

Defining the molecular action of HDAC inhibitors and synergism with androgen deprivation in ERG-positive prostate cancer

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Gene fusions between prostate-specific, androgen responsive *TMPRSS2* gene and oncogenic ETS factors, such as *ERG*, occur in up to 50% of all prostate cancers. We recently defined a gene signature that was characteristic to prostate cancers with *ERG* activation. This suggested epigenetic reprogramming, such as upregulation of histone deacetylase 1 (*HDAC1*) gene and downregulation of its target genes. We then hypothesized that patients with *ERG*-positive prostate cancers may benefit from epigenetic therapy such as HDAC inhibition (HDACi), especially in combination with antiandrogens. Here, we exposed *ERG*-positive prostate cancer cell lines to HDAC inhibitors Trichostatin A (TSA), MS-275 and suberoylanilide hydroxamic acid (SAHA) with or without androgen deprivation. We explored the effects on cell phenotype, gene expression as well as ERG and androgen receptor (AR) signaling. When compared with 5 other prostate cell lines, *ERG*-positive VCaP and DuCaP cells were extremely sensitive to HDACi, in particular TSA, showing synergy with concomitant androgen deprivation increasing apoptosis. Both of the HDAC inhibitors studied caused repression of the *ERG*-fusion gene, whereas the pan-HDAC inhibitor TSA prominently repressed the *ERG*-associated gene signature. Additionally, HDACi and flutamide caused retention of AR in the cytoplasm, indicating blockage of androgen signaling. Our results support the hypothesis that HDACi, especially in combination with androgen deprivation, is effective against *TMPRSS2-ERG*-fusion positive prostate cancer *in vitro*. Together with our previous *in vivo* observations of an “epigenetic reprogramming gene signature” in clinical *ERG*-positive prostate cancers, these studies provide mechanistic insights to *ERG*-associated tumorigenesis and suggest therapeutic paradigms to be tested *in vivo*.

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Key words: ERG; HDAC inhibitors; prostate cancer; AR

Our understanding of the molecular pathogenesis of prostate cancer was recently reshaped by Chinnaiyan and coworkers by their finding of recurrent oncogenic fusions between an androgen receptor- (AR) regulated transmembrane protease (*TMPRSS2*) and various ETS-transcription factors.¹ The most frequent of these ETS fusion partners is the v-ets erythroblastosis virus E26 oncogene homolog (*ERG*), followed by the related ETS-proteins *ETV1*, *ETV4* and *ETV5*. Since this discovery, evidence on the causative oncogenic role of ETS-fusion genes in prostate cancer has started to accumulate.^{2–5} *ERG*, together with other ETS factors, is also a well-known translocation partner in nonepithelial cancers such as Ewing’s sarcoma and acute myeloid leukemia.⁶ We recently defined a gene signature that correlated with *ERG*-fusion gene expression in prostate cancers.⁷ This *ERG*-related gene set may reflect the downstream effects of ectopic *ERG* oncogene expression, and could therefore contribute prostate cancer initiation and/or progression. Interestingly, we observed evidence for epigenetic reprogramming in *ERG*-positive tumors, including upregulation of the histone deacetylase 1 (*HDAC1*) gene and downregulation of its targets. We then hypothesized that patients with *ERG*-positive prostate cancers may benefit from epigenetic therapy such as HDAC inhibitor treatment, especially in combination with androgen deprivation. As the *ERG*-fusion gene is a transcriptional activator, its direct targeting by small molecule compounds may be challenging. Therefore, identification of surrogate and parallel drug treatment options targeting this molecularly and etiologically defined prostate cancer group would be very important.

Here, we exposed 2 normal prostate epithelial cell lines, 2 *ERG*-fusion gene positive and 3 fusion gene negative prostate cancer cell lines to HDAC inhibitors and androgen deprivation and studied their effect on growth response and induction of apoptosis. MS-275, an HDAC1 specific inhibitor (EC₅₀ = 181 nM) currently in phase I/II clinical trials, and the nonspecific pan-HDAC inhibitor Trichostatin A (TSA)^{8,9} were used. We explored the effects of both HDAC inhibition (HDACi) on mRNA gene expression profiles and AR activity in the *ERG*-positive prostate cancer cell line VCaP and the *ERG*-negative cell line LNCaP. In our article, we show that both androgen deprivation and HDACi repressed *TMPRSS2-ERG*-fusion gene significantly in VCaP cells. Furthermore, HDAC inhibitor treatment *in vitro* reversed the gene signature linked to the oncogenic *ERG* expression seen *in vivo* in prostate cancers. Additionally, we showed that *TMPRSS2-ERG* expressing prostate cancer cells, especially androgen deprived, were highly sensitive to HDAC inhibitors including SAHA, which is already clinically approved. This supports the hypothesis that patients with this genetic alteration would benefit from HDAC inhibitor treatment in combination with the traditional androgen deprivation therapy.

Material and methods

Prostate cancer cell lines

The prostate cell lines RWPE-1, LNCaP, PC-3 and DU-145 were purchased from ATCC (LGC Promochem AB, Borås, Sweden) and grown in the media recommended by the distributor. The prostate carcinoma cell lines VCaP and DuCaP were received from Adrie van Bokhoven (University Medical Center Nijmegen, Netherlands). All tumor cell lines were grown in the RPMI-1640 medium. EP156T cells were received from Varda Rotter (Weizmann Institute, Israel) and grown in modified MCDB-153 medium (Biological Industries, Kibbutz Beit Haemek, Israel). All the media were supplemented either with 1% or 10% FCS, L-glutamine and penicillin/streptomycin (all reagents Sigma, Munich, Germany). Androgen-ablated cells were grown in 10% charcoal-stripped FCS or in the presence of AR antagonists bicalutamide (Sequoia Research Products, Pangbourne, UK) or flutamide (Sigma).

Additional Supporting Information may be found in the online version of this article.

Matthias Nees and Olli P. Kallioniemi contributed equally to this work.

Abbreviations: AA, androgen ablation; AR, androgen receptor; *ERG*, the v-ets erythroblastosis virus E26 oncogene homolog; HDAC, histone deacetylase; HDACi, HDAC inhibition; SAHA, suberoylanilide hydroxamic acid; *TMPRSS2*, transmembrane protease, serine 2; TSA, trichostatin A.

