14-3-3 Proteins Modulate the ETS Transcription Factor ETV1 in Prostate Cancer

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Abstract
Overexpression of the ETS-related transcription factor ETV1 can initiate neoplastic transformation of the prostate. ETV1 activity is highly regulated by phosphorylation, but the underlying mechanisms are unknown. Here we report that all 14-3-3 proteins, with the exception of the tumor suppressor 14-3-3σ, can bind to ETV1 in a condition manner dictated by its prominent phosphorylation site S216. Non-σ 14-3-3 proteins synergized with ETV1 to activate transcription of its target genes MMP-1 and MMP-7, which regulate extracellular matrix in the prostate tumor microenvironment. S216 mutation or 14-3-3 downregulation was sufficient to reduce ETV1 protein levels in prostate cancer cells, indicating that non-σ 14-3-3 proteins protect ETV1 from degradation. Notably, S216 mutation also decreased ETV1-dependent migration and invasion in benign prostate cells. Downregulation of 14-3-3 reduced prostate cancer cell invasion and growth in the same manner as ETV1 attenuation. Finally, we showed that 14-3-3 proteins were overexpressed in human prostate tumors. Taken together, our results showed that non-σ 14-3-3 proteins are important modulators of ETV1 function that promote prostate tumorigenesis. Cancer Res; 73(16): 1–10. © 2013 AACR.

Introduction
ETS variant 1 (ETV1) belongs to the family of ETS transcription factors that is characterized by a winged helix-turn-helix DNA-binding motif (1). ETV1 ablation in mice resulted in limb ataxia and premature death around 1 month after birth, attesting to its crucial developmental role. Furthermore, ETV1 is implicated in tumor formation. A chromosomal translocation with the Ewing sarcoma gene causes the formation of Ewing tumors. Mostly children and adolescents are afflicted by this aggressive disease that leads to the death of nearly half of all patients with Ewing tumor (2). More recently, ETV1 amplification was observed in 40% of all melanomas and ETV1 acted as a promoter of melanoma cell growth (3). Yet the most prominent role for ETV1 has been established in prostate cancer. ETV1 activity is highly regulated by phosphorylation, but the underlying mechanisms are unknown. Here, we have identified 1 mechanism by which phosphorylation of ETV1 modulates its transcriptional activity. Multiple routes exist through which MAPKs target ETV1. First, MAPKs directly phosphorylate ETV1 (11). Second, MAPKs phosphorylate and thereby activate MAPK-activated protein kinases (MAPKAPK) such as RSK1 and MSKs, which themselves phosphorylate ETV1 (12, 13). Third, MAPKs stimulate the enzymatic activity of the coactivator p300 that binds to and acetylates ETV1 (14, 15). And fourth, MAPKs phosphorylate and activate steroid receptor coactivators, which form complexes with ETV1 and thereby stimulate ETV1-dependent gene transcription (16).

Currently, we do not understand how MAPK-induced phosphorylation of ETV1 modulates its transcriptional potential. Here, we have identified 1 mechanism by which phosphorylation of ETV1 does so through facilitating an interaction with 14-3-3 proteins. Although 7 paralogous 14-3-3 proteins exist in mammals that can regulate cell growth and survival (17, 18), their role in prostate cancer has remained largely unexplored.

Materials and Methods
Coimmunoprecipitation assays
Human embryonic kidney 293T cells [CRL-11268; obtained from American Type Culture Collection (ATCC)] were transfected with the calcium phosphate coprecipitation method (15). Two hundred nanograms pcDNA3-14-3-3 expression plasmid or empty vector pcDNA3, 2 μg 6Myc-tagged ETV1 expression plasmid or empty vector pCS3⁺-6Myc, and 7 μg pBluescript KS⁺ (Stratagene) were used for transfection. Coimmunoprecipitations were carried out as detailed in Supplementary Methods and described before (19). For coimmunoprecipitation of endogenous proteins, approximately 10⁷ LNCaP (CRL-1740; obtained from ATCC) or PC3 (CRL-1435; obtained from ATCC) cells were used.
Luciferase assays

293T cells grown in 12-wells were transfected with 0.5 μg CMV-ETV1 expression plasmid or empty vector pEV3S, 200 ng pcDNA3-14-3-3t, and/or 1 μg HER2/Neu-V664E expression plasmid. Thirty-six hours after transfection, RNA was isolated using TRIzol reagent (Invitrogen) and dissolved in 25 μL H2O, of which 0.1 μL was used in a 25 μL reaction using the AccessQuick reverse transcription PCR (RT-PCR) kit (Promega). Details about primers and PCR programs can be found in Supplementary Methods.

