Supplementary Materials and Methods

Immunoprecipitation

To investigate an AR-SET9 interaction, LNCaP cells were grown in RPMI-1640 media containing 10% steroid-depleted (SD)-feotal calf serum (FCS) for 40-hours prior to addition of 10 nM DHT for 8-hours and then subject to immunoprecipitation (IP) using an anti-SET9 antibody (Millipore). Immunoprecipitates were boiled in SDS-sample buffer (10% β-mercaptoethanol, 125 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.005% bromophenol blue) for 10 minutes and subject to Western analysis, as described in Brady *et al*., (1999) using an anti-AR antibody (BD Pharmingen). For immunoprecipitation of ectopically expressed proteins, 5 x 10^5 HEK293T cells were plated per 90-mm dish (Corning) in FCS-containing RPMI-1640 media, and transfected as above with 1 µg of either pFlag-AR or pFlag-AR_{K632R} together with 1 µg wild-type or mutant pFlag-SET9 expression vectors. 48-hours post-transfection, cells lysates were subject to immunoprecipitation using an anti-AR antibody (Santa Cruz Biotechnology: C-19) followed by immunoblotting with an anti-Flag antibody (Sigma: M2). 10 nM DHT was routinely used for all experiments. Duration of each treatment is indicated in corresponding figures and figure legends.

To assess AR methylation in LNCaP cells, AR protein was immunoprecipitated using anti-AR antibodies (C-19: Santa Cruz Biotechnology and BD Calbiochem) and samples subjected to immunoblotting using a pan-methyl-lysine antibody (Abcam) or methyl-lysine antibody (Abcam) (see supplementary Figure S1). The experiment was repeated in HEK293T cells using the 90-mm dish transfection procedure described above and cell lysates immunoprecipitated with anti-AR antibodies and analysed by Western blot using the pan-methyl-lysine antibody (Abcam). For *in vitro* SET9-AR IP, cold methylation reaction

mixtures were immunoprecipitated using an anti-SET9 antibody (Millipore) and lysates subject to Western analysis using a Penta-His antibody (Qiagen).

SET9 knockdown

The siRNA duplex for SET9 knockdown has been previously described and is used in all of the transient SET9 knockdown experiments (Chuikov *et al*., 2004). LNCaP cells were transfected with either the SET9 oligonucleotide or the non-silencing (N/S) oligonucleotide (UUCUCCGAACGUGUCACGUDTCT) using Lipofectamine RNAiMax (Invitrogen) according to manufacturers recommendations. Transfected cells were incubated for 72-hours in FCS-containing RPMI-1640 prior to either Western, immunoprecipitation or quantitative PCR analyses. Proliferation assays, incorporating siRNA-transfected LNCaP cells, were performed using WST-1 reagent according to manufacturer's recommendations (Roche). For quantitative PSA expression analysis, 1 x 10^5 N/S or SET9 siRNA-transfected LNCaP cells or pFlag-AR or pFlag-AR_{632} transfected PC3 cells were lysed in Trizol reagent (Invitrogen), cDNA synthesised as described (Logan *et al*., 2006) and incorporated into real-time PCR analysis using the following primer combinations, PSA-F: ATGTGGGTCCCGGTTGTCT; PSA-R: AGCGCCAATCCACGTCA, and GAPDH-F: CGACCACTTTGTCAAGCTCA; GAPDH-R: GGGTCTTACTCCTTGGAGGC, to determine relative PSA expression. Quantitative PCR and analysis was performed as described (Logan *et al*., 2006). Data represents the mean of three independent experiments performed in triplicate +/- standard error. For caspase 3 expression analysis by flow cytometry, LNCaP cells transfected with siRNA against SET9 or p53 (GACUCCAGUGGUAAUCUAC) (Chen *et al*.) for 48 hours prior to treatment with or without 0.5 μM doxorubicin for 24 hours were subject to caspase 3 analysis according to the manufacturer's recommendations (Becton Dickinson) using a FACScan flow cytometer (Becton Dickinson).

