% confluency [42] and then transfected with 100 ng indicd luciferase reporter plasmid, 900 ng pBluescript KS+ as a carrier, 30 ng of empty vector pEV3S or indicated ETV1 expression plasmids, and 4 ng empty vector pQCXIP or pQCXIP-H-Ras-G12V utilizing 2 µg polyethylenimine [43]. After 8 h, cells were washed once with phosphate-buffered saline and then incubated for another 36 h in appropriate media before lysis with 300 µl of 25 mM Tris, 2 mM EDTA (pH 7.8 adjusted with H3PO4), 10% glycerol, 1% Triton X-100, 2 mM DTT [44]. Luciferase activities were then measured in a Berthold Lumat LB9507 luminometer as described [45]. In case of LNCaP cells, they were grown in poly-L-lysine-coated 6-wells to ~30% confluency and transfected with 1 µg luciferase reporter plasmid, 1 µg pBluescript KS+, indicated amounts of pEV3S or ETV1 expression plasmids, and indicated amounts of pEV3S or JMJD2A expression vector utilizing 6 µg polyethylenimine.

**RT-PCR**

293T cells were transfected in 12-wells as above utilizing the calcium phosphate coprecipitation method and RNA prepared employing 0.5 ml Trizol [46]. After isopropanol precipitation and washing with 75% ethanol, RNA was dried and dissolved in 40 µl H2O [47]. Then, RT-PCR was conducted as described [48] with MMP1 (5’-GTTCAAGACAGAAGCTCTAG-3’ and 5’-CTGCAAGTTGACCCAGCATTTAG-3’) or GAPDH (5’-GAGCCACATCGCTCAGACACC-3’ and 5’-TGACAAGCTCCGGTCTCACG-3’) primers. Resultant DNA fragments were separated on agarose gels [49] and visualized by staining with ethidium bromide [50].

**Cycloheximide treatment and cellular fractionation**

Human 293T cells grown in poly-L-lysine-coated 6-cm plates were transfected with 3.6 µg pBluescript KS+ and either 0.4 µg wild-type or 4xR ER81/ETV1 expression plasmid as above. The next day, cells were split into 12-wells and another day later treated with 100 µg/ml cycloheximide (diluted from a 100 mg/ml stock in DMSO) for 0-6 h. Thereafter, cells were lysed and boiled in Laemmli buffer and lysates subjected to SDS-PAGE and western blotting with rabbit anti-ETV1 (Abcam ab81086) and anti-actin (Sigma A2066) antibodies. Quantitation of signal intensities was done with a LICOR Odyssey imager. Similarly transfected 293T cells were fractionated with the NE-PER nuclear and cytoplasmic extraction kit (Pierce) essentially as described [51].

**Coimmunoprecipitation and GST pulldown assays**

293T cells, which were grown in poly-L-lysine-coated 6-cm plates to ~30% confluency [52], were transfected with 0.5-1 µg of 6Myc-ER81/ETV1 (wild-type or 4xR mutant), 1.5 µg Flag-JMJD2A, or 3 µg Flag-HDAC1 or Flag-HDAC3 expression vectors. Total DNA was made up to 4 µg with pBluescript KS+ and then 8 µg polyethyleneimine was employed for transfection [43]. 10 h later, cells were washed once with 3 ml phosphate-buffered saline, incubated for another 36 h, and then lysed as described [53]. Immunoprecipitations were then conducted with anti-Flag monoclonal antibody M2 (Sigma-Aldrich) and coprecipitated proteins detected by anti-Myc western blotting essentially as described [54]. For GST pulldown assays, GST and GST-SMAD4 proteins were produced in Escherichia coli and purified with the help of glutathione agarose [55]. Binding of ETV1 present in protein extracts from transfected 293T cells was then assessed in the presence of 10 mM NEM as previously described [56].

**Cell growth assay**

LNCaP cells were seeded into poly-L-lysine coated 12-wells [57] and infected thrice every 12 h with retrovirus (pQCXIH system, Clontech), which was produced in 293T cells essentially as described [58] and expressed untagged wild-type or 4xR ER81/ETV1. Then, cells were cultivated for two days without selection before seeding at a density of 3000 cells/well into 96-well plates [59]. Relative cell growth was determined with the PrestoBlue cell viability kit (Thermo Fisher) by measuring the difference of absorbance at 570 nm and 595 nm according to the manufacturer’s recommendations.

