Published in final edited form as:

Oncogene. 2011 May 12; 30(19): 2207–2218. doi:10.1038/onc.2010.600.

INHIBITION OF ANDROGEN RECEPTOR ACTIVITY BY HISTONE DEACETYLASE 4 THROUGH RECEPTOR SUMOYLATION

Yonghua Yang[1,†,#], **Anfernee Kai-Wing Tse**[1,#], **Pengfei Li**[1], **Qiuping Ma**[1], **Shengyan Xiang**[1], **Santo V. Nicosia**[1,2,4], **Edward Seto**[2,3], **Xiaohong Zhang**[1,2,3], and **Wenlong Bai**[1,2,3]

[1]Department of Pathology and Cell Biology, USF College of Medicine, Tampa, Florida 33612-4799

[2]Department of Oncological Sciences, USF College of Medicine, Tampa, Florida 33612-4799

[3]Program of Molecular Oncology, H. Lee Moffitt Cancer Center, Tampa, Florida 33612-4799

[4]Program of Experimental Therapeutics, H. Lee Moffitt Cancer Center, Tampa, Florida 33612-4799

Abstract

The transcriptional activity of the androgen receptor is regulated by both ligand binding and posttranslational modifications including acetylation and SUMOylation. Histone deacetylases are known to catalyze the removal of acetyl groups from both histones and non-histone proteins. In the present study, we report that histone deacetylase 4 (HDAC4) binds to and inhibits the activity of the androgen receptor (AR). This inhibition was found to depend on the SUMOylation, instead of deacetylation, of the AR. Consistently, HDAC4 increases the level of AR SUMOylation in both whole cell and cell-free assay systems, raising the possibility that the deacetylase may act as an E3 ligase for AR SUMOylation. Knock down of HDAC4 increases the activity of endogenous AR and androgen induction of prostate specific antigen expression and prostate cancer cell growth, which is associated with decreased SUMOylation of the receptor. Overall, the studies identify HDAC4 as a positive regulator for AR SUMOylation, revealing a deacetylase-independent mechanism of histone deacetylase action in prostate cancer cells.

Keywords

Androgen receptor; Androgens; HDAC4; Prostate cancer; SUMOylation; SUMO E3 ligase

Correspondence should be addressed to W.B: Department of Pathology and Cell Biology, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., MDC 11, Tampa, Florida 33612-4799; T: 813-974-0563; F: 813-974-5536; wbai@health.usf.edu.

†Current address: Medical College of Georgia Cancer Center, 1120 15th Street, CN2101A, Augusta, GA 30912

#The authors contribute equally.

Conflict of Interest: The authors declare no conflict of interests.

Introduction

Androgens are the male hormone responsible for the development, maintenance and regulation of male phenotype and reproduction. Besides the male reproductive system, target tissues for androgens also include external genitalia, sebaceous glands, muscle, and neuronal tissues. In addition to these established physiological functions, androgens are implicated in multiple pathological processes including androgen insensitivity syndrome, 5α-reductase deficiency, X-linked spinal bulbar muscular atrophy (Kennedy's disease), benign prostate hyperplasia and prostate cancer. Androgens, particularly 5α-dihydrotestosterone (DHT), are known to stimulate the proliferation and inhibit apoptosis of normal prostate epithelial and prostate cancer cells.

The effects of androgens are mediated through the AR (Chang *et al.*, 1988; Lubahn *et al.*, 1988), a member of the steroid/thyroid nuclear receptor superfamily (Evans, 1988; Tsai and O'Malley, 1994). It directly binds androgen response elements and regulates the transcription of androgen target genes. Similar to other steroid hormone receptors, human AR is composed of a N-terminal A/B region containing the major activation function (AF-1), a DNA binding domain (DBD) with two C_2C_2 zinc fingers, a hinge region and a C-terminal ligand binding domain (LBD) that contains another activation function (AF-2). In the absence of androgens, LBD represses the activities of both AFs (Brinkmann *et al.*, 1999). After androgen binding, the repression is relieved through the formation of a conformation suitable for interaction with a complex of transcriptional coactivators that often are histone acetyl transferase (HAT) themselves or associated with such activity (Onate *et al.*, 1995; Xu and O'Malley, 2002). These coactivators mediate the effect of the AR on gene transcription by directly remodeling the chromatin structure through the acetylation of core histones.

Besides androgen binding, the transcriptional activity of the AR is also regulated by posttranslational modifications such as phosphorylation, acetylation and SUMOylation. Phosphorylation occurs on multiple serine/threonine (Blok *et al.*, 1996) and tyrosine (Guo *et al.*, 2006) residues. They positively regulate the AR activity by either enhancing the receptor's response to low levels of androgens or by causing androgen independent activation. Similar to phosphorylation, acetylation on lysine residues in the hinge region also increases AR activity (Fu *et al.*, 2000; Fu *et al.*, 2004). To the opposite of acetylations, SUMOylation on two lysine residues in the N-terminal A/B region represses the AR activity (Poukka *et al.*, 2000) through mechanisms that likely involve co-repressor recruitment (Dotzlaw *et al.*, 2002; Lin *et al.*, 2004).

Different from the androgen-bound form, ligand-free or antiandrogen-bound AR interacts with co-repressor complexes containing histone deacetylases (HDACs) that remodel chromatin structure through the removal of acetyl groups from histones (Jones and Shi, 2003). Besides histones, HDACs also suppress androgen action by catalyzing AR deacetylation (Fu *et al.*, 2006; Gaughan *et al.*, 2002). In the present study, we present evidences that HDAC4, a class II HDAC, inhibits AR activity through a mechanism that independent of AR deacetylation. Instead, our studies demonstrate that the inhibitory effect

of HDAC4 is mediated mainly through enhanced AR SUMOylation. The relevance of these findings to androgen signaling in prostate cancer cells is discussed.

Results

HDAC4 inhibits AR activity

HDACs are known to deacetylate both histone and non-histone proteins. HDACs such as HDAC1 have been shown to inhibit AR activity through AR deacetylation (Gaughan *et al.*, 2002). To test the effect of different HDACs on AR activity, the receptor was transfected into AR-negative 293 cells together with members of classes I and II HDACs. Its transcriptional activity was then measured in the presence or absence of synthetic androgen R1881 with a co-transfected AR reporter gene, ARR_3TKLuc in which three copies of the -244/-96 DNA fragment of the probasin gene were placed in front of thymidine kinase promoter followed by the firefly luciferase cDNA (Kasper *et al.*, 1999). Among all tested HDACs, HDAC1, HDAC4 and HDAC7 consistently decreased AR activity with HDAC4 showing the strongest repression (Figs 1A). The repression depended on the amounts of co-transfected HDAC4 (Fig 1B) and was not due to a decrease in the level of AR expression (Fig 1A). Comparison among selected members of class II HDACs revealed that HDAC5 and HDAC10 did not inhibit AR activity at levels comparable to HDAC4 (Figs 1C and 1D), showing that the ability to inhibit AR is a not a general feature for members of class II HDACs.

Further analyses showed that the dose dependent inhibition occurred with two additional AR reporters (Figs. 2A and 2B), ARE_2e1bLuc in which firefly luciferase gene is under the control of two copies of synthetic non-selective AREs and adenoviral E1b promoter (Nawaz *et al.*, 1999) and p(-286/+28)PBLuc in which the luciferase reporter gene is under the control of the -286/+28 fragment of the probasin gene promoter (Zhang *et al.*, 2004), and that the inhibition was reversed by co-transfection with HDAC4 siRNA but not GFP siRNA (Fig 2C). The HDAC4 vector expressed HDAC4 protein in a dose dependent manner and did not decrease the level of ectopic AR (Figs 2B). More importantly, HDAC4 siRNA decreased the level of endogenous HDAC4 protein and increased the activity of the AR (Fig 2D) without an effect on its level of expression (Fig 2E), showing that endogenous HDAC4 in 293 cells is also an AR repressor.

AR and HDAC4 form a complex *in vivo* and *in vitro*

To determine whether HDAC4 and AR interact, they were ectopically expressed in 293 cells and co-immunoprecipitations performed. As shown in Figure 3, panel A, AR and Flag-tagged HDAC4 were co-precipitated in cells expressing both proteins. In cells that express either AR or HDAC4 with control vector pcDNA3-Flag, no co-precipitations were detected, showing that the anti-Flag and anti-AR antibodies did not cross react and that the co-precipitations were not due to the interaction between AR and Flag tag.

Next, we tested whether the complex formation occurs *in vitro*. AR, His-HDAC4 and His tag were produced by transcription-coupled translation reactions in test tubes. The complex formation was analyzed by pull down assays using Ni-NTA resin. As shown in Figure 3,

panel B, immunoblotting analyses detected the AR protein, in addition to HDAC4, in the precipitates. No AR was precipitated with Ni-NTA resin in reactions containing no His-HDAC4 but with His tag, showing that the pull down assays detected a specific interaction between AR and His-HDAC4. Co-precipitations were detected in reactions containing either vehicle or R1881, suggesting a constitutive interaction independent of AR ligand binding.

For the complex formation to be meaningful, it should occur between endogenous AR and HDAC4. LNCaP cellular extracts were subjected to co-precipitation analyses with an anti-HDAC4 antibody along with rabbit IgG as a negative control. As shown in Figure 3, panel C, the AR was co-precipitated with HDAC4 by the anti-HDAC4 antibody but not by the IgG, showing a specific interaction between endogenous AR and HDAC4. As expected, synthetic androgen R1881 increased the expression of AR, but not HDAC4, protein as well as the amount of the AR precipitated by anti-HDAC4 antibody. β-actin immunoblotting showed that comparable amounts of proteins were used for precipitations.