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EC₅₀ determination, cell proliferation, synergy determination and apoptosis assays

EC₅₀ assays were performed on 384-well plates with 4,000 cells per well plated in their respective growth media. Cells were briefly spun (1,000g, 1 min) and left to attach overnight. A 10-fold dilution series (100 pM–10 μM) of HDAC inhibitors was added to the cells, and the plates were incubated for 48 hr. Growth response and caspase-3 and -7 assays to measure the combined effects of HDACi and flutamide were performed on 384-well plates, in which 2,000–4,000 cells per well were plated depending on the cell line. Cells were left to attach overnight and 10 μM flutamide was added for 24 hr. The following day, medium containing 15 nM TSA and 600 nM MS-275 was added for 48 hr. Cell viability and caspase-3 and -7 activity were measured by homogenous, fluorometric CellTiter-Blue or Apo-One assays (Promega, Madison, WI), according to the manufacturer's instructions. The Envision Multilabel Plate Reader (Perkin-Elmer, Massachusetts, MA) was used for signal quantification. The EC₅₀ data were analyzed with GraphPadPrism 4 software (GraphPad Software, San Diego, CA). The mean values of at least 3 independent measurements were used, and Student's *t*-test was applied for calculating the significance of the changes.

The nature of interaction and the degree of synergy between flutamide and the HDACis were analyzed using the combination index (CI) method of Chou and Talalay.¹⁰ The concentration dependence of antiproliferative effects was determined for each compound, either alone or in combination. Synergy was then analyzed according to the fixed concentration method (*i.e.*, fixed concentration of flutamide together with increasing concentrations of HDACi), thus providing a measure of the enhancement of HDACi activity at various levels of diminished AR-related signaling. The data were analyzed with Calcsyn software (Biosoft, Cambridge, UK), and the CI was calculated from the median effect plots according to equation $CI = (D1)/(D \times 1) + (D2)/(D \times 2)$, where (D×)1 and (D×)2 are the concentrations of compounds D1 and D2 needed to produce a given level of antiproliferative effect when used alone, whereas (D)1 and (D)2 are their concentrations that produce the same effect when used in combination. A CI value of 0.9–1.1 indicates additive interaction, values below 0.9 indicate synergism and values over 1.1 indicate antagonism.

The induction of cell death in VCaP cell line response to 10 μM flutamide (Sigma) and 30 nM TSA, 50 nM MS-275 or 250 nM suberoylanilide hydroxamic acid (SAHA, vorinostat) treatments was confirmed with propidium iodide (PI) staining, followed by flow cytometry analysis using the BD FACSAry Bioanalyzer (Becton & Dickinson, Franklin Lakes, NJ). VCaP cells were grown on 6-well plates and were pretreated with flutamide for 72 hr. HDACi was added for the last 16 hr, samples were collected, stained and analyzed according to the manufacturer's instructions.

Gene expression analysis by beadarrays

Early passage VCaP cells were grown to ~70% confluence prior to treatments with 300 nM TSA (Sigma) or 500 nM MS-275 (Sigma) for 1, 6 and 12 hr. The cells were androgen ablated with 10% charcoal-stripped FCS for 72 hr. In combination treatment, 300 nM TSA (Sigma) or 500 nM MS-275 (Sigma) were added to ablated cells for 6 and 12 hr before harvesting. Total RNA was extracted using Trizol reagent (Invitrogen). Integrity of the RNA prior to hybridization was monitored using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Five hundred nanograms of purified total RNA was amplified with the TotalPrep Kit (Ambion, Austin, TX), and the biotin-labeled cRNA was hybridized to Sentrix HumanRef-8 Expression BeadChips (Illumina, San Diego, CA). The arrays were scanned with the BeadArray Reader (Illumina).

Statistical analysis

The raw data were quantile-normalized and analyzed with the R/Bioconductor software.¹¹ Differentially expressed genes from >3 independent microarray hybridizations were identified using

the significance analysis of microarrays program (SAM),¹² with a false discovery rate set to 0 and a minimum fold change of ≥2-fold. To define the genes altered exclusively by cotreatment of androgen ablation (AA) and HDACi, we used multiclass SAM.¹² Finally, the functional gene ontology (GO) and pathway annotations were analyzed for the sets of differentially expressed genes by EASE score (<http://niaid.abcc.ncifcrf.gov>).¹³ Gene set enrichment analysis (GSEA) was performed for the HDAC inhibitor treatments and AA data with the GSEA software v2.0.1 and MSigDB gene set database¹⁴ (<http://www.broad.mit.edu/gsea/>). Our previously defined *ERG* gene signature gene set was included to the analysis.⁷ Heatmaps and hierarchical clustering (Euclidean distance, complete linkage) were performed with the TIGR Multiexperiment Viewer 4.0 (MeV 4.0) software (<http://www.tm4.org/mev.html>).

Real-time qPCR

TaqMan gene expression probes and primers from the Universal Probe Library (Roche Diagnostics, Espoo, Finland) were used for validation of *TMPRSS2-ERG*-fusion gene and *AR* expression. Real-time quantitative PCR was done using ABI Prism 7900 (Applied Biosystems, Foster City, CA). Quantitation was carried out using the $\Delta\Delta$ CT method with RQ manager 1.2 software (Applied Biosystems). Average expression of the untreated control samples was considered for the calculation of the fold changes. mRNA expression of 2–4 replicate samples was studied.

Western blot analysis and subcellular proteome extraction

For protein extraction and Western blot analysis, cells were plated at 70% confluence and left to attach over night. VCaP cells were first pretreated with 10 μM bicalutamide or 10 μM flutamide for 48 hr. Cell culture medium containing 250 nM or 2.5 μM SAHA, 50 or 500 nM MS-275, 1 nM dihydrotestosterone (Sigma) or vehicle (0.1% DMSO) was then added, and the cells were incubated for the additional 24 hr. Subcellular protein extraction was performed with the Compartment Protein Extraction Kit (Chemicon, Billerica, MA). The cytoplasmic and nuclear protein fractions were analyzed. Protein concentrations were determined using a DC Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Ten micrograms of total protein was taken up in Laemmli buffer containing 3% of β-mercaptoethanol, denatured for 5 min at 95°C, separated on a 7% SDS-polyacrylamide gel and transferred to a Protran nitrocellulose transfer membrane (Schleicher & Schuell, Niedersachsen, Germany). We used antibodies against AR (1:1,000 dilution, mouse monoclonal, Labvision, Fremont, CA), β-Actin (1:5,000 dilution, mouse-monoclonal, Becton Dickinson, Franklin Lakes, NJ) and Histone H3 (1:5,000 dilution, rabbit polyclonal, Abcam, Cambridge, UK). Signal was detected with a 1:5,000 dilution of Alexa Fluor-680 and -800 goat anti-mouse and Alexa Fluor-680 and -800 conjugated goat anti-rabbit secondary antibodies (all from Invitrogen Molecular Probes, Carlsbad, CA) and scanned with an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, IN). Equal loading and purity of the cytoplasmic and nuclear fractions were confirmed with β-Actin and Histone H3 antibodies. Only little nuclear Histone H3 contamination was detected for some of the cytoplasmic fraction samples, whereas nuclear fractions did not show any detectable β-Actin staining.