The stable doxycycline-inducible N/S and SET9 knockdown LNCaP cell lines LNCaP-$N/S_{K/D}$ and LNCaP-$SET9_{K/D}$ respectively, were generated by lentiviral transduction of LNCaP cells with either pTRIPZ-N/S or pTRIPZ-SET9 (target sequence: AGCACTTTATGGGAAATTTATT) shRNA vectors (as recommended by the manufacturers, Open Biosystems) prior to 10 µg/ml puromycin selection for 2-weeks. Inducible expression of both shRNAs by 1 µg/ml doxycycline treatment was analysed by fluorescent imaging of the co-expressed diagnostic marker Turbo-RFP protein after 72 hour doxycycline treatment. Western analysis of doxycycline-treated LNCaP-$SET9_{K/D}$ cells confirmed approximately 80% knockdown of SET9, but not in the LNCaP-$N/S_{K/D}$ as expected.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed in LNCaP cells and PC3 cells as described (Gaughan *et al*., 2005). For immunoprecipitation, 2 µg of each of the following antibodies were used: AR (Santa Cruz Biotechnology), SET9 (Millipore), mono-methylated histone H3 lysine 4 (H3K4me1) (Abcam) and non-specific isotype control antibodies (Dako). Recovered DNA was subject to quantitative PCR analysis as described (Logan *et al*., 2006) using the following primer combinations, ARE I-F: CCTAGATGAAGTCTCCATGAGCTACA; ARE I-R: GGGAGGGAGAGCTAGCACTTG, and ARE III-F: GCCTGGATCTGAGAGAGATAT CAT C; ARE III-R: ACACCTTTTTTTTCTGGATTG. Data represents the average percentage input from three individual ChIP assays.

For ChIP analysis using either the LNCaP-$N/S_{K/D}$ and LNCaP-$SET9_{K/D}$ cell lines 3 x 10^6 cells were plated onto 150-mm dishes in SD-FCS-containing RPMI-1640 media in the absence of puramycin and left to grow for 24-hours prior to the addition of doxycycline for 72-hours.

After confirming shRNA expression using live cell fluorescence imaging, cells were pelleted and subject to ChIP analysis as described above using AR, H3K4me1 and control antibodies.

AR stability and movement assays

HEK293T cells grown in FCS-containing RPMI-1640 media were transfected with expression vectors for pFlag-AR and either SET9 or $SET9_{H297A}$ and after 39-hours, were treated with 1 μM cycloheximide (CHX) for 0-9-hours prior to Western analysis using an anti-AR antibody. N/S or SET9 siRNA-transfected LNCaP cells were grown for 64-hours and then treated with CHX for 0-8-hours prior to AR analysis as above.

To assess androgen-dependent AR movement in response to SET9 knockdown, LNCaP cells grown in SD-FCS containing RPMI-1640 media were transiently transfected with either N/S or SET9 oligonucleotides as described above for 66-hours and then treated with or without 10 nM DHT for 6-hours prior to nuclear-cytoplasmic extraction (NE-PER, Pierce). Extracts were separated by PAGE and subject to immunoblotting with antibodies to AR and respective controls for nuclear and cytoplasmic compartments; PARP1 and GAPDH. HEK293T cells were transiently transfected with pFlag-AR or pFlag-AR_{K632R} for 42-hours in SD-FCS-containing RPMI-1640 media and then treated with 10 nM DHT for a 0-6 hour time-course (as indicated in Figure 5D). Cells were subject to nuclear-cytoplasmic extraction and Western analysis as described for LNCaP cells.

Cell culture and DNA transfection

Cell culture and DNA transfection were performed as described previously (Brady *et al*., 1999). HEK293T, LNCaP and PC3 cells were maintained in RPMI-1640 media containing 10% FCS (Sigma). For reporter assays, 1 x 10^4 HEK293T cells were routinely plated per well in 24-well microtitre plates (Corning). After 24-hours, the cells were transfected using

Lipofectamine LTX (Invitrogen) according to the manufacturer's recommendations and after 48-hours were harvested and assayed for luciferase and β-galactosidase activity as described previously (Brady *et al*., 1999). For androgenic stimulation experiments, cells were grown in media supplemented with 10% serum-stripped FCS (Hyclone) prior to transfection as above. 24-hours prior to harvesting, cells were treated with or without 10 nM dihydrotestosterone (DHT). Unless indicated, co-transfection experiments for reporter assays using HEK293T and LNCaP cells incorporated 20 ng of pFlag-AR or pFlag-AR_{K632R} (HEK293T cells only) together with 25-100 ng wild-type or mutant SET9 expression vector and 100 ng of each reporter construct. Fold increases were determined by comparing activity with empty pCMV-driven vector. Each experiment was performed in quadruplicate and repeated a minimum of three times. Data represents an average of three repeats +/- standard error.

