Sulforaphane destabilizes the androgen receptor in prostate cancer cells by inactivating histone deacetylase 6

Angela Gibbs¹, Jacob Schwartzman¹, Vivianne Deng, and Joshi Alumkal²

Division of Hematology and Oncology, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239

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High consumption of cruciferous vegetables is associated with a lower risk of prostate cancer in epidemiological studies, although the precise constituents that may mediate this observation are unknown (1–4). Sulforaphane, a derivative of glucoraphanin found in crucifers, has heterogeneous biological activities including Phase 2 enzyme induction, cell cycle arrest, and apoptosis, but data on pathways that mediate effects on cell growth, survival, and differentiation are incomplete (5). Of note, several reports show that sulforaphane treatment of prostate cancer cells in vitro leads to reduced prostate cancer cell survival, and treatment of xenograft implants or transgenic animal models of prostate cancer with sulforaphane inhibits tumor formation and metastases (6–8).

Recently, sulforaphane was shown to inhibit histone deacetylase (HDAC) proteins, which are up-regulated in cancer (6, 9–11). Despite their name, some HDAC proteins remove acetyl groups from histone proteins whereas others act on non-histone proteins. Histone deacetylation reduces gene expression whereas non-histone protein deacetylation changes protein function (11). Histone deacetylase 6 (HDAC6) is a cytoplasmic non-histone protein deacetylase whose substrates include alpha-tubulin and the HSP90 chaperone protein (12–14). When HDAC6 deacetylates HSP90, this leads to activation of HSP90, enhanced binding of HSP90 to client proteins including the androgen receptor (AR) protein, and consequently attenuated degradation of client proteins including AR (13). Conversely, HSP90 hyperacetylation after treatment with certain HDAC inhibitors results in dissociation of HSP90 from client proteins such as AR and AR degradation (12, 13, 15, 16).

Indeed, AR, which is activated by androgens, is the most therapeutically relevant target in malignant prostate epithelial cells (17). Both preclinical studies and clinical trials have demonstrated the usefulness and effectiveness of targeting AR with hormonal agents, which act by reducing androgen production or binding to AR for prostate cancer prevention and treatment (17–19). Hormonal agents targeting AR in recurrent prostate cancer are commonly used but are not curative, and resistance mechanisms including AR gene amplification, AR mutations, and up-regulation of pathways that activate AR independent of androgens eventually develop in patients (20). In addition, recent work also demonstrates that androgen levels sufficient for AR activation persist in prostate cancer cells in man despite the use of hormonal agents, and that AR transcript variants, which encode for AR proteins, which are active even in the absence of androgens, exist (21, 22).

Thus, the destabilization and degradation of AR protein represents a rational strategy to interfere with AR signaling and to overcome the aforementioned resistance mechanisms. The present study was designed to test the hypothesis that sulforaphane would inhibit the function of HDAC6, and that this would suppress the stability or function of AR. Sulforaphane's ability to suppress HDAC6 was unknown, and its effects on AR signaling remained poorly characterized. We describe herein experimental results that confirm that sulforaphane destabilizes AR protein and disrupts AR signaling by inactivating HDAC6.

Results

Sulforaphane Treatment Increases HSP90 Acetylation and Leads to Dissociation of AR from HSP90. We treated LNCaP prostate cancer cells with sulforaphane and showed increased enrichment of acetylated lyses on HSP90 compared with vehicle 4 h after treatment (Fig. 1A). In the input samples, HSP90, HDAC6, and alpha-tubulin levels were similar between vehicle and sulforaphane-treated cells; levels of acetylated alpha-tubulin, a HDAC6 target, increased in sulforaphane-treated samples, indicating inhibition of protein deacetylation (Fig. 1A). Similar results were seen in VCaP prostate cancer cells (Fig. S1). Sulforaphane treatment also disrupted the interaction between HSP90 and AR

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1A.G. and J.S. contributed equally to this work.

2To whom correspondence should be addressed: Oregon Health and Science University, 3303 SW Bond Avenue, CH14R, Portland, OR 97239. E-mail: alumkalj@ohsu.edu.

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Phane increased levels of acetylated alpha-tubulin (Fig. 1) between vehicle and sulforaphane-treated cells whereas sulforaphane receptor were similar in both conditions (lanes 3 and 7). In the input treatment (lanes 1 and 5). Levels of acetylation of the androgen setting of decreased HSP90–AR interaction with sulforaphane blotting for AR demonstrates similar AR protein levels between pathway (Fig. S2).