**Statistical analysis**

Data were analyzed with Prism 6 for Mac OS X (GraphPad Software, Inc.) and the significance
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level was set at $P<0.05$. Averages with standard deviations are displayed. Statistical tests being utilized are detailed in the figure legends.

Results

Sumoylation of ETV1

To analyze whether ETV1 is sumoylated, we chose human embryonic kidney 293T cells as a study object since they robustly express all SUMO pathway components. We expressed ETV1 without and with HA-tagged SUMO1 in 293T cells. Cell extracts were then prepared in the presence or absence of NEM and anti-Myc western blotting performed. Arrows point at non-sumoylated ETV1, while asterisks mark sumoylated forms of ETV1. Expression of HA-SUMO1 led to a dose-dependent appearance of higher molecular weight forms of ETV1 (marked by asterisks in Figure 1A), whose quantities were considerably reduced in the absence of NEM. Likewise, expression of SUMO1 carrying three copies of a Flag-tag resulted in higher molecular weight forms of ETV1 (Figure 1A), and these were slightly larger than the corresponding bands upon HA-SUMO1 expression, since the three Flag tags add more molecular weight onto SUMO1 than one HA tag. The fact that up to four higher molecular weight forms of ETV1 were observed upon SUMO1 expression suggested the presence of four sumoylation sites in ETV1. Please note that each sumoylation event added approximately 30-45 kDa apparent molecular weight onto ETV1, which is much more than expected from the molecular weight of ~10 kDa for the tagged SUMO1 proteins. Such a phenomenon has often been observed for sumoylation in the literature, as this large modification leads to branched polypeptide chains whose shape greatly hampers their mobility in SDS polyacrylamide gels [60].

To evaluate how efficiently ETV1 became sumoylated, we compared it to SMAD family member 4 (SMAD4), a transcription factor that can be sumoylated on two lysine residues [61-63], and the EWS-ETV1 fusion protein that is present in Ewing tumors and encompasses the C-terminal 164 ETV1 amino acids [13]. We observed that ETV1 was much more sumoylated than EWS-ETV1 or SMAD4 (Figure 1B), and only mono-sumoylation was detectable for SMAD4 despite the presence of two sumoylation sites in this protein. Next, we assessed if endogenous ETV1 would be sumoylated. Sumoylation is a highly dynamic process and the ratio of SUMO-modified to non-sumoylated endogenous protein is normally tiny [64]. Hence, we were never able to detect endogenous sumoylated ETV1 in western blots of cell extracts, which is why we resorted to immunoprecipitation to enrich for sumoylated ETV1. And indeed, when we immunoprecipitated with

![Figure 1. Sumoylation of ETV1. A. 6Myc-tagged ETV1 was coexpressed with HA- (two different amounts) or Flag-tagged SUMO1 in 293T cells. Cell extracts were prepared in the presence and absence of NEM and anti-Myc western blotting performed. Arrows point at non-sumoylated ETV1, while asterisks mark sumoylated forms of ETV1. B. 6Myc-tagged SMAD4, EWS-ETV1 and ETV1 were expressed without or with HA-SUMO1 in 293T cells and protein extracts prepared in the presence of NEM. Shown is an anti-Myc western blot. C. Sumoylation of endogenous ETV1 in HeLa and SW620 cells. Protein extracts were subjected to immunoprecipitation with indicated antibodies and the presence of ETV1 was revealed by anti-ETV1 western blotting.](image-url)
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anti-SUMO1 antibodies, we observed that ETV1 was pulled down in both human HeLa cervical carcinoma and SW620 colorectal cancer cells (Figure 1C). The size of the pulled-down molecule at ~80 kDa is consistent with monosumoylation of the ~60 kDa endogenous ETV1 (note that 6Myc-tagged ETV1 runs at ~80 kDa in Figure 1A, 1B due to its large N-terminal 6Myc tag). This suggests that under physiologic conditions, ETV1 is unlikely to be poly-sumoylated, in contrast to when SUMO1 is overexpressed as shown in Figure 1A, 1B. We then mutated K89, K228, K257 and/or K317 to arginine and determined how this would affect total sumoylation of ETV1. With the exception of the R257 mutant, mutation of individual lysine residues had barely an effect on sumoylation (Figure 2B). However, mutation of two lysine residues together always had a noticeable effect on reducing total sumoylation, most drastically in the R89/257 combination mutant that was devoid of detectable sumoylation. Of the triple mutants, only R89/228/317 and R228/257/317 were monosumoylated, while the other two triple mutants as well as the quadruple R89/228/257/317 mutant were not sumoylated (Figure 2B). These results suggest that K257 and K89 are the most important sumoylation sites, and absence of sumoylation at these two lysine residues might prevent SUMO1 modification on K228 and K317.