Overall, these binding analyses show that AR and HDAC4 interact *in vitro* and *in vivo*. Ectopic AR in 293 cells appears to bind HDAC4 more efficiently in the presence of R1881, which did not occur with endogenous AR in LNCaP cells. The effect of ligand observed in 293 cells could simply be an artifact of protein overexpression. Alternatively, it may reflect a cell type specific relationship between the two molecules.

The inhibition of the AR by HDAC4 is mainly mediated through AR SUMOylation

Because HDAC4 is a deacetylase, we then investigated the possibility that the inhibition by HDAC4 was due to AR deacetylation. We transfected HDAC4 and HDAC1 into 293 cells and tested their effect on the transcriptional activity of the K632A/K633A mutant AR, in which the two known acetylated lysines were mutated to alanines (Thomas *et al.*, 2004). Contrary to our expectations, the acetylation mutant AR was significantly suppressed by HDAC4 (Fig 4A) and the degree of suppression was comparable to that of the wild type AR. HDAC1 inhibited the activity of wild type AR but not that of the acetylation mutant. Both HDACs did not significantly decrease the levels of AR protein.

Using the same assay system, we then tested the involvement of AR SUMOylation in the repression. As shown in Figure 4, panel B, HDAC4 inhibited the activity of wild type AR in a dose dependent manner but the inhibitory effect was significantly compromised by the mutation of the two known AR SUMOylation sites, K386 and K520 (Poukka *et al.*, 2000). Immunoblotting analyses showed that the levels of the wild type and mutant AR proteins were not decreased by the ectopic expression of HDAC4. Overall, these data suggest that the inhibitory effect is mainly mediated through AR SUMOylation instead of deacetylation.

HDAC4 is a positive regulator of AR SUMOylation

Because HDAC4 bound to and inhibited AR activity and the inhibition was compromised by the mutation of the known AR SUMOylation sites, it is conceivable that HDAC4 may function as an E3 ligase for AR SUMOylation. Therefore, we next tested the effect of ectopic HDAC4 on AR SUMOylation in co-transfection assays. Ectopic HDAC4 expression in 293 cells increased AR SUMOylation in a dose-dependent manner, as evidenced by the

appearance of two slower migration AR forms recognized by the anti-SUMO1 antibody (Fig 5A). The two slower migration forms were not detected in cell transfected with the SUMOylation mutant AR, confirming that they represented AR proteins that were SUMOylated at K386 and K520 (Fig 5B). Immunoblotting with anti-HA antibody detected the presence of SUMOylated AR together with HA-SUMO1, showing that SUMOylated AR was the predominant protein modified by the ectopic HA-SUMO1 (Fig 5B). Although the mature SUMO1 protein is about 11 kD in molecular weight, it migrates in SDS-PAGE much slower than expected and usually adds about 20 kD to their substrates, explaining why the two SUMOylated forms of the AR are about 120 and 180 kD in size. Immunoblotting showed that the HDAC4 vector expressed HDAC4 protein and slightly enhanced the level of AR expression, clearly to a lesser degrees than the increase in the level of AR SUMOylation. SUMO1 immunoblotting showed that HDAC4 had no effect on the level of SUMO1 expression. Subsequent pull down assays with NI-NTA resin under denatured conditions confirmed the positive effects of HDAC4 on AR SUMOylation (Fig 5C).

To test the ability of *in vitro* produced HDAC4 to stimulate AR SUMOylation, recombinant AR protein was added to SUMOylation reactions containing E1, E2, and SUMO1. AR SUMOylation was determined in the presence of control rabbit reticulocyte lysates or lysates containing HDAC4 produced by transcription-coupled translations. As shown in Figure 5, panel D, SUMOylated AR was detected in reactions containing HDAC4 and wild type AR but not in reactions with the SUMOylation mutant. The degree of AR SUMOylation depended on the amounts of HDAC4 in the reactions. In the absence of HDAC4, SUMOylated AR was weak (Fig 5D).

The AR expressed in prostate cancer cells is SUMOylated and its activity inhibited by HDAC4

To test whether the stimulation of AR SUMOylation by HDAC4 also occurs in prostate cancer cells, AR was transfected into AR-negative PC3 cells together with different amounts of HDAC4 and its levels of SUMOylation were measured. In this co-transfection experiment, the HDAC4 vector increased HDAC4 protein expression and the level of AR SUMOylation in a dose-dependent manner without an obvious effect on the level of AR or SUMO1 expression (Fig. 6A). β-actin controls showed that comparable amounts of cellular extracts were used for the analyses. Consistent with the positive effect on AR SUMOylation, HDAC4 inhibited the activity of the AR on p(-286/+28)PBLuc (Fig 6B) and ARR_3TKLuc (Fig 6C) reporters in a dose-dependent manner.

To extend our studies to endogenous AR in prostate cancer cells, we first tested the SUMOylation of endogenous AR in LNCaP cells. As shown in Figure 7, panel A, reciprocal co-immunoprecipitation analyses with anti-AR and anti-SUMO1 antibodies, not IgG, showed that the endogenous AR was SUMOylated. The endogenous AR appears to be predominantly SUMOylated on both K386 and K520 sites, which is different from ectopic AR in 293 cells and recombinant AR SUMOylated *in vitro*. Similar to ectopic AR expressed in 293 cells, the SUMOylation of endogenous AR was further increased by ectopic HDAC4 in a dose dependent manner (Fig 7B). Consistent with the increased SUMOylation, the activity of the endogenous AR on probasin (Fig 7C) and prostate specific antigen (PSA) (Fig

7D) promoter-based reporter genes was suppressed in a dose-dependent manner by increasing amounts of HDAC4. More importantly, the ectopic expression of HDAC4 suppressed the PSA induction by DHT in LNCaP cells (Fig 7E), showing that the inhibition of AR transcriptional activity by HDAC4 in reporter assays can be translated into the suppression of the biological activity of androgens.

To determine whether endogenous HDAC4 contributes to the SUMOylation of endogenous AR, LNCaP cells were transfected with various amounts of HDAC4 siRNA and the AR SUMOylation was determined. As shown in Figure 7, panel F, HDAC4 siRNA decreased the levels of endogenous HDAC4 but not AR protein, which was associated with a decrease in the SUMOylation of the endogenous AR. Consistent with the decrease in AR SUMOylation, HDAC4 siRNA, in a dose dependent manner, increased the activity of the endogenous AR on both probasin (Fig 7G) and PSA (Fig. 7H) promoters. More importantly, the stable expression of HDAC4 shRNA decreased HDAC4 expression, which was accompanied by an increase in the growth response of LNCaP cells to R1881 (Fig. 7I). These data show that endogenous HDAC4 suppresses androgen action in prostate cancer cells.

In our analyses, ectopic HDAC4 had little effect on the level of AR protein expression and androgens increased the level of the AR as well as the level of AR SUMOylation. Overall, these analyses show that HDAC4 suppresses the function of endogenous AR in prostate cancer cells through receptor SUMOylation.

Discussion

In summary, we show here that HDAC4 represses the transcriptional activity of the AR mainly through enhanced SUMOylation, instead of deacetylation. We provide multiple lines of evidences to support such an action for HDAC4. First, HDAC4 decreases the activity of the AR in reporter assays whereas other members of the class II HDACs have little effect. The inhibition occurs with multiple AR reporters and is not due to decreased level of the AR protein expression. Secondly, the HDAC4 siRNA reverses AR repression by ectopic HDAC4 and, by itself, increases the transcriptional activity of the receptor, showing the involvement of endogenous HDAC4 in the repression. Thirdly, the mutation of lysine residues in the AR that are known to be SUMOylated diminishes the inhibitory effect of HDAC4 whereas the mutation of AR acetylation sites has a modest effect, suggesting the involvement of AR SUMOylation. Consistently, HDAC4 binds to AR and increases its level of SUMOylation in intact cells and in a cell-free system. Finally, endogenous HDAC4 positively regulates the SUMOylation of endogenous AR in prostate cells and suppresses the induction of PSA expression and prostate cancer cell growth by androgens.

The AR is acetylated and its transcriptional activity regulated by histone deacetylases such as HDAC1 (Cheng *et al.*, 2004; Gaughan *et al.*, 2002) and SIRT1 (Dai *et al.*, 2007; Fu *et al.*, 2006). In the case of HDAC1, it is known that its deacetylase activity is required for the AR repression. It is not directly demonstrated whether such regulation is mediated through deacetylation of the receptor at the known acetylation sites although such an action is clearly implicated (Cheng *et al.*, 2004; Gaughan *et al.*, 2002). SIRT1, on the other hand,

deacetylates the AR at the known sites and inhibits its transcriptional and biological activities (Dai *et al.*, 2007; Fu *et al.*, 2006). Our studies are different in the sense that a deacetylase is shown to inhibit AR mainly through enhanced AR SUMOylation. It is however important to point out that our data do not totally exclude the involvement deacetylation process or the protein-protein interact between HDAC4 and AR in the repression. Our data in Figures 3 and 4 show that HDAC4 interacts with and inhibits the activity of SUMOylation-deficient mutant AR but apparently to a much lesser degree than the inhibition of wild type AR. This suggests that enhanced SUMOylation is not the sole mechanism for AR repression by the deacetylase. Previous studies have described the involvement of HDAC4 in AR repression (Jeong *et al.*, 2004; Karvonen *et al.*, 2006) and in Hela cells, it was shown that the inhibition of AR activity by HDAC4, not by HDAC7, was relieved by trichostatin (Karvonen *et al.*, 2006). Overall, these studies suggest that the mechanism underlying AR inhibition by HDAC4 may be cell type specific.