For mammalian two-hybrid reporter analysis, HEK293T cells grown in SD-FCS-containing RPMI-1640 media were transiently transfected with pM-AR-DBD/LBD and pVP16-AR-TD together with either wild-type SET9, methylase-dead $SET9_{H297A}$ or SRC-1 expression vectors for 24-hours prior to 10 nM DHT stimulation for an additional 24-hours before harvesting and analysis. Data represents the average of three independent experiments performed in quadruplicate +/- standard error.

Mass spectrometry

1 µg $AR_{623-640}$ peptide was incorporated into an *in vitro* methylation assay with and without 1 µg SET9 for 1 hour. 2 µl of the reaction mixture was injected on an Ultimate3000 system via a 10mm PepMap trap column (Dionex, UK) and separated on a 15 cm PepMap column (Dionex, UK) with a linear gradient from 4% acetonitrile, 0.1% formic acid to 65% acetonitrile/0.1% formic acid over 42 min at 300 nl/min. The eluent was sprayed online into an HCT Ultra Proteome Discovery System (Bruker, UK), MS data were acquired in the

standard/enhanced mode and MSMS data were collected in ultra scan mode. Ions for the AR_{WT} peptide were on an inclusion list for CID fragmention in the 3D ion trap of the HCT mass spectrometer. MSMS spectra were collected with an ICC of 300000. Data were analysed manually using Bruker DataAnalysis 4.0 and in-house written spreadsheets.

Supplementary Figure legends

Figure S1. LNCaP cells grown in serum-containing media were transiently transfected with either non-silencing (N/S) or SET9 siRNAs for 72 hours prior to immunoprecipitation using an anti-AR antibody followed by Western analysis using a methyl-lysine antibody (Abcam).

Figure S2. A. CID MSMS spectrum of the non-methylated peptide. B. CID MSMS spectrum of the methylated peptide. Both peptides yielded excellent low energy CID fragmentation spectra. The zoomed in spectral region in panes C and D shows that the b7, b8 and b9 ions are identical for both peptides, indicating that the methylation has not occurred on lysine 630. All fragment ions beyond b10 are 14 Da higher for the methylated peptide (D) than for the non-methylated peptide (C), indicating that the methylation has occurred on lysine 632.

Figure S3. A. HEK293T cells grown in serum-containing media were transiently transfected with AR and either SET9 or $SET9_{H297A}$, together with the PSA luciferase and β-galactosidase reporters. After 48 hours, cells were harvested and luciferase and β-galactosidase analyses performed. B. U2OS cells were transiently transfected as in (A), but included the androgen-responsive ARE III reporter. Data in (A) and (B) represents the mean of three independent experiments performed in quadruplicate +/- standard error.

Figure S4. LNCaP cells grown in steroid-depleted media for 72-hours were treated with 10 nM DHT over a time-course of 0-120 minutes and then subject to chromatin immunoprecipitation (ChIP) analysis using an anti-AR antibody, or non-specific isotype

control, followed by quantitative PCR analysis using primers specific to the proximal (ARE I) (left panel) and distal (ARE III) (right panel) regions of the PSA promoter.

Figure S5. ChIP analysis of SET9 at ARE I and ARE III in PC3 cells treated with and without 10 nM DHT for 0 and 120 minutes.

Figure S6. A. ChIP analysis of histone H3K4 mono-methylation (H3K4me1) at ARE I in LNCaP-SET9$_{K/D}$ cells treated with and without doxycycline for 48 hours and 10 nM DHT for 0 and 120 minutes. B. ChIP analysis of AR at ARE I in LNCaP-SET9$_{K/D}$ cells treated with and without doxycycline for 48 hours and 10 nM DHT for 0 and 120 minutes.

Figure S7. LNCaP cells transiently transfected with either non-silencing (N/S) or SET9 oligonucleotides were subject to histone extraction and Western analysis using anti-H3K4me1, -H3K9me2, pan-methyl-lysine and H3 antibodies.

Figure S8. PC3 cells were transiently transfected with either wild-type AR, AR$_{632}$ or empty vector for control and incubated for 72 hours in steroid-depleted media prior to 8 hour DHT stimulation and quantitative PSA expression profiling. Data represents the mean of three independent experiments (+/- standard error).

Figure S9. A. U2OS cells were transiently transfected with SET9, SET9$_{H297A}$ or empty vector for 48 hours and then treated with 0.5 μM doxorubicin treatment for an additional 24 hours prior to caspase 3 analysis. Data represents the average of three independent experiments +/- standard error (* represents statistical significance of < 0.05). B. SET9

Western analysis from LNCaP cells transiently transfected with either non-silencing (N/S) or SET9 siRNAs and treated with and without 0.5 μM doxorubicin. NT represents a non-transfected control that received no oligonucleotides.