**Impact of sumoylated lysine residues on ETV1 transcriptional activity**

The ΨKXE consensus sumoylation motif is very similar to the synergy control motif, (I/V)KXE, where additional proline residues are present at least one side spaced by 0-3 amino acids [65, 66]. Such synergy control motifs have been demonstrated to be modified by SUMO, and the ability to repress transcription through a synergy control motif was in some cases shown to be dependent on sumoylation [66, 67]. Moreover, synergy control motifs require for transcriptional inhibition the presence of multiple DNA-binding sites that facilitate the interaction of several copies of a transcription factor at a particular gene promoter [67].

**Identification of ETV1 sumoylation sites**

Lysine residues that become sumoylated are often localized within a ΨKXE consensus motif, where Ψ is a bulky aliphatic residue, frequently leucine, isoleucine or valine, and X any amino acid [60]. Analysis of ETV1 revealed four such lysine residues at positions 89, 228, 257 and 317 (Figure 2A). Consistent with all four of these lysine residues being sumoylation sites, ETV1 amino acids 1-182 displayed only one higher molecular weight form upon SUMO1 overexpression, while amino acids 1-249 and 249-477 displayed two and amino acids 333-477 none (Figure 2A). Also, the fact that EWS-ETV1, which encompasses ETV1 amino acids 314-477, was seemingly monosumoylated (see Figure 1B) is consistent with K317 being a site for SUMO1 attachment. We then mutated K89, K228, K257 and/or K317 to arginine and determined how this would affect total sumoylation of ETV1. With the exception of the R257 mutant, mutation of individual lysine residues had barely an effect on sumoylation (Figure 2B). However, mutation of two lysine residues together always had a noticeable effect on reducing total sumoylation, most drastically in the R89/257 combination mutant that was devoid of detectable sumoylation. Of the triple mutants, only R89/228/317 and R228/257/317 were monosumoylated, while the other two triple mutants as well as the quadruple R89/228/257/317 mutant were not sumoylated (Figure 2B). These results suggest that K257 and K89 are the most important sumoylation sites, and absence of sumoylation at these two lysine residues might prevent SUMO1 modification on K228 and K317.

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**Figure 2.** Identification of sumoylation sites. A. Indicated ETV1 amino acids fused to a 6Myc-tag were coexpressed without and with SUMO1 in 293T cells. Shown are anti-Myc western blots. Arrowheads point to unmodified and asterisks at sumoylated proteins. On the left, a sketch of the human ETV1 protein depicting its ETS DNA-binding domain as well as the two acidic domains (ac) that contribute to transactivation. Lysines being part of a sumoylation consensus motif (ΨKXE) are pointed out. B. 6Myc-ETV1 that was mutated at the indicated site(s) from lysine to arginine was coexpressed with SUMO1 in 293T cells. Shown is an anti-Myc western blot.
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from K228, all other ETV1 sumoylation sites are part of such a synergy control motif, suggesting that sumoylation may repress ETV1 transcriptional activity. To test this hypothesis, we utilized the E74₃-tk80-luc luciferase reporter construct that contains three E74 sites to which ETV1 can bind [27]. When transfected into 293T cells, the E74₃-tk80-luc reporter was not affected by cotransfected wild-type ETV1 or ETV1 mutated at any single sumoylation site (lysine to arginine mutations; Figure 3A). However, three double and all four triple mutants displayed transcriptional stimulation, and the highest activation was observed with the R89/228/257/317 quadruple mutant (4xR; Figure 3A). This indicates that sumoylation represses transcription mediated by ETV1. Because ETV1 can be activated by phosphorylation triggered through the Ras oncoprotein [27], we also analyzed ETV1-dependent transcription upon cotransfection of oncogenic Ras (Figure 3B). Again, we observed that mutation of ETV1 sumoylation sites led to transcriptional activation under this condition.
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Since the transcription repression function of synergy control motifs is reportedly dependent on multimerized binding sites [67], we assessed how ETV1 sumoylation would affect transcription mediated by a single E74 site in the corresponding E74-tk80-luc luciferase reporter (Figure 4A). Unexpectedly, we observed that inhibition of sumoylation had similar stimulatory effects as with the E74-tk80-luc luciferase reporter in the absence and presence of oncogenic Ras, implying that ETV1 does not require multimerization in order to be transcriptionally inhibited by sumoylation in 293T cells. We then tested a proven target of ETV1, the matrix metalloproteinase 1 (MMP1) gene [28], and found that its promoter was also stimulated upon mutation of sumoylation sites, but only in the presence of cotransfected Ras (Figure 4B). Interestingly, another target of ETV1, the Yes-associated protein 1 (YAP1) gene promoter [68], was unaffected by mutation of ETV1 sumoylation sites (Figure 4C), implicating that posttranslational modification of ETV1 with SUMO1 has promoter-specific effects on its transcriptional activity. We extended our analysis also to the endogenous MMP1 gene in 293T cells and, as reported before [28], found activation of MMP1 gene transcription by ETV1 only upon stimulation with the receptor tyrosine kinase HER2/ERBB2 (that induces phosphorylation of ETV1 similar to oncogenic Ras); notably, mutation of all four sumoylation sites led to superactivation of MMP1 gene transcription (Figure 5A). As sumoylation may affect protein stability or intracellular localization [60, 69], we also analyzed whether this would be the case for ETV1. However, mutation of all four ETV1 sumoylation sites did not significantly affect protein stability nor ETV1 distribution between the cytoplasm and cell nucleus (Figure 5B, 5C).