Imidazole that, like sulforaphane, acts via the Nrf2-Keap1 inhibitor trichostatin A (TSA) and the triterpenoid CDDO-Similar results were seen for both cell lines with the HDAC out after treatment with sulforaphane (SFN) 20 μM (Fig. 2). AR and GAPDH levels by Western blot were quantified.

AR and GAPDH levels by Western blot were quantified. (Fig. 2A). The AR immunoprecipitation followed by Western blotting for AR demonstrates similar AR protein levels between vehicle (lane 2) and sulforaphane-treated (lane 6) cells in the setting of decreased HSP90–AR interaction with sulforaphane treatment (lanes 1 and 5). Levels of acetylation of the androgen receptor were similar in both conditions (lanes 3 and 7). In the input samples, levels of HSP90, AR, and alpha-tubulin were similar between vehicle and sulforaphane-treated cells whereas sulforaphane increased levels of acetylated alpha-tubulin (Fig. 1B).

Sulforaphane Treatment Lowers AR Protein Levels. Using later time points, we treated LNCaP cells with sulforaphane and found that AR protein levels decreased at 12 h for the highest doses of sulforaphane (10–20 μM) (Fig. 2A). In VCaP cells, at 24 h, AR protein levels declined for all sulforaphane dose levels (Fig. 2B). Similar results were seen for both cell lines with the HDAC inhibitor trichostatin A (TSA) and the triterpenoid CDDO-Imidazole that, like sulforaphane, acts via the Nrf2-Keap1 pathway (Fig. S2). AR transcripts were not consistently suppressed by sulforaphane treatment, emphasizing that posttranscriptional mechanisms are involved (Fig. S3). Thus, across both AR-expressing cell lines, sulforaphane treatment reduced AR proteins levels.

Sulforaphane Treatment Lowers AR Target Gene Expression. We next determined the functional consequences of AR protein depletion by performing real-time PCR for several AR target genes, PSA and the TMPRSS2-ERG gene fusion, which is present in VCaP cells (Fig. S4A–C). There was a dose-dependent reduction in gene expression in both cell lines. Similar results were seen with TSA. Thus, there is a high concordance between reduced AR protein levels and reduced AR target gene expression in prostate cancer cells.

Sulforaphane Treatment Reduces AR Occupancy at Its Androgen Response Elements (AREs) and Lowers ERG Protein Levels. To determine whether AR protein depletion from its target genes’ AREs was responsible for reduced target gene expression, we performed chromatin immunoprecipitation (ChIP). Sulforaphane treatment reduced enrichment of AR at the PSA ARE in both cell lines (Fig. S4A and B). Similarly, AR enrichment was decreased with sulforaphane treatment at the TMPRSS2 ARE (Fig. S4C). A Western blot with VCaP protein lysates at this same time point confirms near-absent levels of AR and ERG proteins after sulforaphane treatment (Fig. S4D).

Proteasome Inhibitor Treatment Rescues AR Protein from Sulforaphane-Induced Degradation. Because it is known that hyperacetylation of HSP90 inhibits its function and targets its client proteins to the proteasome for posttranslational degradation, we assessed the effects of treatment with sulforaphane and TSA with the proteasome inhibitor MG132 (Fig. 4). Treatment with sulforaphane (lane 2) or TSA (lane 4) in the absence of MG132 reduced AR protein levels versus the vehicle-treated cells (lane 6). However, simultaneous treatment with MG132 and either sulforaphane (lane 1) or TSA (lane 3) restored the AR steady-state level close to that seen in the vehicle-treated control (lane 5).

Sulforaphane Inhibits HDAC6 Enzymatic Function. We determined recombinant HDAC6 enzymatic activity on its tubulin substrate in a cell-free tubulin deacetylase assay (Fig. 5). Incubation of vehicle-treated recombinant HDAC6 with tubulin dimers (lane...
2) reduced levels of acetylated tubulin versus the vehicle-treated tubulin dimers incubated without HDAC6 (lane 1), which indicated that the HDAC6 enzyme was functional. However, incubation of HDAC6 with sulforaphane (lanes 3 and 4) or TSA (lane 5) led to decreased HDAC6 deacetylase activity as evidenced by increased levels of alpha-tubulin compared with the vehicle-treated HDAC6 control (lane 2).