E3 ligase for SUMOylation usually functions as an adaptor protein that brings E2 conjugating enzyme to substrates to increase their SUMOylation. Our data show that HDAC4 interacts with the AR and stimulates its SUMOylation *in vitro* and in intact cells. Previous studies suggest that HDAC4 interacts with Ubc9, the SUMO E2 (Kirsh *et al.*, 2002). Overall, these analyses raise the possibility for HDAC4 to function as an E3 ligase for AR SUMOylation. This is consistent with several reports describing HDAC4 and related members of class II deacetylases, such as HDAC5, as potential SUMO E3 ligases for MEF2 and LXR (Ghisletti *et al.*, 2007; Gregoire and Yang, 2005; Zhao *et al.*, 2005). We have used HDAC4 produced in a bacterial system and found that it did not increase AR SUMOylation (data not shown). The lack of activity from bacterial HDAC4 could be due to the lack of appropriate posttranslational modifications in bacterial system required for HDAC4 to be functional as a SUMO E3 ligase. Alternatively, HDAC4 may enhance AR SUMOylation through known E3 enzymes such as members of PIAS family, which have been identified as E3 ligases for AR SUMOylation (Kotaja *et al.*, 2002; Nishida and Yasuda, 2002; Poukka *et al.*, 2000). It remains to be determined whether such E3 ligases exist in rabbit reticulocytic lysates.

The fact HDAC4 itself is a SUMOylated protein (Kirsh *et al.*, 2002) raises an interesting possibility that HDAC4 SUMOylation may regulate its ability to enhance AR SUMOylation, creating a SUMOylation signaling cascade. However, in transient co-transfection studies, we found that the SUMOylation-deficient HDAC4, HDAC4(K559R), increased AR SUMOylation and decreased its transcriptional activity to levels comparable to what was caused by wild type HDAC4 (data not shown). The analyses suggest that HDAC4 SUMOylation is required neither for its SUMOylation promoting activity toward the AR nor for its ability to inhibit AR activity. This is different from the requirement of HDAC4 SUMOylation for its full deacetylase activity (Kirsh *et al.*, 2002). It is thus possible that HDAC4 SUMOylation may differentially regulate its deacetylase and SUMOylation-promoting activities by shifting HDAC4 toward protein deacetylation.

Androgens stimulate the growth of prostate cancer cells and the expression of PSA, a serum marker for prostate cancer. Our analyses show that HDAC4 increases and its siRNA decreases the SUMOylation of endogenous AR in prostate cancer cells. The changes in AR

SUMOylation are associated inversely with alterations in the receptor's transcriptional activity and its ability to stimulate PSA expression and prostate cancer growth. These analyses suggest that HDAC4 suppresses the positive effect of androgens in prostate cancer cells, which is consistent with a recent report that SENP1 catalyzes AR de-SUMOylation and helps fine tune the cellular responses to androgens in prostate cancer cells (Kaikkonen *et al.*, 2009). HDAC inhibitors have been actively investigated clinically as anticancer agents for human malignancies including prostate cancers. However, the transcriptional activity of the AR is known to be negatively regulated by HDAC-mediated deacetylation and HDAC inhibitors may potentially enhance androgen induction of prostate cancer growth. Enhancement of AR SUMOylation through targeted increase of the SUMO E3 ligase activity associated with HDAC4 may offer an alternate strategy for prostate cancer intervention.

Materials and Methods

Materials

17β-hydroxy-17α-methyl-19-norandrost-4,9,11-trien-3-one (methyltrienolone or R1881) was from New England Nuclear (Perkin-Elmer Life Sciences, Boston, MA, USA). 5α-androstan-17β-ol-3-one (dihydrotestosterone or DHT) was from Sigma (St. Louis, MO). Charcoal-stripped fetal bovine serum (sFBS) was from HyClone (Logan, UT). 293, PC3 and LNCaP cells are all from American Tissue Culture Collection. 293 and PC3 cells were maintained in Dulbecco' modified Eagle's medium (DMEM) and LNCaP cells in Roswell Park Memorial Institute (RPMI) 1640 with 10% FBS. PG-21 and rabbit anti-AR (Upstate Biotechnology, Lake Placid, NY), M2 and rabbit anti-Flag (Sigma, St. Louis, MO), anti-HDAC4 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-SUMO1 (Zymed Laboratories Incorporation, South California, CA) and anti-β-actin (Sigma, St. Louis, MO) antibodies are all from commercial sources. Plasmids expressing HDACs (Zhang *et al.*, 2004), pCMVβGal (Li *et al.*, 2001), ARE_2e1bLuc (first described as pPRE/GRE.E1b.LUC) (Nawaz *et al.*, 1999), pCMVhAR (Li *et al.*, 2001), K632A/K633A mutant AR (Thomas *et al.*, 2004), pcDNA-Flag-K386R/K520R AR (Poukka *et al.*, 2000), p(-286/+28)PBLuc (Zhang *et al.*, 2004), ARR_3TKLuc (Kasper *et al.*, 1999), PSALuc (Snoek *et al.*, 1998), His-HD4 (Miska *et al.*, 1999), HA-SUMO1 and His-SUMO1 (Pan and Chen, 2005) have previously been described. pSilencer Neo vector and the control GFP siRNA vector were from Ambion, An Applied Biosystems Business (Austin, TX). siRNA vector against HDAC4 was generated by inserting targeting sequence, 5′ GACGGCCAGTGGTCACTG 3′, into the pSilencer Neo vector.

Transient transfections and reporter assays

For reporter assays, cells were plated in 6-well plates (2×10^5 per well) and one day after plating, cells were transfected for 5 hours by Lipofectamine Plus or Lipofectamine 2000 (for LNCaP cells) following the protocol provided by Gibco/BRL. Transfected cells were cultured in medium containing 5% sFBS and treated with androgens or vehicle for 24 hours. Cellular extracts were prepared by directly adding lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N′,N′,N′,N′-tetraacetic acid, 10% glycerol, 0.2% Triton X-100) to the cells on ice. Luciferase activity was determined using the

luciferase assay system or the dual luciferase assay system from Promega Corporation (Madison, WI) following the company's protocol. β-galactosidase (β-gal) activity was determined as previously described (Bai and Weigel, 1996). Duplicate or triplicate samples were assayed for each reporter activity which was normalized with β-gal activity from co-transfected β-gal expression vector to minimize the artifact caused by variation of transfection efficiency.

Immunological assays

For immunoprecipitations, 2×10^6 cells in 100 mm dishes were transfected and/or treated as described above for reporter assays. Cellular extracts were prepared and precipitations performed as described (Li *et al.*, 2003). For immunoblotting, cellular extracts or precipitates were separated on SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, probed with cognate antibodies, and visualized with enhanced chemiluminescence as described (Lee *et al.*, 2000).

Ni-NTA pull-down assays

To assay the *in vitro* interaction between AR and HDAC4, the two proteins were produced *in vitro* using TNT®-coupled Reticulocyte Lysate System (Promega, Madison, WI) following the company's protocol. 6 μl of the expressed HDAC4 and 12 μl of the expressed AR were added to 100 μl of buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.2) and incubated at 4°C for 3 hrs. The reactions were then mixed with 50 μl Ni-NTA resins (buffer: resin at 4:1) and incubated over night with shaking. After washing with buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.2) for 3 times, the proteins eluted with buffer 3 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 7.2) and separated on SDS-PAGE gel for immunoblotting analysis.

In vitro SUMOylation assays

To determine the effect of HDAC4 on AR SUMOylation *in vitro*, Flag-AR, Flag-K386R/K520R AR and HDAC4 were produced *in vitro* using either TNT®-coupled Reticulocyte Lysate System (Promega, Madison, WI). Flag-AR was purified by M2 beads and its SUMOylation but HDAC4 was assayed in an *in vitro* SUMOylation system (LAE Biotech International). 1 μg of purified either wild type or the SUMO mutant AR was added to a 20 μl reaction containing SUMO1, E1, conjugate E2 and control reticulocyte lysates or lysates containing HDAC4. The reactions were carried out at 30°C for 2.5 hours, boiled in SDS sample buffer and analyzed in a 5% SDS–PAGE to better separate SUMOylated and non-SUMOylated AR. AR and modified AR were detected by immunoblotting with the PG-21 anti-AR antibody.

ELISA assays for measuring PSA

LNCaP cells were plated in RPMI 1640 containing 10% FBS. After cells attached, they were starved in RPMI 1640 with 1% charcoal-stripped FBS for 48 hrs to deplete androgens, transfected with HDAC4 and treated with DHT for 24 hrs. The medium was collected and PSA levels were determined using the Tandem-E PSA ImmunoEnzyMetric Assay Kit (Hybritech Inc., San Diego, CA) following manufacturer's protocols. The absorbance at 405

nm was measured using a UV spectrophotometer. PSA concentration (ng/ml) was determined based on a standard curve generated with PSA controls of known concentrations provided by the kit.

Cell growth assays

To test the effect of HDAC4 on androgen induced cell growth, LNCaP cells were transfected with GFP or HDAC4 shRNA vectors (OriGene Technologies, Rockville, MD) and stable pools were generated after selection with puromycin. LNCaP cells and shRNA stable pools were depleted of androgens by culturing in 1% charcoal stripped FBS for two days and treated with 10 nM R1881 for 3 days. Cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Absorption at 595 nm was measured in a MRX microplate reader (DYNEX technologies, Chantilly, VA). Percentage of cell growth was calculated by the subtraction and division with OD_{595} value at time zero. In each growth assay, eight samples were analyzed in parallel for each data point. Student *t* test was applied to derive the *p* values.