Next, we assessed the role of sumoylation in LNCaP prostate cancer cells, since ETV1 especially exerts pro-tumorigenic functions in prostatic adenocarcinoma [8]. As expected from previous analyses showing MMP1 to be regulated by ETV1 in LNCaP cells [33], ETV1 overexpression greatly increased the activity of an MMP1 luciferase reporter construct (Figure 6A). Startlingly, simultaneous mutation of multiple sumoylation sites repressed rather than activated the MMP1 promoter, and the most drastic effect was observed with the 4xR quadruple mutant. A similar repression of luciferase activity was observed with the 4xR mutant at the promoter of the YAP1 gene (Figure 6B), another previously demonstrated ETV1 target gene in LNCaP cells [68]. But no change compared to wild-type ETV1 was observable for the 4xR mutant with the E74-tk80-luc luciferase reporter in LNCaP cells (Figure 6C), which is different from 293T cells described above. Moreover, the known transcriptional synergy
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Figure 5. Hyperactivation of endogenous MMP1 transcription by ETV1-4xR. A. 293T cells were transfected with wild-type or 4xR ETV1. Where indicated, oncogenic HER2/ERBB2 was additionally transfected. MMP1 mRNA levels were assessed by RT-PCR; GAPDH mRNA levels served as controls. B. 293T cells transfected with ETV1 or its 4xR mutant were treated with the protein synthesis inhibitor cycloheximide for up to 6 h and ETV1 protein levels (normalized to actin) monitored. Statistical significance was assessed by two-way ANOVA (Sidak’s multiple comparison test; n=3); n.s., not significant. C. Sumoylation does not affect intracellular distribution in transfected 293T cells. The ratio of cytoplasmic to nuclear signal for wild-type ETV1 was arbitrarily set to 1 and then compared to the 4xR mutant. Statistical significance was assessed by an unpaired, two-tailed Student’s t-test (n=3).

Figure 6. Transcriptional impact of ETV1 sumoylation in LNCaP prostate cancer cells. A. Response of the MMP1 (-525/+15) luciferase reporter upon cotransfection of 30 ng wild-type ETV1 and indicated SUMO site mutant expression vectors; pEV3S denotes the empty expression vector. Differences compared to wild-type ETV1 were evaluated by one-way ANOVA (Dunnett’s multiple comparisons test; n=4); n.s., not significant; **P<0.01; ****P<0.0001. B and C. Likewise for the YAP1 (-496/+22) and E74-3-tk80-luc reporter constructs, respectively. D. Synergy between ETV1 (15 ng expression vector) and JMJD2A (75 ng expression vector) was assessed with the MMP1 (-525/+15) luciferase reporter. One-way ANOVA (Tukey’s multiple comparisons test; n=6).
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between ETV1 and its interaction partner, the histone demethylase and epigenetic regulator JmJC domain-containing 2A (JMJD2A) [68, 70], was reduced upon mutation of the four sumoylation sites (Figure 6D). These data indicate that sumoylation exerts an activating role on ETV1-mediated transcription in LNCaP cells that is opposite to its repressive role in 293T cells.