Supplementary References

Brady, M.E., Ozanne, D.M., Gaughan, L., Waite, I., Cook, S., Neal, D.E. and Robson, C.N. (1999) Tip60 is a nuclear hormone receptor coactivator. *J Biol Chem*, 274, 17599-17604.

Chen, L., Iraci, N., Gherardi, S., Gamble, L.D., Wood, K.M., Perini, G., Lunec, J. and Tweddle, D.A. (2010) p53 Is a Direct Transcriptional Target of MYCN in Neuroblastoma. *Cancer Res*, 70, 1377-1388.

Chuikov, S., Kurash, J.K., Wilson, J.R., Xiao, B., Justin, N., Ivanov, G.S., McKinney, K., Tempst, P., Prives, C., Gamblin, S.J., Barlev, N.A. and Reinberg, D. (2004) Regulation of p53 activity through lysine methylation. *Nature*, 432, 353-360.

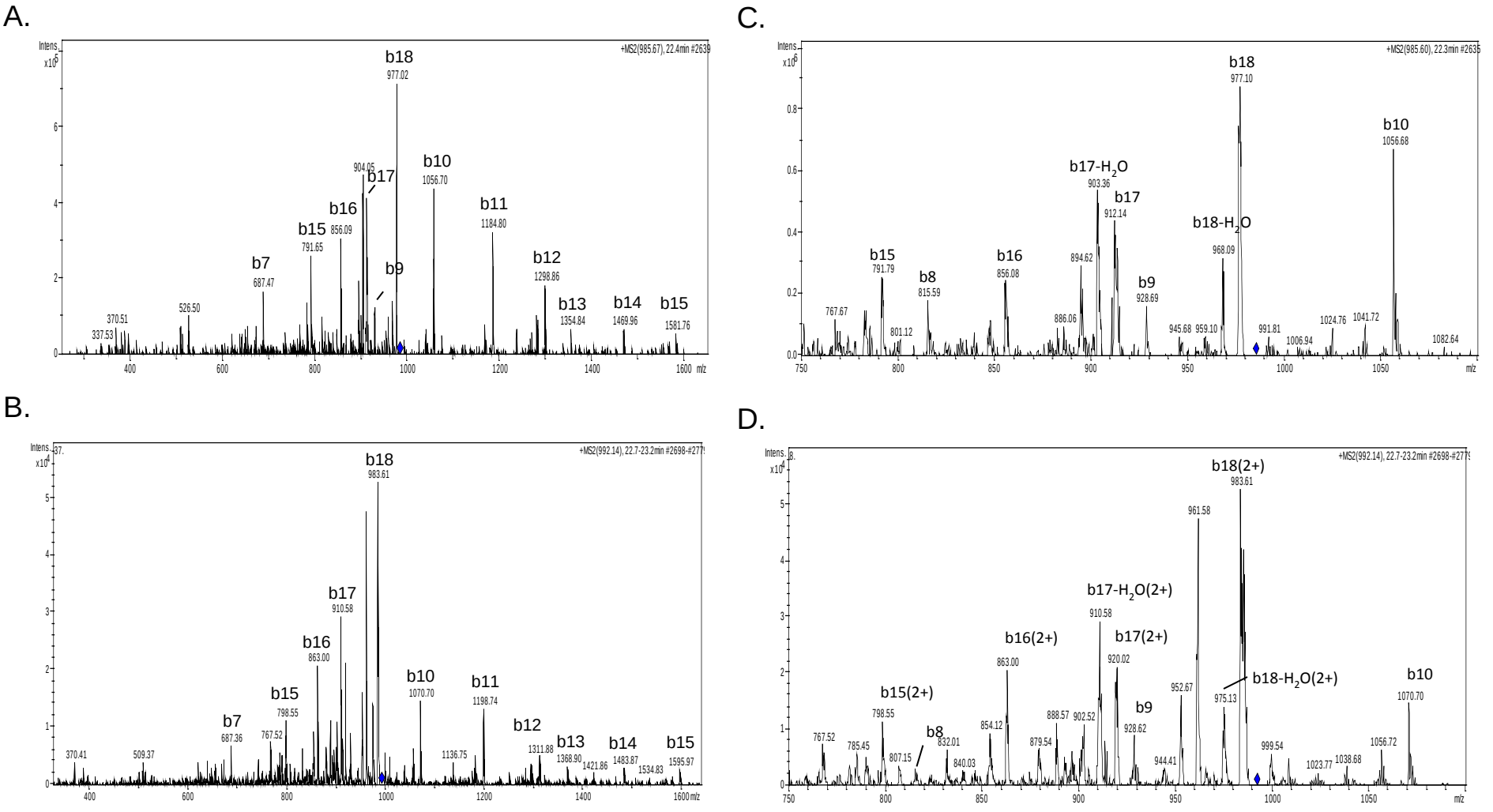
Gaughan, L., Logan, I.R., Neal, D.E. and Robson, C.N. (2005) Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation. *Nucleic Acids Res*, 33, 13-26.