Chromatin immunoprecipitation assay

Four 10-cm dishes of LNCaP cells were processed for formaldehyde crosslinking and DNA shearing essentially as described (8). Cell lysates were pooled and then split into equal aliquots before adding antibodies. After immunoprecipitation, reverse-crosslinking and DNA recovery, PCR was used to amplify promoter fragments as detailed in Supplementary Methods.

Cell growth and invasion assays

Cells were seeded in 96 wells. Growth was monitored using the TACS MTT cell proliferation kit (Treviden). Whereas LNCaP cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, RWPE-1 cells were grown in keratinocyte serum-free media (GIBCO) supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL epidermal growth factor. For RWPE-1 cell invasion, 5 × 10⁴ cells were seeded onto a Matrigel invasion chamber (BD Biosciences, 8 μm pores) in keratinocyte serum-free media containing 0.1% bovine serum albumin and placed into 24-well plates containing normal growth media. After 48 hours, noninvaded cells were removed with a cotton swab and invaded cells fixed with methanol and stained with Hemacolor Stain Set (Harleco). For LNCaP cell invasion, 10⁵ cells (pre-treated with 10 μg/mL mitomycin C for 2 hours) were seeded onto rat-tail collagen I-coated Boyden chambers (BD Biosciences, 8 μm pores) in DMEM plus 0.1% serum and placed in 24-well plates containing DMEM plus 10% serum.

Immunohistochemical staining

Tissue microarrays [AccuMax A302 (IV)] containing 32 prostate tumors (2 sample spots per tumor) and corresponding 32 normal prostate tissues were deparaffinized and then stained with 14-3-3t (C-17; Santa Cruz Biotechnology sc-732) or 14-3-3e (8C3; Santa Cruz Biotechnology sc-23957) antibodies. As normal tissue was lacking in 1 (14-3-3e) or 2 (14-3-3t) cases, only 31 or 30 pairs, respectively, of tumor/normal tissues were included in the analyses. Cytoplasmic and nuclear immunohistochemical staining was graded on a scale of 0 to 3. Tumor staining was defined as the average of the staining grade of two sample spots.

Results

Identification of novel ETV1 interaction partners

To identify novel ETV1 cofactors, we fused ETV1 to two affinity tags, expressed it in 293T cells and isolated native protein complexes containing ETV1 by two consecutive affinity purifications. Subsequent mass spectrometry identified 6 different 14-3-3 proteins (β, γ, ε, η, τ, ζ) that interacted with ETV1 (Supplementary Fig. S1).

To confirm this interaction with 14-3-3 proteins, we probed their binding to ETV1 in coimmunoprecipitation experiments. While 14-3-3t, ζ, and ε communoprecipitated with ETV1, 14-3-3e did not (Fig. 1A). These results suggest that ETV1 interacts with all 14-3-3 proteins with the exception of 14-3-3ε. Also, we confirmed the ability of endogenous ETV1 to bind to endogenous 14-3-3ε in 2 different human prostate cancer cell lines, LNCaP and PC3 (Fig. 1B). Consistently, ETV1 also colocalized with 14-3-3t in LNCaP cell nuclei (Supplementary Fig. S2).