Results

ERG-positive cell prostate cancer cells are highly sensitive to HDACi growth suppression and this sensitivity increases upon AA

To evaluate the impact of the suggested epigenetic deregulation in the *ERG*-positive prostate cancers,⁷ we determined the EC₅₀ values for TSA and MS-275 after 48 hr exposure in a panel of prostate cell lines (Table I). The *ERG*-fusion gene positive prostate cancer cell lines VCaP and DuCaP were >50-fold more sensitive to TSA (EC₅₀ = 13 and 7.9 nM, respectively) and >10-fold more sensitive to MS-275 (EC₅₀ = 620 and 580 nM, respectively)

compared with the *ETS* fusion gene negative PC3 and DU145 cells lines (TSA: DU-145 EC_{50} = 670 nM, PC-3 EC_{50} = 1.6 μ M; MS-275 DU-145 EC_{50} = 8.7 μ M, PC-3 EC_{50} = 6.2 μ M) (Table I). LNCaP cells, which contain the *ETV1* fusion gene,⁵ were sensitive to moderate concentrations of HDAC inhibitors, displaying >10-fold and 3.5-fold lower sensitivity to TSA (EC_{50} = 140 nM) and MS-275 (EC_{50} = 2.2 μ M) treatment compared with VCaP cells. Interestingly, normal prostate epithelial cells EP156T and RWPE-1 were unresponsive to both HDAC inhibitors (Table I).

We then studied whether androgen deprivation would further enhance the HDACi effects on cell proliferation and apoptosis in a panel of prostate cancer cell lines. VCaP, DuCaP, LNCaP, DU-

145 and PC3 cells were androgen deprived using 10 μ M AR antagonist flutamide for 24 hr prior to subsequent 15 nM TSA and 600 nM MS-275 for the last 48 hr. These were the EC_{50} concentrations determined to VCaP cell line. The given concentrations of TSA and MS-275 reduced cell proliferation only in the *ERG*-positive VCaP and DuCaP cells and showed significant synergism with flutamide (Figs. 1a and 1b). The effects of flutamide and HDACi were determined to be synergistic by the CI method in VCaP (TSA CI = 0.81, MS-275 CI = 0.47) and DuCaP cells (TSA CI = 0.95, MS-275 CI = 0.21). The potentiation of TSA by flutamide was moderate compared with strong synergism between MS-275 and flutamide. This may point to the fact that the mechanism of action of the different HDAC inhibitors is strikingly different and not all of them might be effective in combination with ablation.

The observed growth inhibition and synergy was confirmed to be partially mediated through apoptosis in both VCaP and DuCaP cells. Caspase-3 and -7 activities increased by 30% in response to both TSA and MS-275 in combination with flutamide in VCaPs cells and over 20% in response to MS-275 and flutamide in DuCaP cells when compared with cells treated with HDACi alone (Figs. 1c and 1d). Additionally, the number of apoptotic cells increased by 2- to 3-fold in response to cotreatment of flutamide and MS-275, TSA or a 3rd well-established HDAC inhibitor SAHA, as measured by PI staining followed by flow cytometer analysis (FACS) in VCaP cells (Fig. 2). The synergy in growth inhibition and apoptosis induction in response to flutamide and HDACis was confirmed to be related to functional suppression of AR by studying the combined effects of AA by charcoal-stripped serum and TSA or MS-275 in VCaP cell line (Supp. Info. Fig. 1).

TABLE I – EC_{50} VALUES FOR HDAC INHIBITORS TSA AND MS-275 IN VARIOUS PROSTATE CELL LINES

Cell line	<i>ERG</i> status	TSA		MS-275	
		EC_{50} (nM)	Ratio to VCaP	EC_{50} (nM)	Ratio to VCaP
VCaP	+	13 ± 2	1	620 ± 95	1
DuCaP	+	7.9 ± 0.6	0.61	580 ± 61	0.95
LNCaP	–	140	10.8	2,200 ± 220	3.5
PC3	–	1,630	125	6,230	10.0
DU145	–	670	52	8,780	14.1
RWPE1	–	>10,000	>770	>10,000	>16
EP156T	–	>10,000	>770	>10,000	>16

ERG-negative LNCaP, PC3 and DU145 and *ERG*-positive VCaP and DuCaP prostate cancer cell lines were treated with increasing concentrations from 10 pM to 10 μ M of HDAC inhibitors TSA and MS-275 for 48 hr. Cell viability was assayed and the EC_{50} values were determined for each cell line. Each concentration of HDACi involved 6 replicates and the results represent repeated experiments.