**HDAC6 Overexpression Rescues AR and HDAC6 Proteins from Sulforaphane Treatment.** We overexpressed empty vector or FLAG-HDAC6 in LNCaP cells followed by treatment with sulforaphane or vehicle (Fig. 6A). Overexpression of HDAC6 led to a detectable anti-FLAG signal and higher levels of HDAC6 versus empty-vector-transfected cells, whereas levels of acetylated alpha-tubulin declined, indicating the presence of functional HDAC6 protein. Surprisingly, in empty-vector-transfected cells, HDAC6 protein levels were reduced with sulforaphane treatment; although sulforaphane does reduce HDAC6 transcript levels, this does not fully account for the observed HDAC6 protein depletion (Fig. S5A). Concomitant proteasome inhibitor and sulforaphane treatment (lane 4) restored HDAC6 protein levels versus sulforaphane-treated cells (lane 3) whereas TSA had minimal effects on HDAC6 protein levels (lanes 5 and 6) (Fig. S6). Additionally, in empty-vector-transfected cells, sulforaphane treatment lowered AR protein and increased acetylation of alpha-tubulin. However, in HDAC6-overexpressing, sulforaphane-treated cells, HDAC6 protein expression was restored, AR protein depletion was attenuated (without changing AR transcript levels), and alpha-tubulin acetylation was blunted versus the effects seen in empty-vector, sulforaphane-treated cells (Fig. 6A, Fig. S5B).

**HDAC6 siRNA Recapitulates Sulforaphane’s Effect on AR Protein Levels.** We transiently transfected LNCaP cells with either a control or HDAC6 siRNA. HDAC6 siRNA depleted cells of HDAC6, which was most pronounced at 72 h (Fig. 6B). At this later time point, depletion of AR protein and acetylation of alpha-tubulin in the HDAC6 knockdown cells were most pronounced versus the control siRNA cells (Fig. 6B).

**Discussion**

High consumption of cruciferous vegetables is associated with a lower risk of prostate cancer development, but the precise constituents that may mediate this association are unknown. One possible candidate is sulforaphane, derived from glucoraphanin found in crucifers, which has been shown to have effects as a chemopreventive and anticancer agent in multiple preclinical systems through Phase 2 enzyme induction, cell cycle arrest, and apoptosis (1–4, 8, 23–33). However, the molecular mechanisms that

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Fig. 3. Sulforaphane treatment of prostate cancer cells reduces AR target gene expression. (A) Real-time PCR of PSA expression from LNCaP cells treated with vehicle, increasing doses of sulforaphane, or TSA at the indicated time points. (B and C) Real-time PCR of PSA (B) and TMPRSS2-ERG (C) gene expression from VCaP cells at 24 h. The vehicle-treated sample was set to 1. 18S was used as an endogenous control in all assays.

Fig. 4. Proteasome inhibitor treatment of prostate cancer cells rescues AR protein from sulforaphane treatment. LNCaP cancer cells were treated for 24 h with 20 μM sulforaphane with or without 10 μM MG132, 300 nM TSA with or without 10 μM MG132, 10 μM MG132, or vehicle. AR levels and GAPDH levels by Western blot were quantified.

Fig. 5. Sulforaphane treatment inhibits HDAC6. Tubulin dimers were either incubated without recombinant HDAC6 or with recombinant HDAC6 in the presence of vehicle, sulforaphane, or TSA. Levels of HDAC6, acetylated tubulin, and tubulin by Western blot were quantified.
underlie the previously observed antitumor effects of sulforaphane in prostate cancer models have not been fully clarified.

The AR protein is the central, therapeutically relevant pathway in prostate cancer, and hormonal deprivation strategies are commonly used in the treatment of all phases of this disease and work by interfering with androgen production or binding to AR, which is an activating event (17). Thus, interference with the state of AR activation or AR protein levels represents a rational strategy to ablate AR signaling for the prevention and treatment of this disease (17–19).