We next explored whether 14-3-3 proteins would affect transcription of the matrix metalloproteinase-1 (MMP-1) gene that is targeted by ETV1 (11). First, we analyzed the activation of an MMP-1 luciferase reporter gene in 293T cells. ETV1 strongly stimulated MMP-1 luciferase activity, which was further enhanced by approximately 3-fold upon coexpression of 14-3-3ε (Fig. 1C). Then, we studied how 14-3-3t affects endogenous MMP-1 gene transcription in 293T cells. Robust activation of endogenous MMP-1 transcription requires stimulation of ETV1 through the MAPK pathway, which can be accomplished by coexpression of the HER2/Neu receptor tyrosine kinase (11). We did not observe any activation of MMP-1 transcription in the presence of ETV1 or HER2/Neu alone, but their joint expression led to detectable MMP-1 mRNA levels (Fig. 1D). Importantly, 14-3-3t synergized with ETV1 and HER2/Neu in the activation of MMP-1 transcription; similar results were also observed in case of 14-3-3ε (see Fig. 2D). We conclude that 14-3-3t and ε stimulate ETV1-dependent gene transcription.

Apart from full-length ETV1, splice variants as well as a truncated ETV1 (ΔETV1) were found in prostate tumors. In particular ΔETV1, which lacks the first 131 amino acids due to gene translocation, is frequently overexpressed in prostate cancer (4–6). Therefore, we assessed whether ΔETV1 would also cooperate with 14-3-3 proteins. Indeed, 14-3-3t communoprecipitated with ΔETV1 (Fig. 1E) and both 14-3-3t and 14-3-3ε synergized with ΔETV1 to induce MMP-1 transcription (Fig. 1F).

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Phosphorylation-dependent interaction between ETV1 and 14-3-3 proteins

14-3-3 proteins often bind phosphoserine-containing motifs (17) and thus potentially also one or more of the seven MAPK-dependent phosphorylation sites within ETV1 (Fig. 2A). To test this, we first delineated the region within ETV1 that mediates interaction with 14-3-3. Thus, we expressed ETV1 truncations together with 14-3-3 and conducted coimmunoprecipitation experiments (Fig. 2B). The N-terminal activation domain (amino acids 1–182) did not interact with 14-3-3, nor did the C-terminal half of ETV1 (amino acids 249–477). However, amino acids 1–249 and 182–477 interacted, suggesting that amino acids 182–249 are critical for binding to 14-3-3 proteins.

ETV1 amino acids 182–249 contain 2 MAPKAPK phosphorylation sites, S191 and S216. Therefore, we mutated both S191 and S216 to alanine and noted that the corresponding ETV1-A191/216 molecule was no longer binding to 14-3-3 (Fig. 2B). Indeed, while wild-type 14-3-3 coimmunoprecipitated strongly with ETV1, the R56,60A mutant did not (Fig. 2C), further implicating that the interaction between 14-3-3 and ETV1 is mediated by phosphorylation on S216.

To further corroborate this notion, we mutated 14-3-3 at 2 conserved arginine residues (R56 and R60) that are crucial for binding of 14-3-3 proteins to phosphorylated ligands (22). Indeed, while wild-type 14-3-3 coimmunoprecipitated strongly with ETV1, the R56,60A mutant did not (Fig. 2C), further implicating that the interaction between 14-3-3 and ETV1 is mediated by phosphorylation on S216.

Phosphorylation of S216 of ETV1 was also found to be crucial for the ability of ETV1 to activate MMP-1 transcription in 293T cells. As a control, neither mutation of all 4 MAPK sites (A94/139/143/146) nor of another MAPKAPK site (A334) affected ETV1 binding to 14-3-3; please note that ETV1 is phosphorylated on S191 and S216 due to constitutive MAPKAPK activity in 293T cells (13, 14). These data indicate that 14-3-3 interacts with ETV1 via phosphorylated S216.

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observable for the A216 mutant. Altogether, these data indicate that MAPKAPK-mediated phosphorylation on S216 is required for ETV1’s ability to cooperate with 14-3-3 proteins.