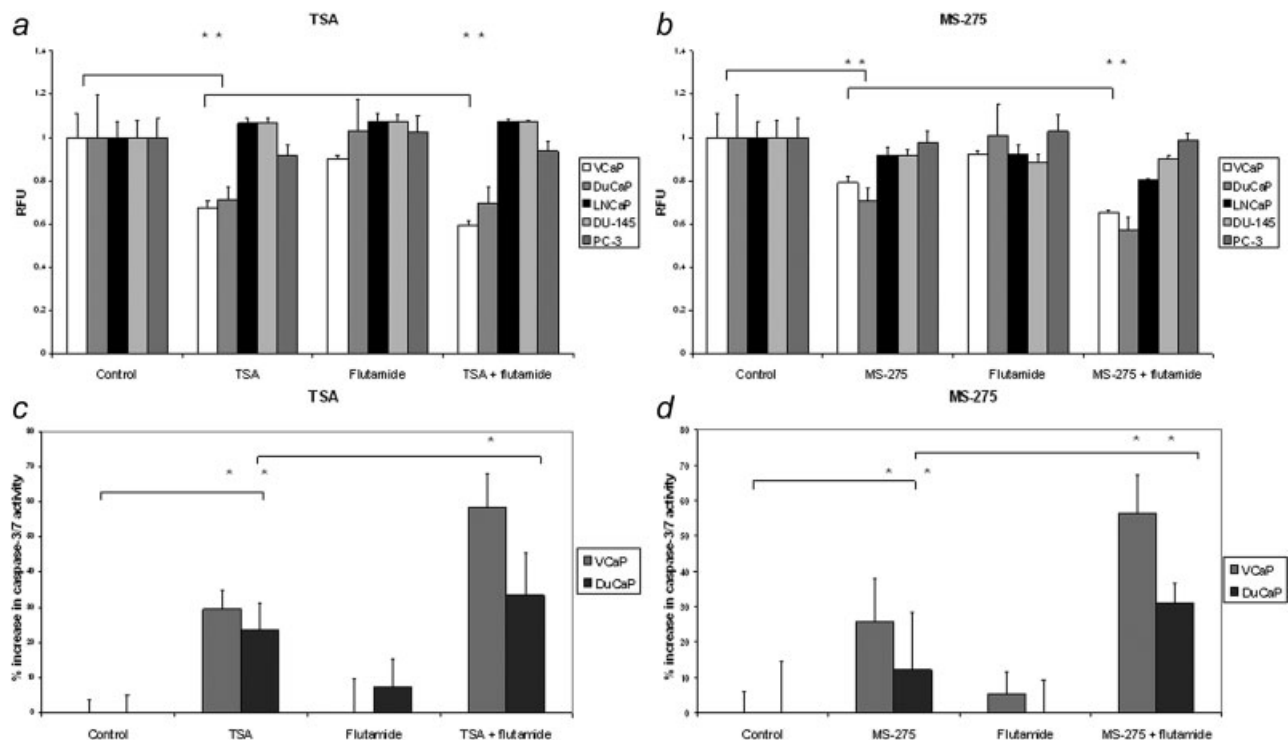


FIGURE 1 – HDACi-mediated growth arrest is enhanced by androgen deprivation and mediated through apoptosis in *ERG*-positive prostate cancer cells. The cell proliferation was studied with CellTiterBlue assay (Promega) in response to TSA (a) and MS-275 (b) with and without flutamide in a panel of prostate cancer cell lines. The prostate cancer cells were treated with 10 μ M flutamide for 72 hr followed by 15 nM TSA or 600 nM MS-275 for the last 48 hr. The signal was normalized to untreated controls. The caspase-3 and -7 activity was measured in *ERG*-positive VCaP and DuCaP cell lines in response to TSA (c) and MS-275 (d). The significance of proliferation reduction and increase in caspase activity in response to various treatments was analyzed by *t*-test (* p < 0.003). Each bar represents the median of >6 replicate measurements, and the error bars represent standard deviation.

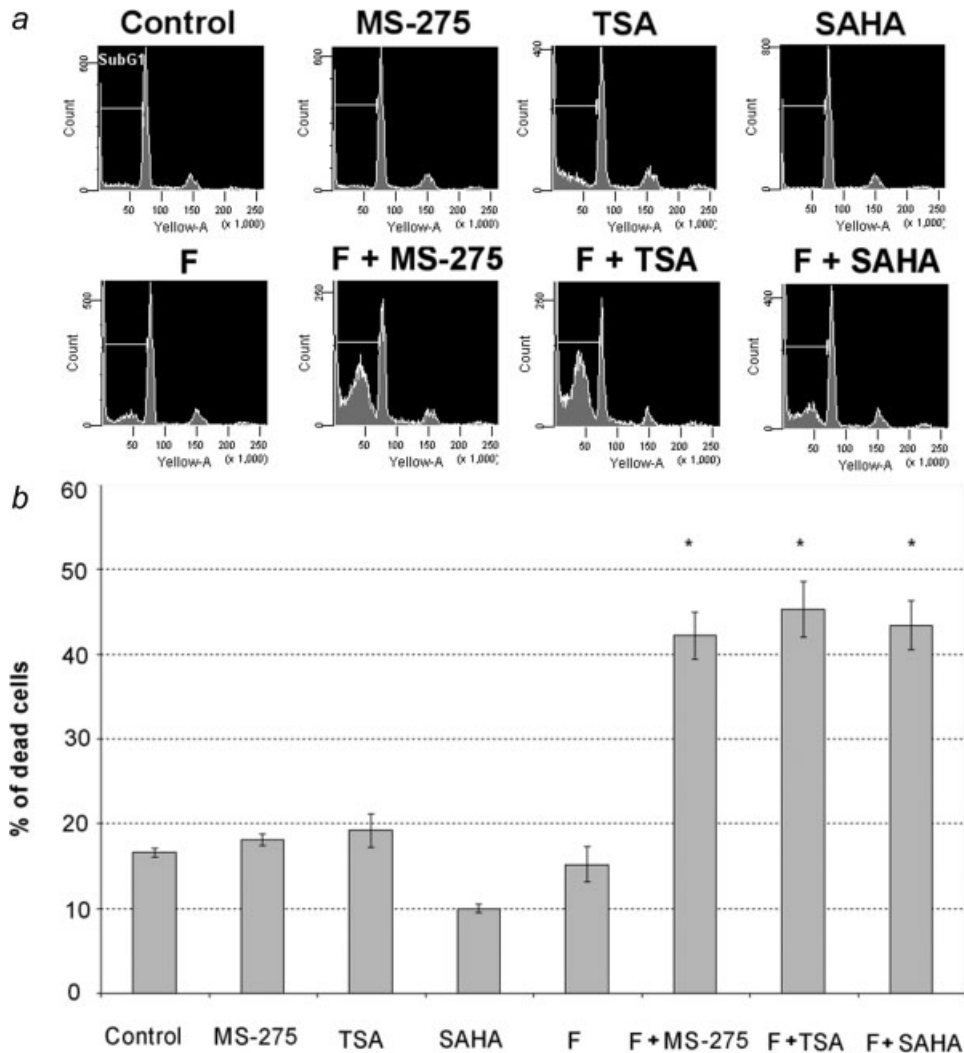


FIGURE 2 – The effect of HDAC inhibitors and AR antagonists increase the amount of apoptotic cells in the VCaP prostate cancer cells. VCaP cells were treated with 50 nM MS-275, 30 nM TSA or 250 nM SAHA for 16 hr. AR was inhibited by 10 μ M flutamide (F) for 72 hr. In combination treatments, the cells were treated with 10 μ M flutamide for 72 hr before adding HDAC inhibitors for the last 16 hr. Cell death was measured by propidium iodide staining and flow cytometry analysis. (a) The amount of dead cells was determined as a sub-G1 portion (white vertical line) from the total cell pool, as indicated in the DNA histograms. (b) Comparison of the subG1 dead cells populations in response to various treatments applied. The figure represents the mean of 3 independent experiments. The significance of change in the sub-G1 population in response to various treatments was analyzed by *t*-test (* $p < 0.05$). The error bars represent SEM.