We show here that sulforaphane treatment increases acetylation of two HDAC6 target proteins: HSP90 and alpha-tubulin. At early time points, in the setting of unchanged HDAC6 protein levels, HSP90 becomes hyperacetylated, and the AR protein dissociates from HSP90, which matches an earlier report with the HDAC inhibitor LAQ824 (Fig. 1, Fig S1) (15). At later time points, after treatment with sulforaphane or the HDAC inhibitor TSA, AR protein levels decline, and this decline is attenuated by inhibition of proteasomal degradation, which suggests that sulforaphane treatment targets AR for proteolytic degradation (Figs. 2 and 4). This same effect has been demonstrated for other HSP90 client proteins including ErbB2, bcr-abl and c-kit after interference with HSP90 function with pharmacological HDAC inhibitors (12, 15, 34).

Although AR protein levels declined without reductions in AR transcript levels for most doses of sulforaphane, for the highest doses of sulforaphane (20 μM in LNCaP cells and 15–20 μM in VCaP cells) AR transcript levels also declined. We found that the HDAC inhibitor TSA also suppressed AR transcripts, a response that has been reported by others and with other HDAC inhibitors (Fig. S3) (35, 36). Consequently, the overall effect of sulforaphane and other agents with HDAC inhibitory properties on AR levels may not be solely posttranscriptional. It is clear, however, that a posttranslational effect is a critical control point in light of our observations that AR protein levels are rescued by simultaneous sulforaphane and proteasome inhibitor treatment or overexpression of HDAC6, and that most sulforaphane doses do not lower AR transcript levels (Figs. 4 and 6 and Figs. S3 and S5).

We confirmed that the disappearance of AR corresponded to reduced AR binding to and expression of its target genes (Fig. 3, Fig S4). The reduced enrichment of AR at its target genes by ChIP demonstrates that AR depletion is mediating the reduced AR target gene expression. Notably, we found that levels of TMPRSS2-ERG, a fusion of the AR-regulated TMPRSS2 gene and the ERG transcription factor, which is commonly overexpressed in human prostate cancer, are reduced (37). ERG overexpression in normal prostate cells leads to enhanced invasiveness and growth, and ERG knockdown in VCaP prostate cancer cells by siRNA leads to decreased invasiveness (38). ERG overexpression under an AR-regulated promoter in a transgenic murine model was recently shown by two independent groups to transform prostate cells and to induce formation of prostate cancer precursor lesions highlighting ERG’s importance in early stages of prostate tumorigenesis (38, 39). Thus, therapies such as sulforaphane, which deplete cells of AR and ERG protein, may hold promise for the prevention and treatment of prostate cancer.

We focused on HDAC6 as a key candidate for a sulforaphane target because others have shown that HDAC6, specifically, interacts with HSP90 and alpha-tubulin and deacetylates these proteins and that hyperacetylation of HSP90 cripples its chaperone function and leads to client protein dissociation and degradation (12–16). We demonstrated, by using a cell-free system, that incubation of recombinant HDAC6 with sulforaphane inhibits HDAC6 deacetylase activity. In cells, we showed that sulforaphane treatment inhibits HDAC6 function without changing its levels at early time points (4 h) (Figs. 1 and 5). However, at later time points (16 h), we have shown that sulforaphane leads to reduced HDAC6 protein levels (Fig. 6A, Fig. S6). The related compound CDDO-Imidazole also depletes HDAC6 in a dose-dependent manner, which parallels reduced AR protein levels and increased tubulin acetylation (Fig. S2). CDDO-Imidazole was previously shown to deplete Her2Neu protein levels in breast cancer cells through proteasomal degradation, although the effect of this agent on HDAC6 was not explored in that report (40). Given that CDDO-Imidazole, like sulforaphane, acts via the Nrf2 pathway, it is possible that the effect of these agents on HDAC6 protein levels is mediated by activation of gene targets of the Nrf2–Keap1 pathway.

There are several possible mechanisms for the reduced HDAC6 protein levels (beyond reduced HDAC6 transcript levels) (Fig. 6A, Fig. S5A); however, our data with proteasome inhibitor rescue suggests that proteasomal degradation is principal (Fig. S6). The importance of reduced HDAC6 levels with sulforaphane treatment is highlighted by the fact that overexpression of HDAC6 attenuates sulforaphane-mediated AR and HDAC6 protein depletion, and HDAC6 siRNA recapitulates the findings seen with sulforaphane. (A) LNCaP cells were transfected with pCDNA3.1 or FLAG-HDAC6. Cells were then treated with either vehicle or 15 μM sulforaphane. Intensity values for bands by Western blot in the respective vehicle controls were set to 1. (B) LNCaP cells were transfected with siRNA to either the luciferase gene (si LUC) or HDAC6 gene (si HDAC6). Levels of protein expression by Western blot were quantified, and the bands from the HDAC6 siRNA samples were compared to the luciferase control samples from the same time point.