Acknowledgments

The authors thank Drs. X. J. Yang for HDAC4 vector, R. J. Matusik for probasin based AR reporter genes, P. R. Rennie for the PSA based reporter gene, G. Jenster for GST-AR-NT construct, J. J. Palvimo for AR SUMOylation mutants, and A. P. Lieberman for AR acetylation mutants. The work was supported by the Public Health Service grant CA93666 and CA111334 (W.B), a DOD Prostate Cancer grant DAMD17-02-1-0140 (W.B) and grants 09KT-03 and 06BCBG1 (W.B) from the Florida Department of Health.

References

Bai W, Weigel NL. Phosphorylation of Ser211 in the chicken progesterone receptor modulates its transcriptional activity. J Biol Chem. 1996; 271:12801–6. [PubMed: 8662804]

Blok LJ, de Ruiter PE, Brinkmann AO. Androgen receptor phosphorylation. Endocr Res. 1996; 22:197–219. [PubMed: 8875135]

Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, et al. Mechanisms of androgen receptor activation and function. J Steroid Biochem Mol Biol. 1999; 69:307–13. [PubMed: 10419007]

Chang CS, Kokontis J, Liao ST. Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. Proc Natl Acad Sci U S A. 1988; 85:7211–5. [PubMed: 3174628]

Cheng J, Wang D, Wang Z, Yeh ET. SENP1 enhances androgen receptor-dependent transcription through desumoylation of histone deacetylase 1. Mol Cell Biol. 2004; 24:6021–8. [PubMed: 15199155]

Dai Y, Ngo D, Forman LW, Qin DC, Jacob J, Faller DV. Sirtuin 1 is required for antagonist-induced transcriptional repression of androgen-responsive genes by the androgen receptor. Mol Endocrinol. 2007; 21:1807–21. [PubMed: 17505061]

Dotzlaw H, Moehren U, Mink S, Cato AC, Iniguez Lluhi JA, Baniahmad A. The amino terminus of the human AR is target for corepressor action and antihormone agonism. Mol Endocrinol. 2002; 16:661–73. [PubMed: 11923464]

Evans RM. The steroid and thyroid hormone receptor superfamily. Science. 1988; 240:889–95. [PubMed: 3283939]

Fu M, Liu M, Sauve AA, Jiao X, Zhang X, Wu X, et al. Hormonal control of androgen receptor function through SIRT1. Mol Cell Biol. 2006; 26:8122–35. [PubMed: 16923962]

Fu M, Wang C, Reutens AT, Wang J, Angeletti RH, Siconolfi-Baez L, et al. p300 and p300/cAMP-response element-binding protein-associated factor acetylate the androgen receptor at sites

governing hormone-dependent transactivation. J Biol Chem. 2000; 275:20853–60. [PubMed: 10779504]

Fu M, Wang C, Zhang X, Pestell RG. Acetylation of nuclear receptors in cellular growth and apoptosis. Biochem Pharmacol. 2004; 68:1199–208. [PubMed: 15313417]

Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. J Biol Chem. 2002; 277:25904–13. [PubMed: 11994312]

Ghisletti S, Huang W, Ogawa S, Pascual G, Lin ME, Willson TM, et al. Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. Mol Cell. 2007; 25:57–70. [PubMed: 17218271]

Gregoire S, Yang XJ. Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. Mol Cell Biol. 2005; 25:2273–87. [PubMed: 15743823]

Guo Z, Dai B, Jiang T, Xu K, Xie Y, Kim O, et al. Regulation of androgen receptor activity by tyrosine phosphorylation. Cancer Cell. 2006; 10:309–19. [PubMed: 17045208]

Jeong BC, Hong CY, Chattopadhyay S, Park JH, Gong EY, Kim HJ, et al. Androgen receptor corepressor-19 kDa (ARR19), a leucine-rich protein that represses the transcriptional activity of androgen receptor through recruitment of histone deacetylase. Mol Endocrinol. 2004; 18:13–25. [PubMed: 14576337]

Jones PL, Shi YB. N-CoR-HDAC corepressor complexes: roles in transcriptional regulation by nuclear hormone receptors. Curr Top Microbiol Immunol. 2003; 274:237–68. [PubMed: 12596910]

Kaikkonen S, Jaaskelainen T, Karvonen U, Rytinki MM, Makkonen H, Gioeli D, et al. SUMO-specific protease 1 (SENP1) reverses the hormone-augmented SUMOylation of androgen receptor and modulates gene responses in prostate cancer cells. Mol Endocrinol. 2009; 23:292–307. [PubMed: 19116244]

Karvonen U, Janne OA, Palvimo JJ. Androgen receptor regulates nuclear trafficking and nuclear domain residency of corepressor HDAC7 in a ligand-dependent fashion. Exp Cell Res. 2006; 312:3165–83. [PubMed: 16860317]

Kasper S, Rennie PS, Bruchovsky N, Lin L, Cheng H, Snoek R, et al. Selective activation of the probasin androgen-responsive region by steroid hormones. J Mol Endocrinol. 1999; 22:313–25. [PubMed: 10343290]

Kirsh O, Seeler JS, Pichler A, Gast A, Muller S, Miska E, et al. The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase. Embo J. 2002; 21:2682–91. [PubMed: 12032081]

Kotaja N, Karvonen U, Janne OA, Palvimo JJ. The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1. J Biol Chem. 2002; 277:30283–8. [PubMed: 12060666]

Lee H, Jiang F, Wang Q, Nicosia SV, Yang J, Su B, et al. MEKK1 activation of human estrogen receptor alpha and stimulation of the agonistic activity of 4-hydroxytamoxifen in endometrial and ovarian cancer cells. Mol Endocrinol. 2000; 14:1882–96. [PubMed: 11075819]

Li P, Lee H, Guo S, Unterman TG, Jenster G, Bai W. AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. Mol Cell Biol. 2003; 23:104–18. [PubMed: 12482965]

Li P, Nicosia SV, Bai W. Antagonism between PTEN/MMAC1/TEP-1 and androgen receptor in growth and apoptosis of prostatic cancer cells. J Biol Chem. 2001; 276:20444–50. [PubMed: 11278645]

Lin DY, Fang HI, Ma AH, Huang YS, Pu YS, Jenster G, et al. Negative modulation of androgen receptor transcriptional activity by Daxx. Mol Cell Biol. 2004; 24:10529–41. [PubMed: 15572661]

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science. 1988; 240:327–30. [PubMed: 3353727]

Miska EA, Karlsson C, Langley E, Nielsen SJ, Pines J, Kouzarides T. HDAC4 deacetylase associates with and represses the MEF2 transcription factor. Embo J. 1999; 18:5099–107. [PubMed: 10487761]

Nawaz Z, Lonard DM, Smith CL, Lev-Lehman E, Tsai SY, Tsai MJ, et al. The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. Mol Cell Biol. 1999; 19:1182–9. [PubMed: 9891052]

Nishida T, Yasuda H. PIAS1 and PIASxalpha function as SUMO-E3 ligases toward androgen receptor and repress androgen receptor-dependent transcription. J Biol Chem. 2002; 277:41311–7. [PubMed: 12177000]

Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science. 1995; 270:1354–7. [PubMed: 7481822]

Pan Y, Chen J. Modification of MDMX by sumoylation. Biochem Biophys Res Commun. 2005; 332:702–9. [PubMed: 15907800]

Poukka H, Karvonen U, Janne OA, Palvimo JJ. Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci U S A. 2000; 97:14145–50. [PubMed: 11121022]

Snoek R, Bruchovsky N, Kasper S, Matusik RJ, Gleave M, Sato N, et al. Differential transactivation by the androgen receptor in prostate cancer cells. Prostate. 1998; 36:256–63. [PubMed: 9719026]