Mechanism of HDACi effects: Silencing AR and ERG-regulated gene profiles

To gain deeper mechanistic insights into therapeutic potential of HDACi and AR suppression in ERG-positive prostate cancers, we studied gene expression patterns in response to TSA, MS-275 and androgen deprivation by charcoal-stripped serum for both individually and in combination with VCaP prostate cancer cells. Gene expression profiles induced by HDAC inhibitors in the ERG-positive VCaP cells were compared with those seen in the ERG-negative prostate cancer cell line LNCaP. Differentially expressed genes in response to each condition were analyzed by the SAM algorithm.¹² TSA and MS-275 caused differential expression (≥ 2 -fold alteration) of 6% ($n = 1,458$) and 5% ($n = 1,136$) of the genes studied in the VCaP cells. The overlap between the genes changed by both HDACi was roughly 25% ($n = 304$). AA alone altered the expression of only 2% ($n = 400$) of the genes. Combining TSA or MS-275 with AA altered the expression of 8% ($n = 1,862$) or 9% ($n = 2,002$) of the genes. Four hundred and

sixteen genes were changed only by the combination of TSA or MS-275 and AA in VCaP cells. The altered gene expression patterns were strikingly different between TSA and MS-275, pointing to partially different mechanism of action between these compounds.

The overlap in gene expression alterations induced by HDACi, AA and AA followed by HDACi (AA + HDACi) in VCaP cells was illustrated by Venn diagram (Fig. 3a). The functional GO and pathway annotations associated to the overlapping genes were studied by EASE score.¹³ Nineteen genes overlapping between HDACi and AA were linked to the annotation “calcium signaling pathway” ($p = 0.03$). Fifty-six genes were overlapping between HDACi and AA + HDACi and were linked to GO annotation “regulation through progressive cell cycle” ($p = 0.04$). Sixteen genes altered by AA + HDACi were also affected by AA alone and linked primarily to “M phase of mitotic cell cycle” ($p = 3.1E-8$). These results indicate that both androgen deprivation and HDACi interfere in particular with cell cycle control and may tar-