![Fig. 6. Ectopic overexpression of HDAC6 attenuates sulforaphane-mediated AR and HDAC6 protein depletion, and HDAC6 siRNA recapitulates the findings seen with sulforaphane.](image-url)

![Fig. 7. Model of sulforaphane attenuation of AR signaling via HDAC6 inactivation. Normally, HSP90 is deacetylated by HDAC6, which enables it to chaperone client proteins such as AR. HDAC6 also deacetylates alpha-tubulin. With sulforaphane treatment, HDAC6 is inhibited or targeted for protein degradation. This leads to hyperacetylated alpha-tubulin; the functional significance of this remains unclear. The HDAC6 inactivation with sulforaphane also leads to hyperacetylated, inactive HSP90 protein, which dissociates from AR, and AR is then targeted for protein degradation. Consequently, AR binding to its target gene androgen response elements (ARE), including TMPRSS2-ERG, is diminished, which reduces their expression.](image-url)
sion of HDAC6 protein, in the presence of sulforaphane, reverses sulforaphane’s effect on depleting HDAC6 and AR proteins and increasing acetylation of alpha-tubulin compared with empty-vector-transfected, sulforaphane-treated cells (Fig. 6A).

Finally, that HDAC6 depletion by siRNA recapitulates the sulforaphane treatment effects and leads to reduced AR protein further corroborates that the effects we have seen with sulforaphane are mediated by inactivation of HDAC6 (Fig. 6B). While it is possible that sulforaphane-mediated HSP90 hyperacetylation and consequent AR degradation occur via inhibition of HSP90 deacetylases besides HDAC6, although none are known, we can state that the influence of this agent on HDAC6 function and protein expression accounts, at least in part, for the influence of sulforaphane on the expression of the AR protein (Figs. 5 and 6).

Prostate cancer remains a common and sometimes lethal cancer, and it is clear that the AR protein is an important target in all phases of this disease (17, 20). Although the preclinical data and limited human data for sulforaphane is promising, including reports of inhibition of histone deacetylase activity and histone hyperacetylation and gene reactivation in xenograft tumors and intact tissues in animal models and peripheral blood mononuclear cell in man, our work elucidates unique mechanisms of action of sulforaphane through non-histone protein deacetylase inhibition—increased protein acetylation of HDAC6 targets such as alpha-tubulin and HSP90, inhibition or reduced levels of HDAC6 protein, and consequently reduced levels of AR protein and target genes including PSA and TMPRSS2-ERG (Fig. 7) (7, 9, 10). Further human testing will be necessary before suggesting whether sulforaphane may play a role in human prostate cancer prevention or therapy, but the studies we describe herein provide a strong rationale and, by clarifying the AR/HSP90/HDAC6 complex as a target of this agent, provide opportunities to conduct target validation studies.

Methods

Cell Culture and Drug Treatment. LNCaP and VCaP cells were grown according to American Type Culture Collection instructions. Sulforaphane (SNF, #54441 Sigma), MG-132 (#102–0005, Biomol), and CDDO-Imidazole (National Cancer Institute/Developmental Therapeutics Program Open Chemical Repository) were resuspended in DMSO. Trichostatin A (TSA, #78552 Sigma) was resuspended in 100% ethanol. Vehicle-treated cells received the respective vector-transfected, sulforaphane-treated cells (Fig. 6).

PSA (Fig. 7) (7, 9, 10). Further human testing will be necessary before suggesting whether sulforaphane may play a role in human prostate cancer prevention or therapy, but the studies we describe herein provide a strong rationale and, by clarifying the AR/HSP90/HDAC6 complex as a target of this agent, provide opportunities to conduct target validation studies.

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Immunoprecipitation and Immunoblot Analysis. Immunoprecipitation and immunoblotting experiments were performed as described (41). X-ray exposures were scanned as uncompressed images and bands were quantified by densitometry with Quantity One software (Bio-Rad). Intensity values were normalized to those of the endogenous controls. The vehicle sample was set to 1. Enrichment was calculated as: (intensity IP/Intensity input)/ (Intensity IgG/Intensity input) or intensity IP/input (Fig. S1). See SI Methods for the antibodies used.