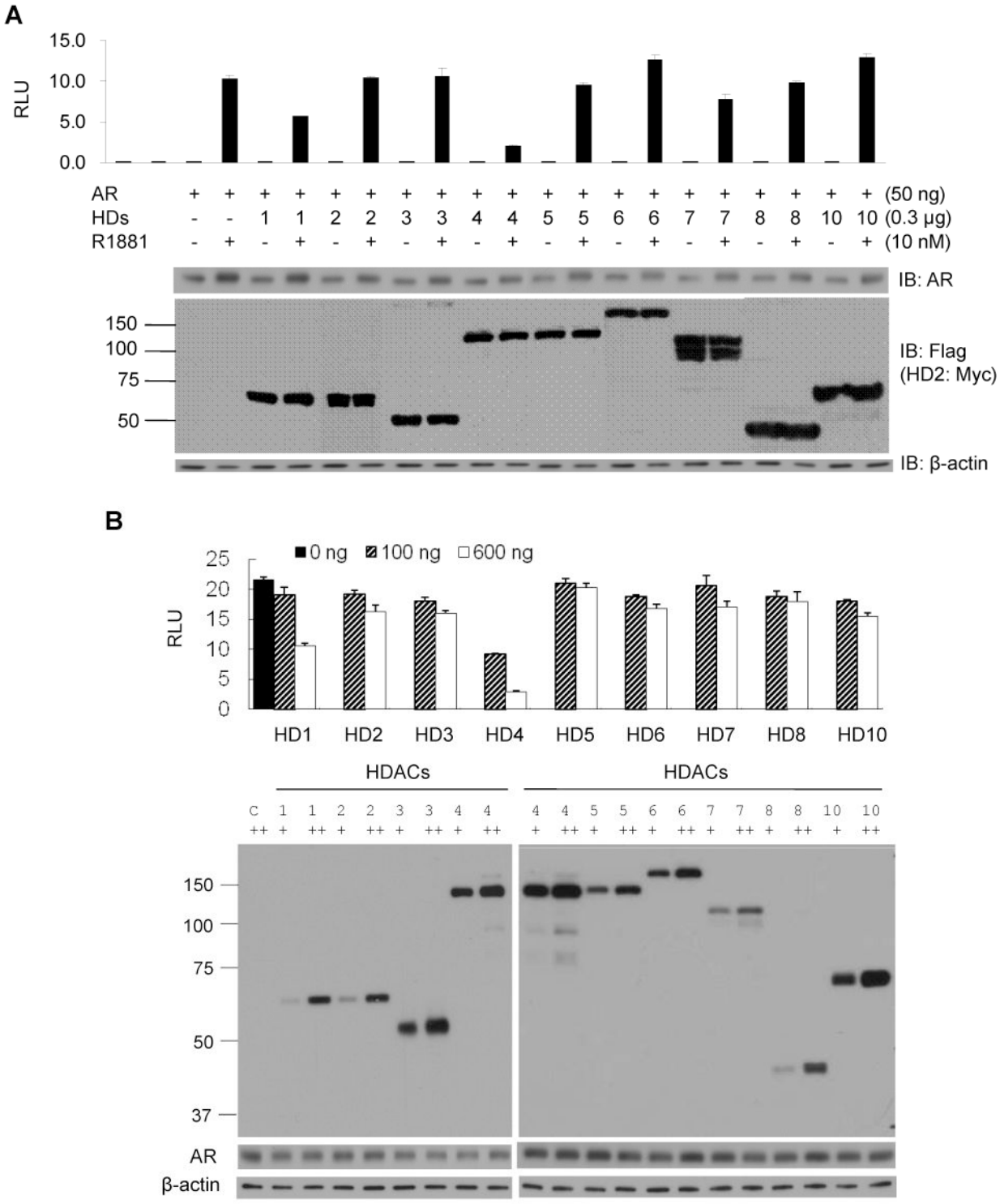
Thomas M, Dadgar N, Aphale A, Harrell JM, Kunkel R, Pratt WB, et al. Androgen receptor acetylation site mutations cause trafficking defects, misfolding, and aggregation similar to expanded glutamine tracts. J Biol Chem. 2004; 279:8389–95. [PubMed: 14670946]

Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem. 1994; 63:451–86. [PubMed: 7979245]

Xu J, O'Malley BW. Molecular mechanisms and cellular biology of the steroid receptor coactivator (SRC) family in steroid receptor function. Rev Endocr Metab Disord. 2002; 3:185–92. [PubMed: 12215713]

Zhang X, Wharton W, Yuan Z, Tsai SC, Olashaw N, Seto E. Activation of the growth-differentiation factor 11 gene by the histone deacetylase (HDAC) inhibitor trichostatin A and repression by HDAC3. Mol Cell Biol. 2004; 24:5106–18. [PubMed: 15169878]

Zhao X, Sternsdorf T, Bolger TA, Evans RM, Yao TP. Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. Mol Cell Biol. 2005; 25:8456–64. [PubMed: 16166628]



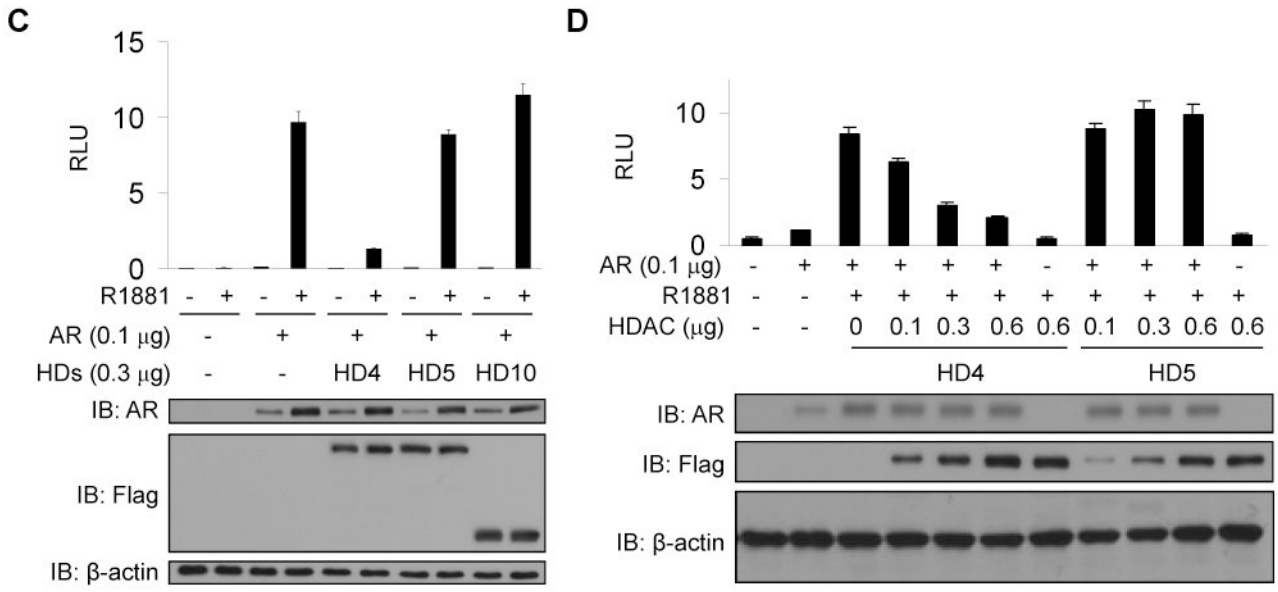


Fig. 1. Differential effects of class I and II HDACs on AR transcriptional activity

(A). 293 cells were transfected with 0.3 μg of pCMVβGal and ARR_3TKLuc together with or without indicated amounts of pCMVhAR and HDAC (HD) vectors. Transfected cells were treated for 24 hours with R1881 (+) or ethanol (-) as indicated. Luciferase activity was normalized with cognate β-gal activity. Three independent experiments were performed and a representative data were shown. Each data point is the average of samples analyzed in triplicates and the error bars denote standard deviations. (B). Cells in were transfected and AR activity determined as in panel A. All transfections included 0.05 μg of pCMVhAR together with either a control vector pcDNA-Flag (C) or two doses of HDACs, 100 ng (+) and 600 ng (++). Transfected cells were treated with 10 nM R1881 for 24 hours. The levels of AR, HDACs and β-actin expression were determined by immunoblotting (IB) with corresponding antibodies. (C) and (D). Cells were transfected and treated and AR activity determined as in panel A. The levels of AR, HDACs and β-actin expression were determined by immunoblotting (IB) with corresponding antibodies.

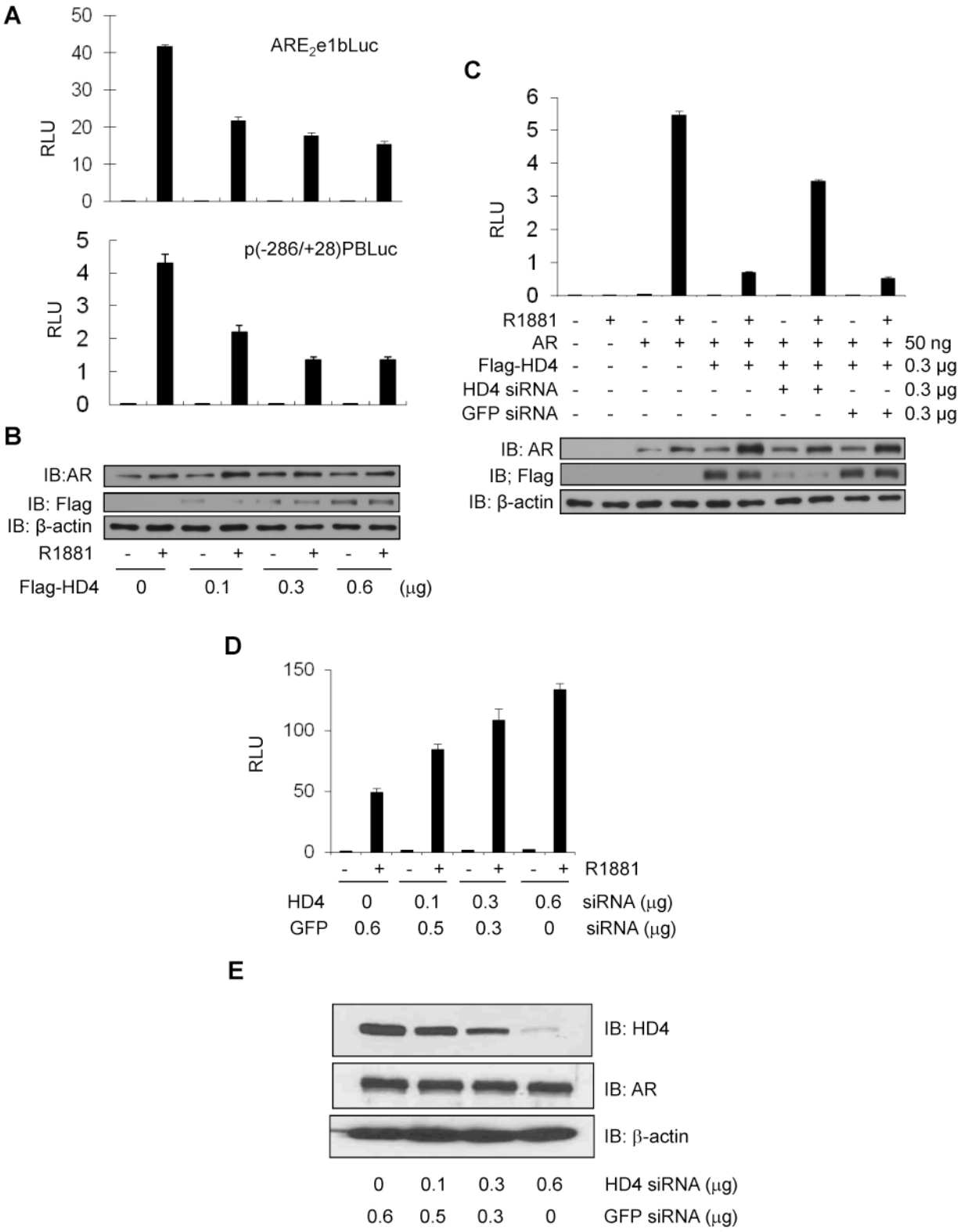


Fig. 2. Dose-dependent inhibition of AR transcriptional activity by ectopic and endogenous HDAC4 in 293 cells