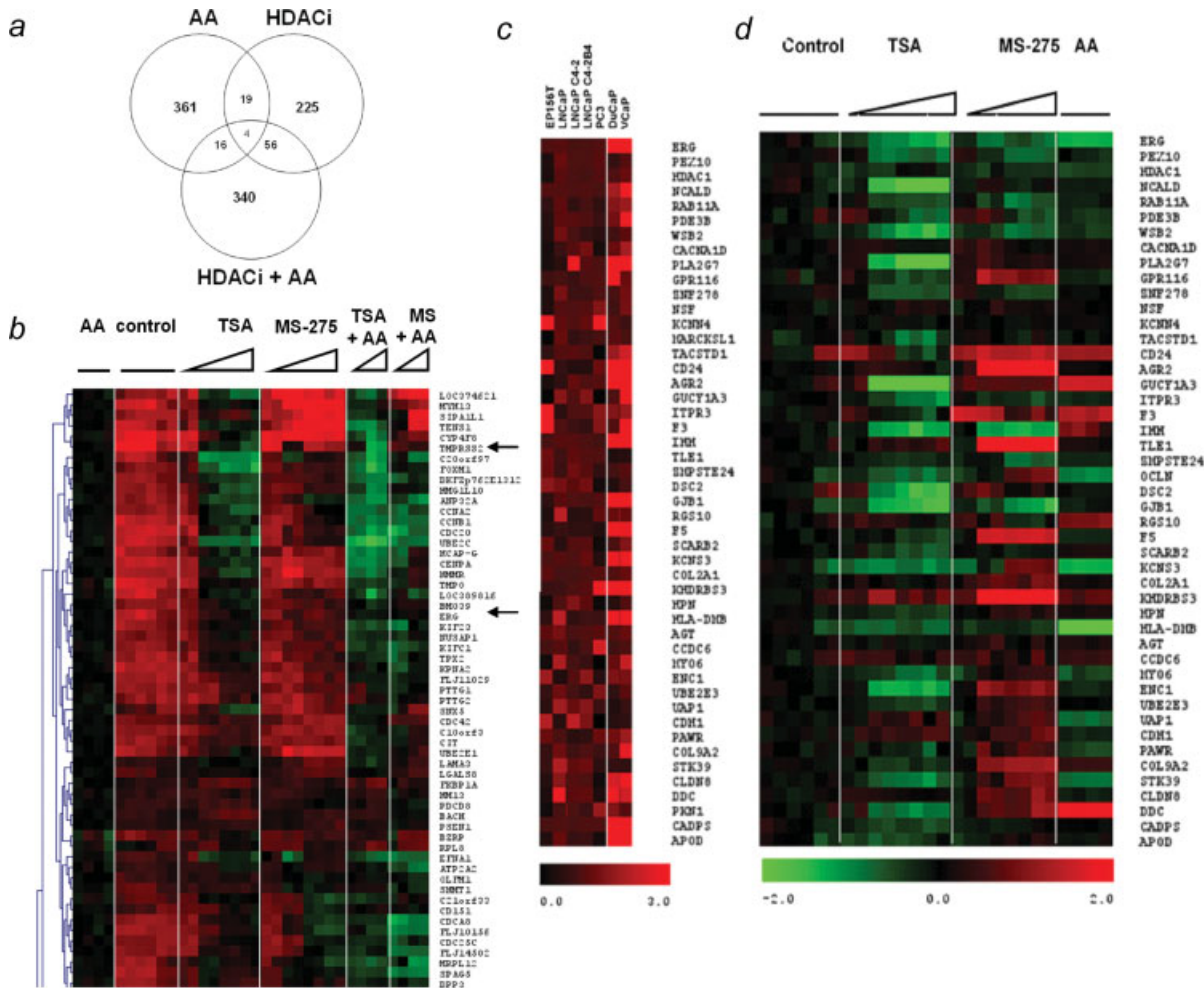


FIGURE 3 – HDACi and androgen deprivation-induced gene expression changes in *ERG*-positive cell lines. (a) Venn diagram, summarizing the ≥ 2 -fold gene expression changes induced by both TSA and MS-275 (HDACi), 72 hr AA by charcoal-stripped serum (AA) and both treatments in combination (HDACi + AA) in VCaP cell line. (b) Heatmap, illustrating the effects of TSA, MS-275 and AA alone or in combination for AR responsive genes. The gene expression data was normalized to the median expression level in androgen-ablated VCaP cells. The triangles represent the length of exposure to HDACi and AA (1–12 hr for HDACi or 6–12 hr for AA). (c) The presence of the previously defined *ERG* gene expression signature was studied in a collection of gene expression data including a panel of *ERG*-positive (VCaP, DuCaP) and *ERG*-negative (EP156T, LNCaP, LNCaP C4-2, LNCaP C4-2B4, PC3) prostate cell lines. The Affymetrix log₂ transformed gene expression data were median centered. The signature was confirmed to be significantly enriched ($p < 0.01$) by binomial distribution exclusively in the *ERG*-positive prostate cancer cell lines. (d) The effects of TSA, MS-275 and androgen ablation (AA) were studied on the *ERG* signature expression in VCaP cell line. The median of untreated samples was set to 1. TSA reversed expression of the *ERG* signature significantly, as confirmed by GSEA (nominal $p < 0.001$; enrichment score, ≥ -0.5). MS-275 and androgen ablation also affected the expression of the *ERG* signature genes, but these effects did not reach statistical significance.

get some similar mechanisms. The lists of the overlapping genes are presented in Supporting Information Table I.

The translocation between *TMPRSS2* and *ERG* genes puts the oncogenic transcription factor *ERG* under the control of AR in prostate cancer.¹ To elucidate the role of the HDACi and androgen deprivation in *ERG*-positive cancers, we studied the expression of the most androgen responsive genes in the VCaP cell line (Supp. Info. Table II). The data for these genes in all of the different conditions were studied by hierarchical clustering. We found that most of the genes suppressed via androgen deprivation, including *ERG* and *TMPRSS2*, were prominently suppressed by TSA, but much less effectively by MS-275 (Fig. 3b). The suppression by TSA was further enhanced by AA. This synergistic effect was much more dramatic in the case of MS-275, which alone had only little effect on AR-regulated genes. However, treating androgen-ablated cells with MS-275 led to much more pronounced downregulation of AR-regulated genes including the oncogenic *ERG*-fusion gene than either of the treatments alone (Fig. 3b). This downregulation of androgen-

mediated gene expression profiles most likely explains the very strong synergy observed between MS-275 and flutamide.

The ERG gene expression signature is repressed by HDACi

We further studied the possible downstream effects of *ERG* suppression in cultured VCaP prostate cancer cells by exploring the effects of HDACi on the *ERG* signature genes.⁷ The key genes of the *in vivo* *ERG* signature in clinical prostate cancers were verified to be significantly expressed only in the *ERG*-positive cell lines VCaP and DuCaP when compared with the *ERG*-negative EP156T, LNCaP, LNCaP C4-2 and PC-3 prostate cell lines (Fig. 3c). Expression of these *ERG*-associated genes was significantly (nominal $p < 0.001$; enrichment score = -0.75) repressed by TSA in the VCaP cell line, as confirmed by GSEA¹³ (Fig. 3d). The MS-275 treatment induced the expression of a subset of the *ERG* signature genes (nominal $p = 0.02$; enrichment score = 0.55). As expected, androgen deprivation also suppressed the

TABLE II – THE COMPARISON OF THE GENE SETS LINKED TO *ERG* OVEREXPRESSION IN CLINICAL TUMORS WITH THE GENES ALTERED BY THE HDACI AND ANDROGEN ABLATION *IN VITRO* IN VCaP CELLS

<i>In vivo</i> (Ijijn <i>et al.</i>) ¹	TSA	MS-275	Androgen ablation
Upregulated WNT pathway (8/22) ¹			Downregulated WNT pathway (11/27)
PITX2 Pathway (6/15)			Downregulated Upregulated by WNT in mammary epithelial cells (13/48)
Downregulated CCR5 Pathway (9/16)	Upregulated CCR5 (3/18)	Upregulated PITX2 (9/16)	
TNF and FAS network (8/16)	TNF alpha (3/10)	Upregulated CCR5 (4/18)	
		TNF alpha (3/13)	

¹Numbers in parentheses: number of genes showing core enrichment/total number of genes in the gene set.