**Sumoylation affects the ETV1 interactome**

To gain insight how sumoylation may affect the transactivation potential of ETV1, we interrogated whether this posttranslational modification would affect the binding of ETV1 with transcriptional cofactors. No substantial change in the ability to interact with JMJD2A was noted upon mutation of the four ETV1 sumoylation sites (Figure 7A, left panels). But we also tested binding of ETV1 with two other proteins, the histone deacetylase (HDAC) 1 and HDAC3, which were hitherto unknown as interaction partners of ETV1. We observed that, like JMJD2A, HDAC3 binding to ETV1 was basically unaffected upon mutation of the four sumoylation sites, whereas complex formation with HDAC1 was greatly enhanced upon mutation of all four ETV1 sumoylation sites (Figure 7A, right panels). This is opposite to what was found for the p68/DDX5 and p72/DDX17 RNA helicases, whose interaction with HDAC1 was increased upon sumoylation [71]. We also analyzed the known in vitro binding of ETV1 to another transcription factor, SMAD4 [72]. Here, we observed that compared to the input control, the sumoylated forms of ETV1 were depleted in the SMAD4-bound fraction (Figure 7B), suggesting that sumoylation prevents the binding of ETV1 to SMAD4. Altogether, these data suggest that ETV1 sumoylation can alter its affinity for other proteins.

**4xR mutant represses LNCaP cell growth**

ETV1 is required for maximal growth of LNCaP prostate cancer cells [33, 73]. Hence, we employed this cell line to assess how ETV1 sumoylation might affect the ability of ETV1 to influence cell growth. To this end, wild-type ETV1 or its 4xR mutant was retrovirally overexpressed in LNCaP cells at comparable levels (Figure 8A). Then, cell growth was examined. Overexpression of wild-type ETV1 had no sig-
signficant impact (Figure 8B), probably because endogenous levels of ETV1 are already at saturating levels in LNCaP cells. But the 4xR mutant significantly reduced cell growth. This is likely due to the fact that ectopic ETV1-4xR outcompetes endogenous ETV1 and thereby blunts the latter’s pro-oncogenic activity. These data indicate that sumoylation promotes the oncogenic activity of ETV1 in prostate cancer cells.

**Overexpression of SUMO pathway components in prostate cancer**

SUMO1 is activated through an enzymatic cascade that starts with the SUMO activating enzyme, one subunit of which is ubiquitin-like modifier activating enzyme 2 (UBA2), and continues with the ubiquitin conjugating enzyme 9 (UBC9) that mediates transfer of SUMO1 to its target proteins [60, 69]. Bioinformatics analysis of published data [74-79] with Oncomine [80] revealed that SUMO1 (Figure 9A), UBA2 (Figure 9B), and UBC9 (Figure 9C) are upregulated in prostate tumors compared to the normal prostate gland. Moreover, available data from metastasized tumors [81, 82] showed a higher expression of SUMO1, UBA2, as well as UBC9 in metastases compared to primary prostate tumors (Figure 9D-F). These data suggest that the SUMO pathway is hyperactivated in pri-
Regulation of ETV1 by SUMO1 modification

Discussion

In this study, we have made the following discoveries: (i) ETV1 can become sumoylated in cells on four lysine residues (K89, K228, K257, K317). (ii) Sumoylation of ETV1 can result into transcriptional activation (LNCaP cells) or repression (293T cells). (iii) A previously unknown interaction of ETV1 with HDAC1 seems to be suppressed by sumoylation, and similarly sumoylation reduces binding of ETV1 to SMAD4, while binding to two other proteins (HDAC3, JMJD2A) is basically unaffected by ETV1 sumoylation. (iv) In LNCaP prostate cancer cells, the quadruple SUMO1 site mutant of ETV1 acts in a dominant-negative manner and represses cell growth; this supports the notion that sumoylation enhances the growth-stimulatory effect of ETV1. (v) Components of the SUMO pathway (SUMO1, UBA2, UBC9) are upregulated in prostate tumors, which may promote sumoylation of ETV1 and thereby stimulate its oncogenic function in this cancer.

Despite the fact that SUMO1 overexpression enforced the sumoylation of more than half of the total ETV1 protein in our experiments, the degree of endogenous sumoylation seems to be much smaller. This is a common phenomenon observed with many other sumoylated proteins, but begs the question why sumoylation would then have any significant effect on a protein's function [60, 69]. One proposed idea is that modification with SUMO1 is a highly dynamic process, where sumoylation and desumoylation are rapidly occurring on the same molecule, leading to an overall low steady-state level of SUMO1 modification, but guaranteeing that a large fraction of a protein is becoming sumoylated at least for a short period of time and that this is sufficient for activity control. Another proposed idea is that sumoylation induces a conformational change in a protein that persists after desumoylation [60, 69]. Future studies should look into these and other possibilities how SUMO1 modification of ETV1 can lead to profound changes in its behavior as observed in this report.