(A). Cells were transfected and treated as in Fig. 1. Luciferase activity was assayed with two different AR reporter genes as indicated and normalized with β-gal activity. (B). Cells were transfected with indicated vectors, treated and the levels of AR, HD4, and β-actin expression determined by IB. (C) Cells were transfected with 0.3 μg of pCMVβGal and ARR_3TKLuc and indicated vectors and the activity of AR was measured. The levels of AR, HD4, and β-actin expression were determined by IB. (D). Cells were transfected and treated as in Fig. 1 except that HD4 siRNA vector was used in the place of HD4 expression vector. Luciferase activity was assayed and normalized with β-gal activity. (E). Cells were transfected as in panel D and the levels of AR, endogenous HD4 and β-actin determined by IB.

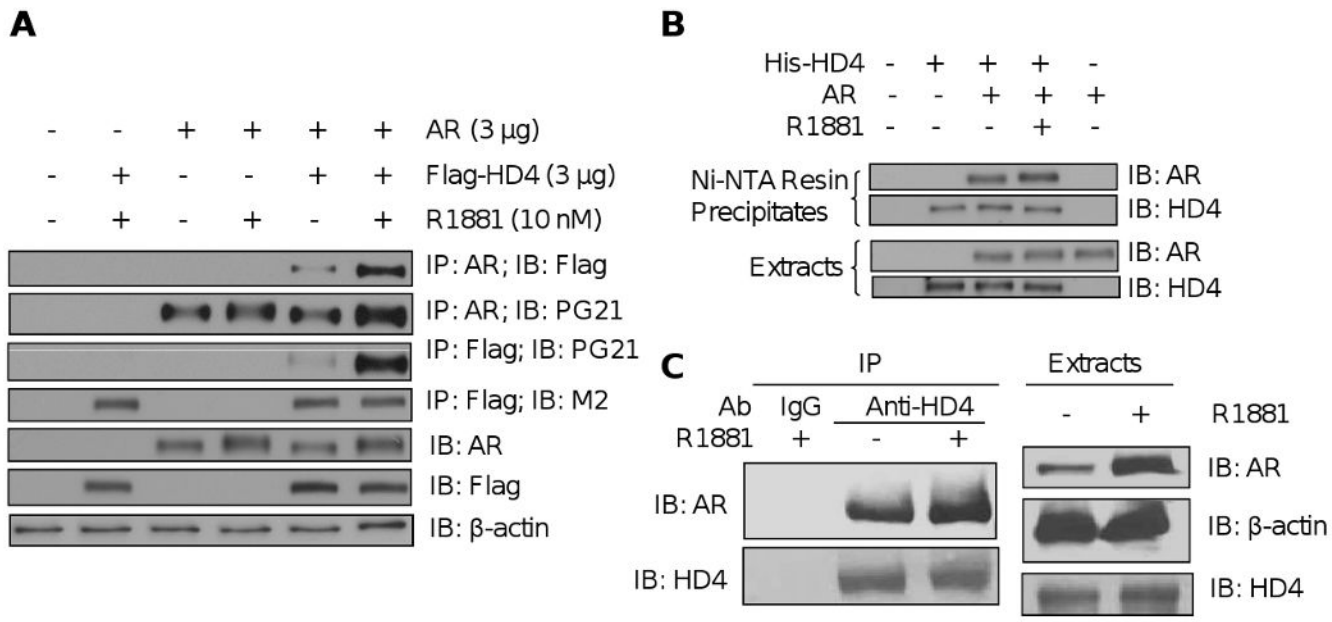


Fig. 3. Interaction between HDAC4 and AR *in vivo* and *in vitro*

(A). 293 cells were transfected with 3 μg of pCMVhAR and Flag-HD4 or pcDNA3-Flag as a control vector. Immunoprecipitations (IPs) were performed with polyclonal anti-AR antibody followed by IB with monoclonal anti-AR (PG-21) and anti-Flag (M2) antibodies. (B). Interaction between AR and HD4 *in vitro*. Interaction of AR and His-HD4 produced by *in vitro* transcription/translation was analyzed by pull down assays as described in the Methods. The AR and HDAC4 in the precipitates (upper panels) were detected by IB with PG-21 and anti-HD4, respectively. pET-30a-His was included in the *in vitro* transcription/translation reactions to serve as a control for His-HD4. The levels of AR and His-HD4 in the input (lower panels) were also shown. (C). Interaction between endogenous AR and HD4 in LNCaP cells. Cells were treated with 10 nM R1881 (+) or vehicle (-) for 24 hours and IPs performed with a rabbit IgG or anti-HD4 antibody. The AR and HD4 in the precipitates and cellular extracts were detected by IB with anti-AR and anti-HD4 antibodies. β-actin blot was included to show even loading.

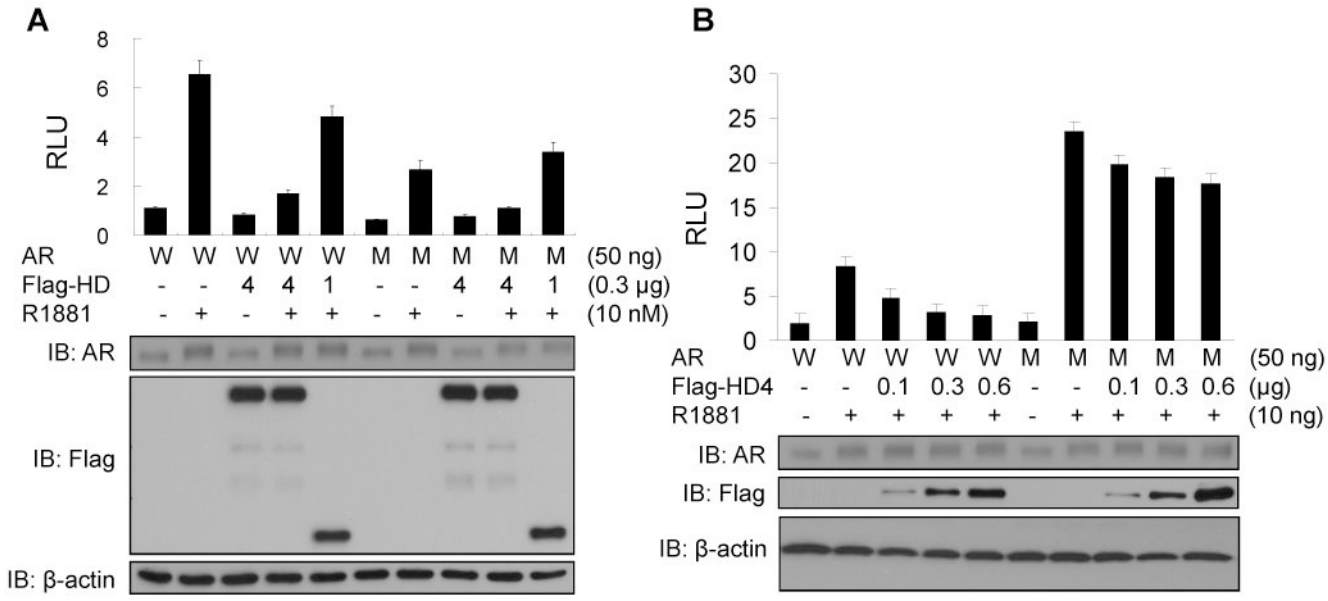


Fig. 4. Effect of HDAC4 on the transcriptional activity of acetylation- and SUMOylation-deficient mutant AR

(A) 293 cells were transfected with indicated amounts of wild type (W) or K632A/K633A mutant (M) AR, Flag-HD4, Flag-HD1 or pcDNA3-Flag control vector together with 0.3 μg ARR_3TKLuc and 0.01 μg pCMVβGal. Transfected cells were treated for 24 hours with 10 nM R1881 (+) or vehicle (-). The activity of the AR and its expression were determined in parallel analyses. For reporter assays, three independent experiments were performed and a representative data were shown. Each data point is the average of samples analyzed in triplicates and the error bars denote standard deviations. (B) 293 cells transfected and treated with R1881 as in panel A except that Flag-AR (W) or Flag-K386R/K520R (M) AR were transfected in the place of K632A/K633A. The activity of the AR and the levels of the AR, HD4 and β-actin proteins were determined.