Logan, I.R., Gaughan, L., McCracken, S.R., Sapountzi, V., Leung, H.Y. and Robson, C.N. (2006b) Human PIRH2 enhances androgen receptor signaling through inhibition of

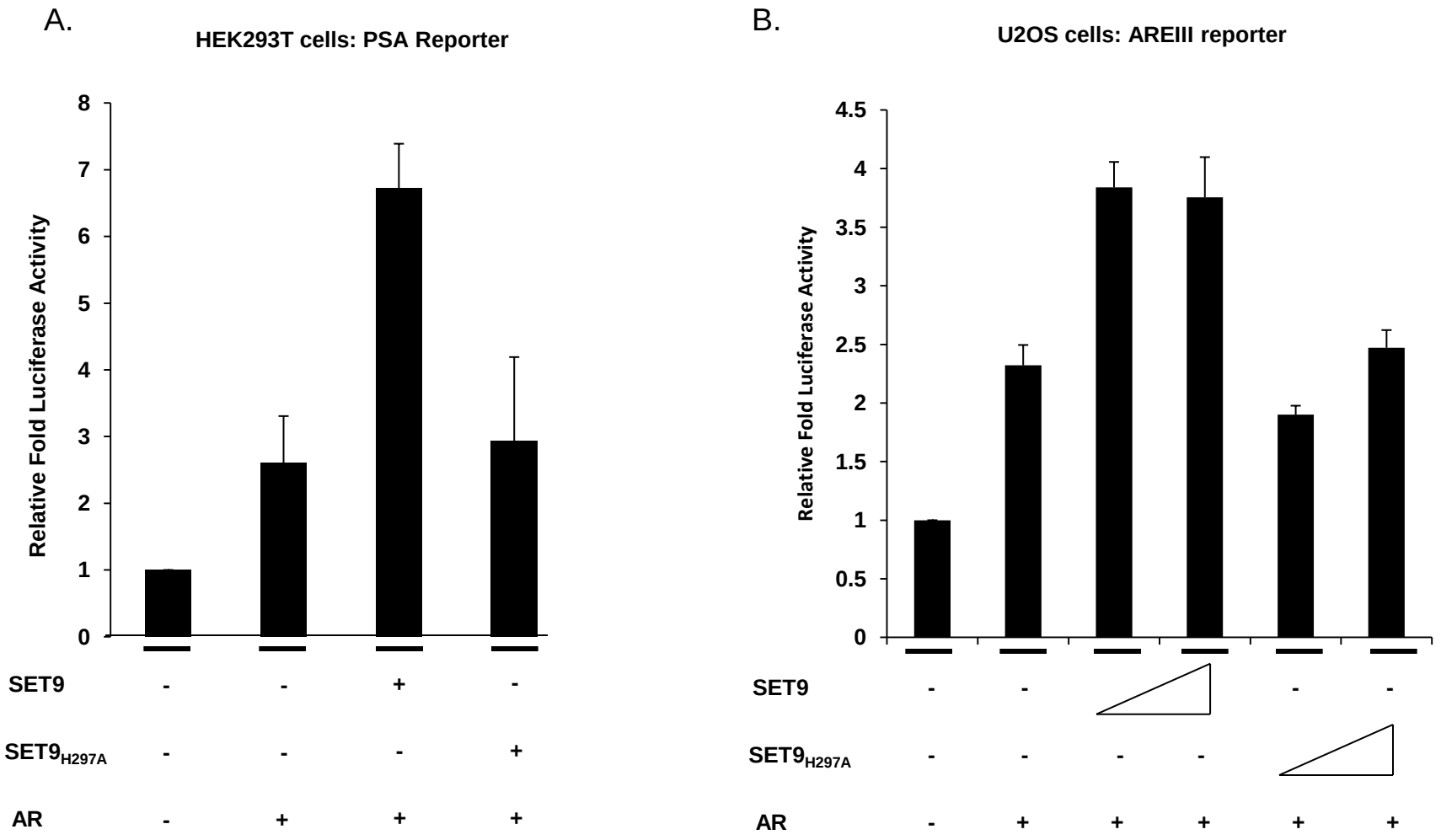
histone deacetylase 1 and is overexpressed in prostate cancer. *Mol Cell Biol*, 26, 6502-6510.