Relationship between ETV1 and 14-3-3 proteins in prostate cancer cells

The most recognized pathologic role of ETV1 is in prostate cancer (2). Therefore, we assessed whether 14-3-3 proteins also cooperate with ETV1 in LNCaP prostate cancer cells. To this end, we studied the expression of ETV1 target genes by RT-PCR. Retroviral delivery of ETV1 shRNA expectedly reduced MMP-1 transcription (Fig. 3A). Importantly, retroviral overexpression of either 14-3-3e or 14-3-3t resulted in enhanced MMP-1 mRNA levels (Fig. 3A). In contrast, 14-3-3e or t had negligible impact on MMP-1 transcription in the presence of ETV1 shRNA, indicating that 14-3-3 proteins act in an ETV1-dependent manner to stimulate MMP-1 transcription. Consistently, the R56,60A mutant of 14-3-3t that does not interact with ETV1 was unable to cooperate with ETV1; it even reduced MMP-1 transcription, likely due to its ability to act as a dominant-negative molecule (22).

We also studied another target gene of ETV1, the matrix metalloproteinase-7 (MMP-7) (21). Downregulation of ETV1 reduced MMP-7 gene transcription, but overexpression of 14-3-3e or t did not significantly stimulate MMP-7 transcription (Fig. 3A). This could be due to the fact that endogenous 14-3-3 protein levels were sufficient to maximally cooperate with ETV1 in MMP-7 transcription. If so, interfering with endogenous 14-3-3 proteins should result in a reduction of MMP-7 mRNA levels. And indeed, expression of dominant-negative 14-3-3t-R56,60A suppressed MMP-7 gene transcription (Fig. 3A). On the other hand, we analyzed how downregulation of 14-3-3t would affect MMP-1 and MMP-7 transcription in LNCaP cells. Similar to downregulation of ETV1, 2 different 14-3-3t shRNAs caused decreased MMP-1 and MMP-7 mRNA levels (Fig. 3B), consistent with 14-3-3t being an important interactant of ETV1.

Next, we assessed whether 14-3-3 proteins would bind together with ETV1 to target gene promoters in chromatin immunoprecipitation (ChIP) assays. Surprisingly, 14-3-3 proteins displayed a promoter-specific behavior. While ETV1 bound to both the MMP-1 and MMP-7 promoter, only the MMP-7 promoter was occupied by 14-3-3 proteins (Fig. 3C). This implies that 14-3-3 proteins indirectly stimulate ETV1-mediated MMP-1 upregulation.

A hint how this may occur came from the analysis of ETV1 proteins levels upon 14-3-3 overexpression and downregulation.

Figure 2. Importance of S216 for the ETV1:14-3-3 interaction. A, sketch of ETV1. AD, activation domain; RD, regulatory domain; ETS, DNA-binding domain. Phosphorylation sites for MAPks (S94, T139, T143, and S146) or MAPKAPks (S191, S216, and S334) are pointed out. B, 6Myc-tagged truncations of ETV1 as well as wild-type or phosphorylation site mutants of full-length 6Myc-ETV1 were coexpressed with 14-3-3t in 293T cells. After anti-Myc immunoprecipitation, coprecipitated 14-3-3t was revealed by Western blotting. C, HA-tagged 14-3-3t, but not its R56,60A mutant, coimmunoprecipitated with 6Myc-ETV1 in 293T cells. Asterisk marks HA-14-3-3t that comigrated with an unspecific band. IgL, immunoglobulin light chain. D, RT-PCR analysis of 293T cells transfected with indicated plasmids.
When overexpressing 14-3-3 proteins in LNCaP cells, we observed that ETV1 mRNA levels were essentially unaffected, but ETV1 protein levels increased (see Fig. 3A and Supplementary Fig. S3A). Conversely, when downregulating 14-3-3, ETV1 protein but not mRNA levels decreased (Fig. 3B and Supplementary Fig. S3B). These data suggest that the ETV1 protein is stabilized through its interaction with 14-3-3 proteins.