There are two close homologs of ETV1: ETV4 and ETV5 [15, 83]. Lysine residues homologous to the four ETV1 sumoylation sites identified here have been shown to become sumoylated in ETV4 and ETV5, indicating that this post-translational modification is conserved. However, while sumoylation of ETV5 solely repressed the activity of this transcription factor, repressing and activating consequences of SUMO modification were reported in case of ETV4 [84-87]. Furthermore, the major sites of acetylation and sumoylation seemingly overlap in ETV4, thereby making these two posttranslational modifications mutually exclusive [88], but this does not hold true for ETV1, whose two acetylation sites, K33 and K116 [35], are different from its sumoylation sites. Hence, despite the fact that homologous lysine residues become sumoylated in ETV1, ETV4 and ETV5, this may have different functional consequences for these three related transcription factors.

Our data indicate that sumoylation can change the interactome of ETV1. In particular, binding of HDAC1 and SMAD4 to ETV1 seems to be suppressed by sumoylation. This may explain how sumoylation raises the transactivation potential of ETV1 in LNCaP cells, since histone deacetylation (as catalyzed by HDAC1) is normally linked to gene repression [89] and SMAD4 binding attenuates the ability of ETV1 to stimulate transcription [72]. However, HDACs have also been found at active genes, and HDAC inhibitors were able to repress transcription at some genes [90, 91], indicating that HDACs do not always act as transcriptional corepressors but also occasionally as coactivators. Such may be the case in 293T cells for HDAC1, which could explain why sumoylation apparently diminishes the ETV1 transactivation potential in this cell line. Also, HDAC3 binding was unaffected by ETV1 sumoylation. Thus, it is possible that SUMO1 modification of ETV1 leads to replacement of HDAC1 by HDAC3 as an interaction partner if their binding to ETV1 is mutually exclusive; and if HDAC3 has a higher corepressor potential than HDAC1, this may also cause a reduction in ETV1-dependent transcription in 293T cells. We do not know how sumoylation affects the binding of ETV1 to many other interactants, whose expression may be different between 293T and LNCaP cells and might thus lead to different outcomes with regard to ETV1-mediated gene regulation.

The upregulation of SUMO1, UBA2, and UBC9 in prostate tumors could lead to enhanced
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sumoylation of ETV1, yet overexpression of the SUMO-specific protease SENP1 that could facilitate desumoylation of ETV1 has also been found in prostate cancer [92]. As such, it is unclear how or whether at all the degree of ETV1 sumoylation is changed during prostate cancer formation. Regardless, we observed that blocking sumoylation on ETV1 reduced LNCaP prostate cancer cell growth, strongly suggesting that inhibiting sumoylation of ETV1 would be beneficial in prostate cancer therapy. However, the androgen receptor is also modified by SUMO and its activity was shown to be increased at selective gene promoters upon mutation of its sumoylation sites or overexpression of SENP1 [93-95]. This would suggest that generic inhibition of sumoylation may dampen ETV1's tumor-promoting activity, but stimulate the pro-oncogenic androgen receptor, and the net effect on prostate tumorigenicity would be uncertain. Further, overexpression of SENP1 reportedly induced prostatic intraepithelial neoplasia and SENP1 was needed for prostate cancer cell metastasis [92, 96], which implies that sumoylation may be beneficial in constraining prostate tumor development. Definitely, more studies are needed to investigate how the SUMO pathway is affected in prostate tumors and whether or not its modulation through SUMO pathway drugs could be translated into clinical care for prostate cancer patients [97].

ETV1 not only plays a pro-tumorigenic role in prostate, but also in other cancers [15, 83]. Given that sumoylation has opposite effects on the transactivation potential of ETV1 in 293T compared to LNCaP cells, it is far from certain that sumoylation will promote the oncogenic potential of ETV1 in non-prostatic tissues. Organ-specific, opposite functions of sumoylation may also be present in case of the androgen receptor, as a recent report suggests that sumoylation of androgen receptor promotes metastasis in endocrine therapy resistant breast cancer [98]. Hence, while our data indicate that blocking ETV1 sumoylation in prostate cancer could suppress growth, this may or may not hold true for melanoma, breast, or colorectal cancer in which ETV1 is also implicated as a tumor promoter.

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Disclosure of conflict of interest

None.

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