Real-time PCR (RNA extraction, cDNA, Real-time PCR). Cells were lysed in TRIzol (Invitrogen) and then purified with the RNeasy Kit (Qiagen). One microgram of RNA was reverse-transcribed using the Omniscript RT kit (Qiagen). See SI Methods for primer and PCR information. Standard curves were generated by measuring expression of target genes and 18S rRNA in serial dilutions of a mock-treated control. All samples were run in triplicate. The vehicle-treated sample was set to 1.

Chromatin immunoprecipitation. Cross-linking and sonication. Cells were cross-linked with formaldehyde and reactions were stopped with glycerine. Crosslinked cells were resuspended in IP buffer with SigmaFast protease inhibitor tablets (Sigma) and sonicated on ice by using a Branson Digital Sonifier model 450. Matrix ChIP. Sonicated cellular lysates were used for Matrix ChIP (42). Immunoprecipitations were performed without adding an antibody or with 500 ng of an anti-AR antibody. PCR. PCRs were amplified using a thermocycler (see SI Methods), and products were run on 2% NaBO3 agarose gels containing GelStar dye (Cambrex) for visualization (43, 44). Bands were imaged for densitometry by using a Gel Doc XR UV transilluminator and Quantity One software (Bio-Rad).

HDAC6 Tubulin Deacetylase (TADAC) Assay. Two micrograms of recombinant HDAC6 (Biomol) were used in a cell-free tubulin deacetylase assay with 25 μg of MAP-rich polymerized tubules (Cytoskeleton), similar to Hubbert et al. (14).

HDAC6 Overexpression. LNCaP cells were transiently transfected with pCDNA3.1 empty vector (Invitrogen) or FLAG-HDAC6 (Addgene) by using Lipofectamine LTX and Plus reagents (Invitrogen). Forty-eight hours later, media was replaced for a 16-h treatment with 15 μM sulforaphane or vehicle.

HDAC6 Knockdown. LNCaP cells were transfected with siRNA by using DharmaFECT 3 transfection reagent for a final concentration of 100 nM (Dharmacon) (14). Cells were harvested at indicated time points posttransfection. Sequences are listed in SI Methods.
Supporting Information

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**SI Methods**

**Antibodies Used in Immunoblotting or Immunoprecipitations (Indicated Dilutions Are for Immunoblotting).** AR (1:1000, #06–680 Upstate); acetylated lysine (#05–515 Upstate); acetylated tubulin (1:2000, #T6793 Sigma); alpha-tubulin (1:10,000, #T6074 Sigma); beta-actin (1:5000, #A5441 Sigma); ERG-1/2/3 (1:5000, #sc-354X Santa Cruz); FLAG epitope (#F7425 Sigma); GAPDH (1:5000, #sc-32233 Santa Cruz); HDAC6 (1:1000, #sc-11420 Santa Cruz); HSP90 (1:1000, #SPA-840 Stressgen); mouse IgG (#sc-2025 Santa Cruz). The following secondary antibodies were used: anti-mouse IgG (1:5000, #A6782 Sigma); anti-rabbit IgG (1:3000, #NA934 Amersham or 1:10,000, #sc-2004 Santa Cruz).

**ChIP PCR Primer Sequences and Conditions.**

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<th>Primer Name</th>
<th>Sequence</th>
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<tr>
<td>PSA ARE ChIP Sense Primer</td>
<td>GCCCTGGATCTGAGAGAGATATCATC</td>
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<tr>
<td>PSA ARE ChIP Anti-Sense Primer</td>
<td>ACACCTTTTTTTT-TCTGGAATTGTC</td>
</tr>
<tr>
<td>TMPRSS2 ARE ChIP Sense Primer</td>
<td>TGGTCCTGGATGATAAAAAAAGTTC</td>
</tr>
<tr>
<td>TMPRSS2 ARE ChIP Anti-Sense Primer</td>
<td>GACATACGC-CCCCACAACAGA</td>
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Primers were synthesized by Integrated DNA Technology (IDT). ChIP PCR program consisted of: 95 °C for 5 minutes for 1 cycle; 95 °C for 30 seconds, 58 °C (TMPRSS2) or 60 °C (PSA) for 30 seconds, and 72 °C for 30 seconds for 35–40 cycles; 72 °C final extension for 5 min.