To investigate this in more detail, we analyzed whether ETV1 protein levels change when S216 phosphorylation is reduced by blocking MAPK signaling in LNCaP cells. Suppressing ERK MAPK activation with U0126 (which blocks RSK1 and MSK activation) as well as inhibiting p38 MAPKs (which are activators of MSK) with SB202190 reduced ETV1 protein levels, whereas ETV1 mRNA levels were basically unaffected (Fig. 3D and Supplementary Fig. S3C). Conversely, overexpression of HER2/Neu or Ras-G12V, which induces the phosphorylation of ETV1 (11), increased ETV1 protein but not mRNA levels (Fig. 3E and Supplementary Fig. S3D). Finally, we compared the stability of wild-type ETV1 and its A216 mutant in LNCaP cells treated with cycloheximide that blocks de novo protein synthesis. We observed that ETV1’s stability decreased upon mutation of S216 (Fig. 3F); the calculated half-lives of wild-type and A216 ETV1 were 3.1 and 1.6 hours, respectively. Collectively, these data indicate that the interaction of phosphorylated S216 with 14-3-3 proteins is a crucial determinant of ETV1 protein stability.

**Physiologic role of the ETV1:14-3-3 interaction**

Next, we analyzed how S216 phosphorylation may affect nontransformed, benign RWPE-1 prostate epithelial cells.
this end, we overexpressed wild-type ETV1 or its A216 mutant at comparable levels (Fig. 4A). Neither wild-type nor mutated ETV1 affected the growth of RWPE-1 cells (Fig. 4B). However, ETV1 reportedly stimulates prostate cell migration and invasion (5, 7, 21). Accordingly, ETV1 overexpression led to faster wound closure and increased invasion through Matrigel (Fig. 4C and D and Supplementary Fig. S4). In contrast, the A216 mutant was less able to promote migration (Fig. 4C) or not at all invasion (Fig. 4D). These data indicate that S216 phosphorylation, and by inference the interaction with 14-3-3 proteins, is important for ETV1’s ability to stimulate cell migration and invasion.

Then, we compared the physiologic functions of 14-3-3 and ETV1 in LNCaP cells. We reasoned that if an ETV1:14-3-3 complex is relevant in these cancer cells, elimination of either component of this complex should show the same effect. And indeed, 2 different 14-3-3 shRNAs phenocopied the reduction of cell invasion upon ETV1 downregulation (Fig. 5A). Likewise, downregulation of either ETV1 or 14-3-3 with 2 different shRNAs resulted in significantly delayed cell growth (Fig. 5B). Altogether, these results strongly suggest that 14-3-3 proteins are crucial for ETV1’s function in prostate cancer cells.

**Overexpression of 14-3-3 proteins in prostate tumors**

To our knowledge, the expression of non-σ 14-3-3 proteins has not been studied in human prostate tumor samples. Therefore, we analyzed publicly available microarray data (23–25) for the expression of 14-3-3σ mRNA. As shown in Fig. 6A, 14-3-3σ mRNA was significantly overexpressed in prostate tumors compared with normal tissues in 3 different datasets. Furthermore, we stained 30 matching pairs of prostate tumors and normal tissues with 14-3-3σ antibodies (Fig. 6B). Supplementary Fig. S5 corroborates the specificity of the used 14-3-3σ antibody. We found that 14-3-3σ staining was significantly (Wilcoxon signed-rank test; \( P < 0.001 \)) enhanced both in the cytoplasm and the nucleus of prostate tumor cells (Fig. 6C). Similar results were obtained for 14-3-3ε overexpression at the mRNA and protein level (Supplementary Fig. S6). Taken together, these data suggest that many prostate tumors are characterized by overexpression of non-σ 14-3-3 proteins.

**Discussion**

In this report, we identified non-σ 14-3-3 proteins as novel interaction partners of ETV1 and ΔETV1, thereby increasing the understanding about the ETV1/ΔETV1 oncoproteins and also shedding new light on the function of non-σ 14-3-3 proteins. Interestingly, while ETV1 and ΔETV1 both stimulated migration and invasion of benign PNT2C2 prostate cells, only ETV1 was capable of inducing anchorage-independent cell growth. Moreover, ETV1 was more active than ΔETV1 when tested with a luciferase reporter gene assay in PNT2C2 cells (5, 6). However, we found that both ETV1 and ΔETV1 synergized with 14-3-3σ or 14-3-3ε in stimulating endogenous MMP-1 transcription in 293T cells, suggesting that the aforementioned
differences between ETV1 and ΔETV1 were not a reflection of different abilities to interact with 14-3-3 proteins. Phosphorylation of ETV1 is a crucial means to regulate its function (9). However, the mechanism by which MAPK-dependent phosphorylation does so has remained unresolved. Here, we show that phosphorylation on S216 facilitates the interaction of ETV1 with non-s14-3-3 proteins. S216 is phosphorylated by MAPKAPKs such as RSK1, MSK1, or MSK2 (12, 13) and both RSK1 (26) and MSK2 (Supplementary Fig. S7) are overexpressed in prostate tumors, suggesting that ETV1 phosphorylation on S216 is enhanced in prostate cancer.