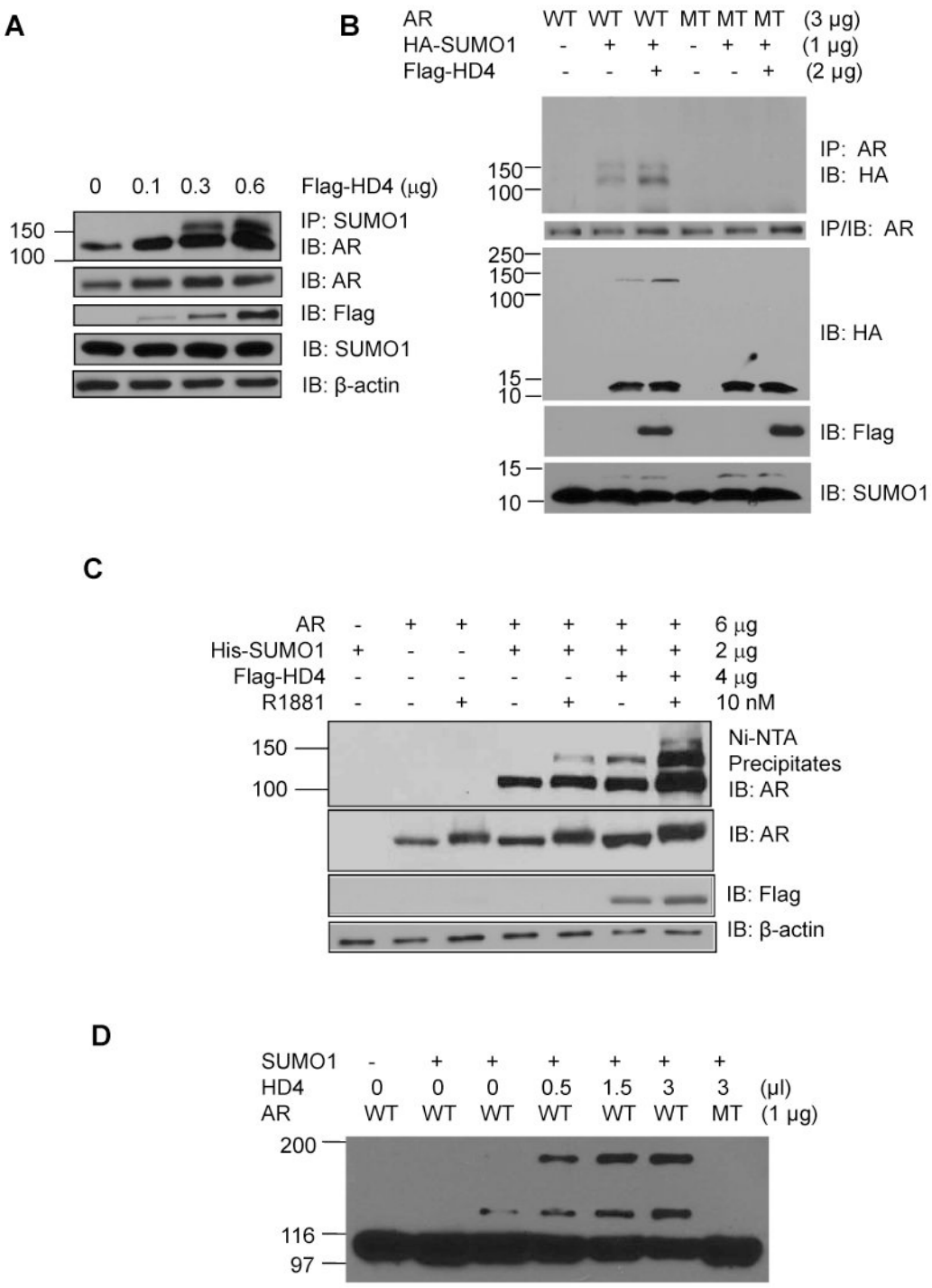


Fig. 5. Enhancement of AR SUMOylation by HDAC4 in intact cells

(A) Enhancement of ectopic AR conjugation with endogenous SUMO1 by HD4. 293 cells were transfected with 1 μg of pCMVhAR and indicated amounts of HD4 and treated for 24 hours with 10 nM R1881. AR SUMOylation was determined by IP with anti-SUMO1 followed by IB with anti-AR antibody (top panel). AR, HD4, SUMO1 and β-actin levels were determined by IB of the extracts with cognate antibodies. (B). Enhancement of AR

conjugation with HA-SUMO1 by HD4. 293 cells were transfected with indicated amounts of Flag-AR (WT) or Flag-K386R/K520R (MT) AR, HA-SUMO1 and HD4 and treated with 10 nM R1881 for 24 hours. AR SUMOylation was determined by IP with anti-AR followed by IB with anti-HA antibody (top panel). AR, HD4, HA-SUMO1, SUMO1 and β-actin levels were determined by IB of the extracts with cognate antibodies. (C). Enhancement of AR conjugation with His-SUMO1 by HD4. Cells were transfected and treated as in panel A except that His-SUMO1 was included in the transfections. AR SUMOylation was determined by pull down assays with Ni-NTA resin followed by IB with anti-AR as described in the Methods. AR, HD4 and β-actin levels were determined by IB. (D). *In vitro* SUMOylation of AR by HDAC4. HD4 and Flag-AR (WT) or Flag-K386R/K520R (MT) AR were produced by *in vitro* transcription-coupled translation in rabbit reticulocytic lysates. I*n vitro* SUMOylation assays were performed in the presence of recombinant SUMO1, E1 and E2. AR and SUMOylated AR were detected by IB with anti-AR antibody.

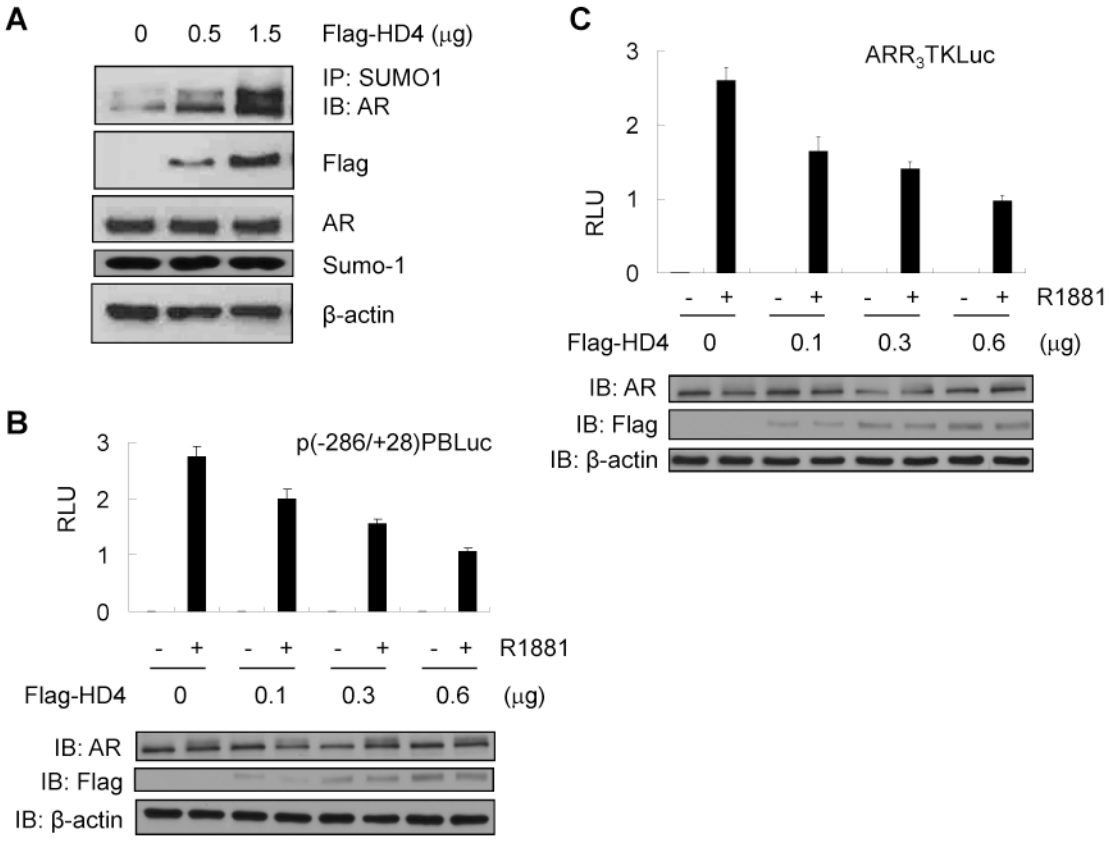
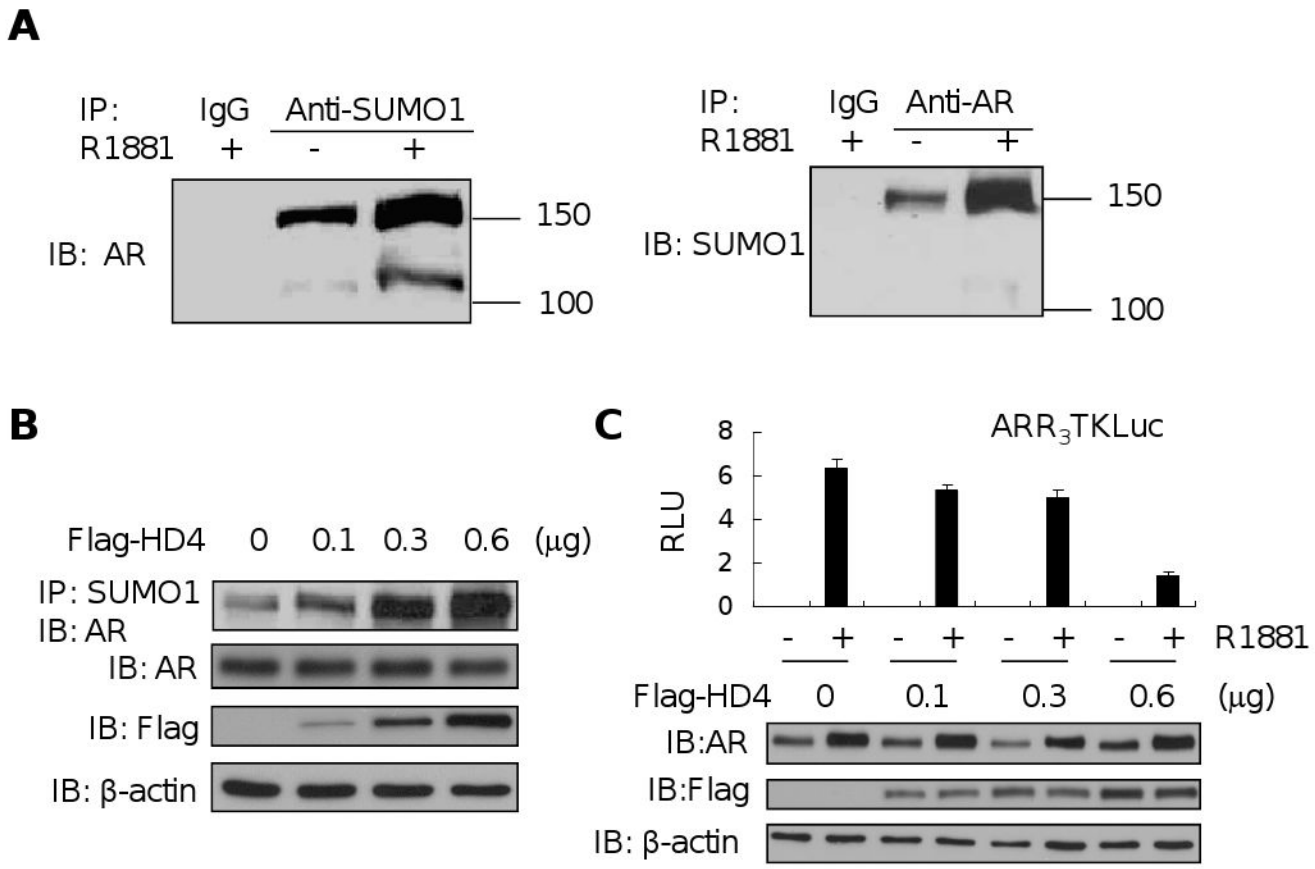