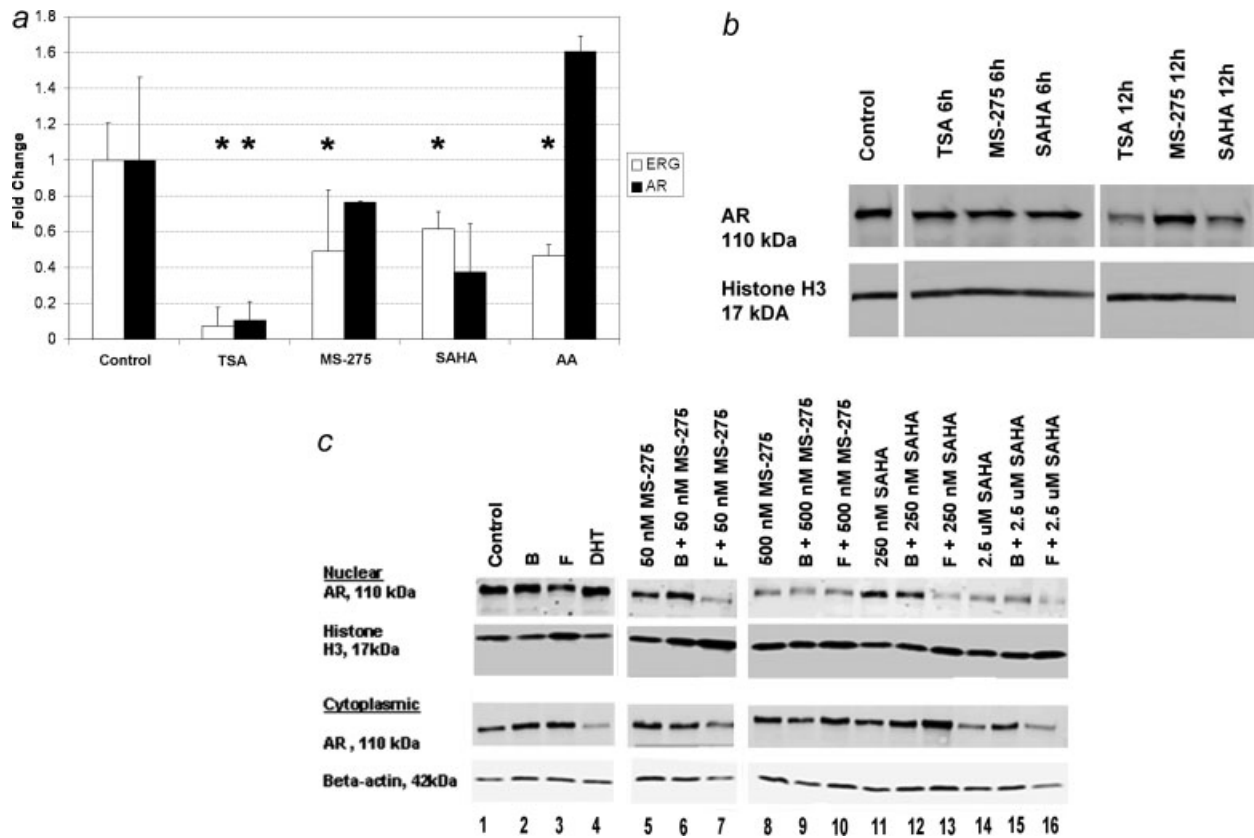


FIGURE 4 – Effects of HDACi to *TPRSS2-ERG* and AR expression and nuclear localization in *ERG*-positive prostate cancer cell line VCaP. (a) Prior to the HDACi treatments, VCaP cells were androgen ablated for 72 hr by using 10% charcoal-stripped serum followed by 12 hr treatment with 300 nM TSA, 500 nM MS-275 or 2,500 nM SAHA. The altered expression of *TPRSS2-ERG* and AR in response to HDACi was confirmed with quantitative real-time PCR (* $p < 0.01$). (b) The AR protein expression was studied after 6 and 12 hr exposure to 300 nM TSA, 500 nM MS-275 or 2,500 nM SAHA. Histone H3 was used as a loading control. (c) The nuclear or cytoplasmic localization of AR was studied by Western blot of both nuclear and cytoplasmic fractions in response to bicalutamide (B, lane 2), flutamide (F, lane 3), dihydrotestosterone (DHT, lane 4), 50 nM and 500 nM MS-275 (lanes 5 and 8), also in combination with bicalutamide and flutamide (6, 7 and 9, 10); and 250 nM and 2.5 μ M SAHA (lanes 11 and 14) in combination with bicalutamide and flutamide (12, 13 and 15, 16). Treatments were compared to untreated control (lane 1). Histone H3 and β -actin antibodies were used as loading controls and as a validation of the purity of nuclear and cytoplasmic fractions, respectively (see Material and methods for details).

expression of *TPRSS2-ERG* and multiple *ERG* signature genes, but the enrichment was only marginally significant (nominal $p = 0.14$; enrichment score = -0.38). In a parallel experiment with *ERG*-negative LNCaP cells, HDACi did not affect expression of the *ERG* signature gene set (data not shown).

ERG suppression in vitro can reverse the pathways associated with in vivo *ERG* overexpression

To further evaluate the previously defined induction of WNT and the related PITX2 pathways in the *TPRSS2-ERG*-fusion gene containing prostate cancers,⁷ we explored if the expression of these gene sets is altered by HDACi and androgen deprivation. The WNT pathway controls organogenesis, tissue differentiation

and maintenance by inducing the PITX2 transcription factor, which is an important modulator of growth control genes.¹⁵ The WNT- and PITX2-related gene sets were found to be significantly (enrichment score < -0.5 ; nominal p -value < 0.001) suppressed by both MS-275 and androgen deprivation (Table II). This finding indicates that suppression of the *ERG* expression can also impact other downstream signaling events. Furthermore, the tumor necrosis factor (TNF) alpha and CCR5 gene sets, previously found to be suppressed in *ERG*-positive prostate cancers, were significantly enriched among the genes induced by TSA and MS-275, specifically in the VCaP cell line (Table II). Overexpression of WNT signaling and suppression of apoptosis, cancer hallmarks linked to prostate cancer development, may therefore be one of the downstream effects of *ERG*, which can be overcome by HDACi treatment.

HDACi interferes with AR expression and localization

Our gene expression data showed that multiple AR-regulated genes were systematically downregulated in response to both AA and HDACi, and that this effect was further enhanced when combined. Because the *TMPRSS2-ERG*-fusion gene is under the control of AR,¹ the effect of androgen deprivation and HDACi on the expression of these 2 genes was studied in detail by quantitative real-time PCR in VCaP cell line. SAHA, a pan-HDAC inhibitor in phase II clinical trials for prostate cancer,⁹ was included in these analyses to evaluate its potential use in *ERG*-positive prostate cancers. Although AA and all of the HDAC inhibitors studied (TSA, MS-275 and SAHA) suppressed *ERG* transcription significantly, only TSA abolished *ERG* expression >90% at nanomolar concentrations (Fig. 4a). Additionally, TSA suppressed *AR* transcription. Analysis of the AR protein levels revealed that both pan-HDACi TSA and SAHA also lowered the level of cellular AR protein at 12 hr treatment (Fig. 4b). To gain mechanistic insight to the HDACi-mediated *ERG* gene expression suppression, we also studied the subcellular localization of AR in response to HDACi, flutamide and bicalutamide in VCaP cells. Surprisingly, 500 nM class I selective MS-275 caused a significant retention of AR protein in the cytoplasm (Fig. 4c, lane 8), suggesting this to be a mechanism that contributes to the observed transcriptional downregulation of the *TMPRSS2-ERG*-fusion transcript (Fig. 3b). In concordance with the previous results, 2.5 μ M of pan-HDAC inhibitor SAHA reduced both nuclear and cytoplasmic AR (Fig. 4c, lane 14). Bicalutamide and flutamide treatment alone had no effect on AR nuclear localization (Fig. 4c, lanes 2 and 3), while dihydrotestosterone (DHT) led to strong nuclear accumulation of the AR (Fig. 4c, lane 4). Surprisingly, low concentrations of either 50 nM MS-275 or 250 nM SAHA together with flutamide inhibited AR nuclear translocation, indicating that cytoplasmic retention of AR may be one reason for the synergy observed with these compounds (Fig. 4c, lanes 7 and 13, respectively).