Phosphorylation at S216 enhances the binding of ETV1 to 14-3-3 proteins. This interaction is cooperative with 14-3-3ε (14) in promoting MMP-1 transcription, but not with 14-3-3σ or 14-3-3δ. Mutation of S216 to alanine reduces ETV1-dependent MMP-1 transcription, while 14-3-3ε synergizes with ETV1 in inducing MMP-1 transcription, no such cooperation was observable with ETV1-A216. Second, mutation of S216 reduced the ability of ETV1 to stimulate RWPE-1 cell migration and invasion. Third, the half-life of the A216 mutant was reduced by half compared with wild-type ETV1, implicating that interaction of ETV1 with 14-3-3 proteins antagonizes ETV1 destruction. As ETV1 can be targeted by COP1 to the proteasomal pathway (30), 14-3-3 proteins may preclude COP1 or other E3 ubiquitin ligases from targeting ETV1 to the proteasome. Such a scenario is not unprecedented. For instance, 14-3-3σ interacts with the p53 tumor suppressor and prevents its ubiquitination by MDM2, thereby also increasing p53-dependent gene transcription (31). Interestingly, 14-3-3 proteins sequester many nuclear proteins upon phosphorylation to the cytoplasm (17, 18). However, ETV1 seems to be a constitutively nuclear protein in LNCaP (Supplementary Fig. S2) and other cell lines (9, 14), indicating that non-s14-3-3 proteins do not regulate ETV1 by affecting its intracellular localization.

While 14-3-3 proteins bound to the same MMP-7 promoter region as ETV1, we did not find binding of 14-3-3 proteins to the MMP-1 promoter, indicating that ETV1 does not necessarily associate with 14-3-3 proteins at gene promoters. But if so, as in
case of the MMP-7 promoter, one way how 14-3-3 proteins might facilitate gene transcription is by binding to phosphorylated S10 on histone H3, which could lead to reduced heterochromatin binding protein-1 interaction and thus enhanced gene transcription (32). Because of the fact that 14-3-3 proteins dimerize and are therefore bivalent (17), it is possible that a monomeric ETV1 molecule recruits a 14-3-3 dimer that simultaneously binds to phosphorylated S10 on histone H3. Notably, this serine residue is also phosphorylated by MSK1 and MSK2 (33), outlining another way how MSKs contribute to ETV1-dependent transcription.

Besides prostate cancer, ETV1 has oncogenic properties in melanomas where it is overexpressed in 40% of all cases (3). In part, this overexpression might be a consequence of enhanced S216 phosphorylation, as constitutive activation of the MAPK pathway by the BRAF-V600E mutation is prevalent in skin tumors. Our results also provide an explanation why the receptor tyrosine kinase KIT, which is another upstream activator of the MAPK pathway, may lead to the reported increase of ETV1 stability in gastrointestinal stromal tumors (34). Thus, ETV1 stabilization through S216 phosphorylation may contribute to the development of many different tumors.