**Real-time PCR Primer Sequences and Conditions.** Taqman gene expression assays for AR (Hs00171172_m1), PSA (KLK3, Hs02576345_m1), HDAC6 (Hs00195966_m1), and 18S endogenous control (4319413E) were purchased from Applied Biosystems.

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<th>Primer Name</th>
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<tr>
<td>TMPRSS2-ERG QRTPCR Sense Primer</td>
<td>CGCGAGCTA-AGCAGGAG</td>
</tr>
<tr>
<td>TMPRSS2-ERG QRTPCR Anti-Sense Primer</td>
<td>CGACTG-GTCCCTCACTCACAA</td>
</tr>
<tr>
<td>TMPRSS2-ERG QRTPCR Probe</td>
<td>FAM5'-CGCGGCAGGAACCTTATCAG-3'TAMRA</td>
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Primers and probe for TMPRSS2-ERG fusion were purchased from IDT. The real-time PCR program consisted of: 50 °C for 2 min for 1 cycle; 95 °C for 10 min for 1 cycle; 95 °C for 15 seconds; and 60 °C for 15 seconds for a total of 40 cycles.

**siRNA Sequences.** HDAC6 gene: CTGCAAGGGATGGATCT-GAAC
Non-targeted control luciferase gene: CGTACGC-GAATACTTCA

Gibbs et al. www.pnas.org/cgi/content/short/0908908106
Fig. S1. Sulforaphane treatment of VCaP prostate cancer cells increases HSP90 acetylation in VCaP cells. An immunoprecipitation with an anti-acetyl lysine antibody was carried out after treatment with sulforaphane (SFN) 20 μM or vehicle for 6 h followed by a Western blot for HSP90. Enrichment was quantified for each immunoprecipitation. Inputs were probed with the indicated antibodies by Western blot and were quantified.
Fig. S2. CDDO-Imidazole recapitulates sulforaphane’s effects. (A) LNCaP and (B) VCaP cells were treated with vehicle or increasing concentrations of CDDO-Imidazole for 24 h. Protein lysates were probed with the antibodies indicated. AR, acetylated tubulin, HDAC6, and actin levels were quantified by Western blot.
Fig. S3. High-dose sulforaphane treatment of prostate cancer cells lowers AR transcript levels. (A) Real-time PCR of AR gene expression with cDNA from LNCaP cells treated with vehicle, increasing doses of sulforaphane, or TSA. (B) Real-time PCR of AR gene expression with cDNA from VCaP cells treated with vehicle, increasing doses of sulforaphane, or TSA at 24 h. The vehicle-treated sample was set to 1. 18S was used as an endogenous control in all assays.
Fig. S4. Sulforaphane treatment of prostate cancer cells depletes AR from androgen response elements (AREs) and lowers ERG protein. LNCaP (A) or VCaP (B and C) cancer cells were treated with vehicle or 20 μM sulforaphane for 24 h. ChIP analysis was used to determine AR occupancy at the indicated gene AREs. Enrichment was calculated compared with the respective input control. (D) Western blot for AR and ERG protein expression from VCaP whole cell lysates treated in parallel with samples from B and C. AR, ERG, and actin levels were quantified by Western blot.
Fig. S5.  Sulforaphane treatment of prostate cancer cells lowers HDAC6 transcript levels whereas HDAC6 overexpression does not increase AR transcript levels. LNCaP cells were transfected with pCDNA3.1 or FLAG-HDAC6. Cells were then treated with either vehicle or 15 µM sulforaphane. (A) Real-time PCR of HDAC6 expression. (B) Real-time PCR of AR expression. The vehicle-treated sample was set to 1. 18S was used as an endogenous control in all assays.
Fig. S6. Proteasome inhibitor treatment of prostate cancer cells rescues HDAC6 protein from sulforaphane treatment. LNCaP cancer cells were treated for 16 h with vehicle without (lane 1) or with (lane 2) 20 μM MG132, 20 μM sulforaphane without (lane 3) or with (lane 4) 20 μM MG132, or 300 nM TSA without (lane 5) or with (lane 6) 20 μM MG132. HDAC6 and actin levels were quantified by Western blot.