Taken together, our study showed that the expression of *TMPRSS2-ERG*-associated genes *in vivo* can be efficiently suppressed by HDACi *in vitro*. *ERG*-fusion gene positive prostate cancer cells were shown to be highly sensitive to HDACi. This sensitivity was further enhanced by concomitant AA, possibly due to inference to AR nuclear localization and protein turnover, suggesting combinational treatment for *ERG*-positive tumors.

Discussion

HDAC inhibitors have been shown to interfere with cell cycle progression and to reduce cell viability in a dose-dependent fashion in a wide range of tumor cell lines.^{16–20} Growth inhibitory activities of HDAC inhibitors, such as SAHA, MS-275 and TSA, have been confirmed both *in vitro* and *in vivo* prostate cancer models and in a number of clinical trials.^{21–27} HDAC inhibitors have also been reported to be equally effective in killing proliferating and nonproliferating tumor or transformed, immortalized cells.²⁸ However, HDAC inhibitors have not yet been clinically approved for prostate cancer treatment. The biological effects of HDAC inhibitors vary, ranging from induction of cell cycle arrest to apoptosis, depending on the type of the inhibitor, concentration as well as cell and tumor types. Here, we show that TSA and MS-275 induced cell death at nanomolar concentrations in *ERG*-positive prostate cancer cells lines (Table I). In accordance with previous data, HDACi had no effect on the growth of the nonmalignant immortalized prostate epithelial cells.²⁹ Moreover, the high sensitivity of the *ERG*-positive prostate cancer cells to HDACi was accompanied by clear synergistic effects with androgen deprivation by both flutamide and charcoal-stripped serum (Figs. 1a and 1b; Figs. S1a and S1b). This sensitivity was linked to the induction of apoptosis in VCaP and DuCaP cells (Figs. 1c and 1d, 2; Figs. S1c and S1d). Although synergy between bicalutamide and micromolar concentrations of SAHA has been previously reported for LNCaP cells,¹⁹ our results indicate that the *ERG*-positive prostate cancer cells are significantly (over 10-fold or more) more sensitive to HDACi and their combina-

tion with androgen deprivation. At the gene expression level, multiple AR-regulated genes (including *TMPRSS2-ERG*) along with the previously defined *in vivo* *ERG* signature were significantly suppressed, especially by TSA. Androgen deprivation enhanced the suppressive effect of HDACi on AR-regulated genes, thereby increasing especially the potency of MS-275 (Fig. 3b). Taken together, our results suggest that the presence of the *ERG*-fusion gene as well as the *ERG* signature might represent an opportunity to select patients that most likely would respond to the combined HDACi and androgen deprivation therapy.

Our study also indicates that the sensitivity of the *ERG*-positive prostate cancers to HDACi could possibly arise from their dependency on both AR and ERG signaling. We found that HDAC inhibitor MS-275 induced AR protein sequestration in the cytoplasm (Fig. 4c). Interestingly, when combined with flutamide, low nanomolar concentrations of HDAC inhibitors MS-275 and SAHA interfered with the AR nuclear transport. Additionally, pan-HDAC inhibitors TSA and SAHA suppressed AR protein levels (Fig. 4b), which has been previously reported to be transcription-dependent in LNCaP cells and related to chaperone Hsp90 acetylation.^{19,30,31} Taken together, these effects reducing transcriptionally active AR were the most likely mechanism behind the observed HDACi induced silencing of the AR-dependent *TMPRSS2-ERG* (Fig. 4a). Furthermore, HDAC inhibitor TSA efficiently suppressed not only the *ERG*-fusion gene but also the *in vivo* defined *ERG* signature; further verifying these genes as being *ERG*-associated (Fig. 3d). Additionally, HDACi reversely activated the apoptotic TNF pathway and suppressed the proliferative WNT pathway in *ERG*-positive cell line (Table II), suggesting that the alterations of these pathways observed in our previous *in vivo* studies⁷ may be due to epigenetic mechanisms.

Taken together, our study provides evidence that both the AR and the *TMPRSS2-ERG*-fusion gene signature and cell proliferation can be efficiently interfered by HDACi, particularly when applied in combination with the androgen deprivation therapy. *ETS* fusion gene overexpressing prostate cancers are highly proliferative and more likely to metastasize, which was recently suggested to be based on ERG-induced cell motility and invasive potential.^{32–35} This cancer subgroup is also strongly associated with prostate cancer related deaths.^{36,37} It would, therefore, be important to eradicate prostate cancers arising from *ETS*-fusion in the early phase, because it has been shown that *ERG*-fusion gene may be bypassed at later stages of cancer progression.^{37–39} Therefore, identification of a more efficient and specific treatment for these aggressive cancers would be of great clinical interest. Because multiple HDAC inhibitors, also in combination with androgen deprivation therapy (<http://clinicaltrials.gov>, NCT00589472), are currently being tested for the treatment of prostate cancers, specific biomarkers to select the patients most likely to benefit as well as those to follow the drug efficacy will be important. Detailed understanding of the mechanisms of action of various HDACi, in particular on AR- and ERG-related pathways, will provide important insights explaining why clinical trials may succeed or fail. Our finding indicates that the *ERG* signature refined here based on *in vivo* associations as well as *in vitro* functional data could represent a possible biomarker for clinical therapeutic trials in *ERG*-positive prostate cancers. Finally, we showed that *TMPRSS2-ERG*-fusion gene expressing cell lines are very sensitive to HDACi, and the fusion gene expression is significantly downregulated in response to HDAC inhibitors *in vitro*. These results indicate that *ERG*-positive prostate cancer patients could benefit from HDAC inhibitor therapy. Finally, our finding of synergy between androgen deprivation and HDACi suggests a combinatorial therapeutic strategy to be the most effective for *ERG* gene fusion positive prostate cancer patients.

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