Fig. 6. AR SUMOylation and inhibition by HDAC4 in prostate cancer cells

(A). HD4 increases the level of AR SUMOylation in prostate cancer cells. PC3 ells were transfected with 1 μg of pCMVhAR and indicated amounts of HD4 and treated with 10 nM R1881. AR SUMOylation was detected by IP with anti-SUMO1 followed by IB with anti-AR antibody (top panel). AR, SUMO1, HD4 and β-actin levels were determined by IB with cognate antibodies. (B) and (C) PC3 cells were transfected with 0.05 μg of pCMVhAR, 0.3 μg of pCMVβGal, 0.3 μg of indicated reporter genes and indicated amounts of Flag-HD4 and treated with ethanol (-) or 10 nM R1881 (+) for 24 hours. Luciferase activity was measured and normalized with β-gal activity. AR, HD4 and β-actin levels were determined by IB with cognate antibodies.



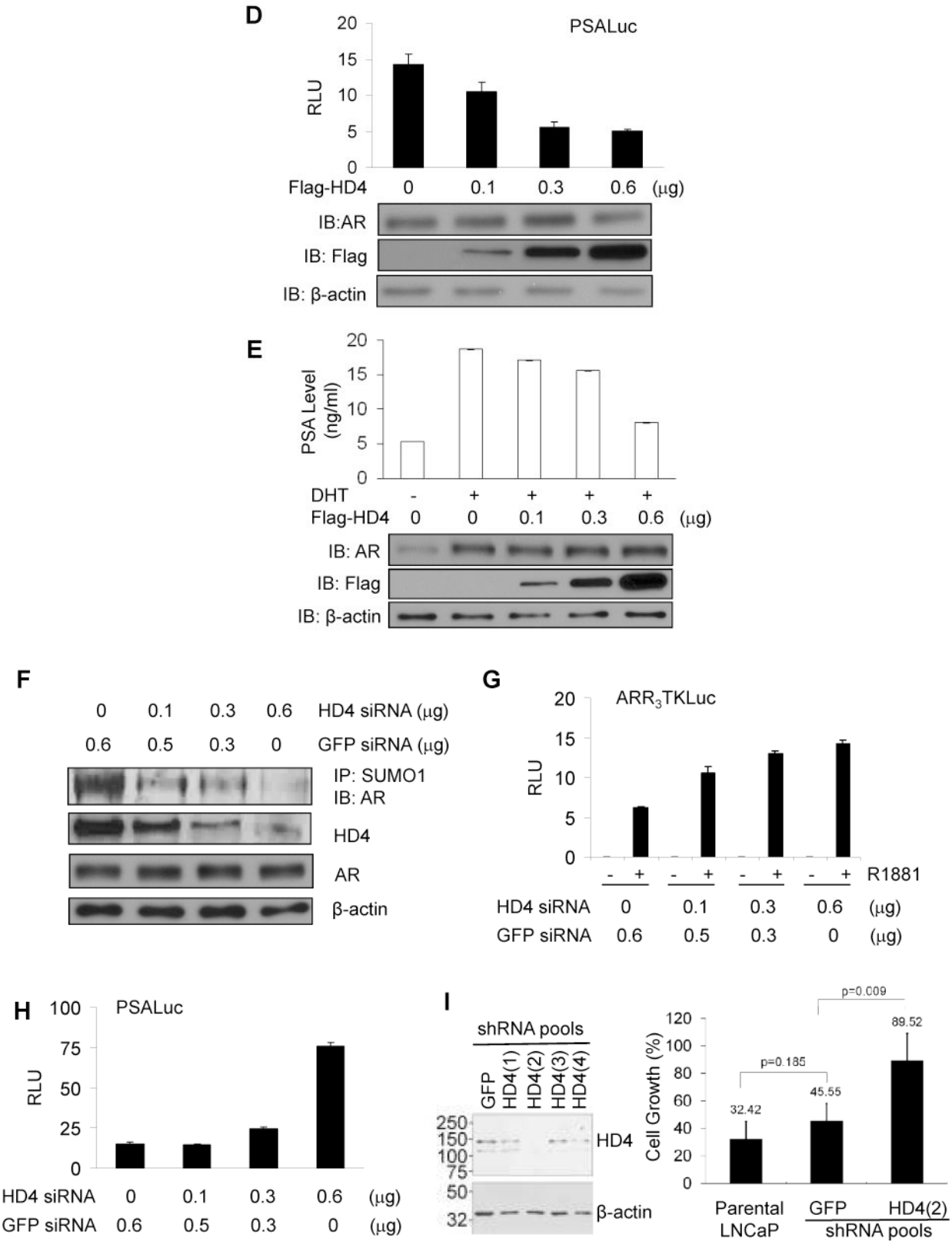


Fig. 7. Regulation of the SUMOylation and activity of endogenous AR by HDAC4

(A). Endogenous AR is SUMOylated by SUMO1. SUMOylation of endogenous AR was determined by reciprocal co-precipitations with indicated antibodies. Mouse IgG was included as a negative control. (B). Induction of endogenous AR SUMOylation by HD4. LNCaP cells were transfected with indicated amounts of Flag-HD4. AR SUMOylation was detected by IP with anti-SUMO1 followed by IB with anti-AR antibody (top panel). AR, HD4 and β-actin levels were determined by IB with cognate antibodies. (C) and (D). Inhibition of the transcriptional activity of endogenous AR by HD4 on reporter genes derived from probasin (panel C) and PSA (panel D) promoters. LNCaP cells were transfected with 0.3 μg of pCMVβGal, 0.3 μg of reporter genes and indicated amounts of Flag-HD4 and treated with or without 10 nM R1881. Luciferase activity was measured and normalized with β-gal. AR, HD4 and β-actin levels were determined by IB with cognate antibodies. (E) Suppression of androgen induction of PSA expression by HD4. Androgen depleted LNCaP cells were transfected with indicated amounts of HD4 and treated with 10^{-6} M DHT for 24 hrs. PSA level was measured as described in the Methods. Triplicate samples were analyzed for each data point and error bars stand for standard deviations. AR, HD4 and β-actin levels were determined by IB with cognate antibodies. (F) LNCaP cells were transfected with indicated amounts of siRNA vectors. AR SUMOylation was detected by IP with anti-SUMO1 followed by IB with anti-AR (the top panel). The levels of endogenous AR, HD4 and β-actin were determined by IB with cognate antibodies. (G) and (H). LNCaP cells were transfected with 0.3 μg of pCMVβGal, 0.3 μg of reporter genes and indicated amounts of siRNA vectors and treated with or without 10 nM R1881. Luciferase activity was determined and normalized with β-gal activity. (I). LNCaP were transfected with GFP and HD4 shRNA vectors and stable pools established after selection in the presence of 2 μg/ml puromycin. HD4 and β-actin protein expression was determined by IB with cognate antibodies. Androgen regulation of cell growth was determined as described in the Methods. Percentages of cell growth were calculated and presented.