A recent report showed that 48 signature genes, including ETV1, were upregulated through the MAPK pathway in melanoma cell lines expressing BRAF-V600E (35). However, the same report also showed that ETV1 mRNA levels were not affected by MEK inhibition in other cancer cells characterized by amplified/mutated HER2/Neu or EGFR. Likewise, inhibiting KIT with imatinib in gastrointestinal stromal tumor cells (34) or blocking the MAPK pathway with U0126 or SB202190 in LNCaP prostate cancer cells (Fig. 3D) did not affect ETV1 mRNA levels, whereas ETV1 protein levels were reduced. On the other hand, activation of the MAPK pathway upon overexpression of HER2/Neu or Ras-G12V increased ETV1 protein but not ETV1 mRNA levels in LNCaP cells (Fig. 3E); similarly, ETV1 transcription was unaffected in PC3 prostate cancer cells upon HER2/Neu or Ras-G12V overexpression (Supplementary Fig. S3E). These data suggest that MAPK-dependent ETV1 overexpression in melanomas is due to both increased ETV1 transcription and enhanced ETV1 stability caused by its interaction with non-σ 14-3-3 proteins, but only due to the latter in prostate and many other tumors.

The role of 14-3-3 proteins in cancer is still a matter of debate. On one side, 14-3-3 proteins are overexpressed, whereas ETV1 protein levels were reduced. On the other hand, activation of the MAPK pathway upon overexpression of HER2/Neu or Ras-G12V increased ETV1 protein but not ETV1 mRNA levels in LNCaP cells (Fig. 3E); similarly, ETV1 transcription was unaffected in PC3 prostate cancer cells upon HER2/Neu or Ras-G12V overexpression (Supplementary Fig. S3E). These data suggest that MAPK-dependent ETV1 overexpression in melanomas is due to both increased ETV1 transcription and enhanced ETV1 stability caused by its interaction with non-σ 14-3-3 proteins, but only due to the latter in prostate and many other tumors.

To our knowledge, the expression and importance of non-σ 14-3-3 proteins in prostate tumors has remained unexplored. Our data show that both 14-3-3α and 14-3-3ε are overexpressed in prostate tumors. Moreover, we found evidence that 14-3-3ζ, γ and β are also upregulated in prostate cancer (Supplementary Fig. S8). Furthermore, 22 of 30 prostate tumors studied in our report displayed overexpression of at least one (τ or η) of the two 14-3-3 proteins analyzed, indicating that probably the vast majority of prostate tumors overexpresses one or more of the 6 non-σ 14-3-3 protein. Thus, ETV1 overexpression will most often be accompanied by non-σ 14-3-3 overexpression,
providing these proteins an opportunity to cooperatively contribute to the causation of prostate cancer. Similar to prostate tumors, overexpression of non-ß 14-3-3 proteins may promote neoplastic transformation in other organs. For instance, 14-3-3ß overexpression was observed in HER2/Neu-positive breast tumors and correlated with reduced survival (38); given that ETV1 is overexpressed in HER2/Neu-positive breast tumors (2), this suggests a potential cooperation of 14-3-3ß with ETV1 in this cancer.

Downregulation of 14-3-3ß in LNCaP prostate cancer cells reduced their growth and invasion. In part, this may be explained by the ability of 14-3-3ß to stimulate ETV1 target genes such as MMP-1 and MMP-7. MMPs have emerged as crucial modulators of tumorigenesis and are not only involved in the remodeling of the extracellular matrix, but have a plethora of other functions, including the processing and shedding of growth factors and cytokines as well as regulating angiogenesis (39). In particular, MMP-1 is often overexpressed in prostate tumors and it promoted the ability of prostate cancer cells to migrate and invade in vitro and grow tumors and metastasize in an orthotopic mouse tumor model (40, 41). Likewise, MMP-1 is overexpressed in melanomas and correlates with reduced patient survival (42). Notably, MMP-1 overexpression conferred an invasive and metastatic phenotype on early-stage melanoma cells, whereas MMP-1 downregulation in late stage melanoma cells curtailed metastasis and angiogenesis (43, 44). Similarly, MMP-7 overexpression is often found in prostate and skin cancer (45–47) and may promote tumorigenesis by enhancing invasion, metastasis, and cell proliferation (48–50).

In conclusion, non-ß 14-3-3 proteins may cooperate with ETV1 not only in prostate tumors, but also in various other neoplasias including melanomas and HER2/Neu-positive breast tumors. Accordingly, obstructing the interaction of ETV1 with non-ß 14-3-3 proteins may be a worthwhile adjuvant therapy in these cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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