Supplementary Data

Supplementary Figure S1

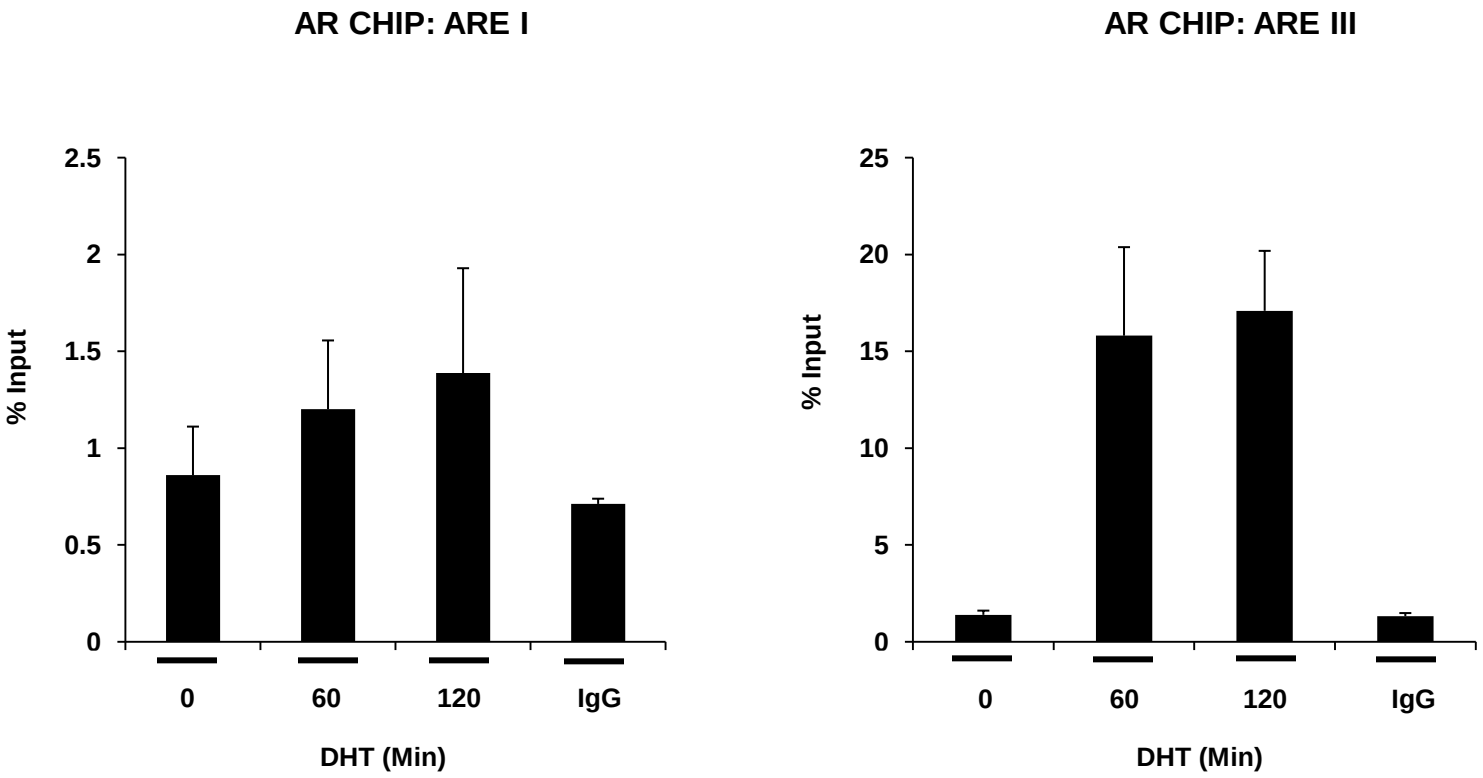


Supplementary Figure S2



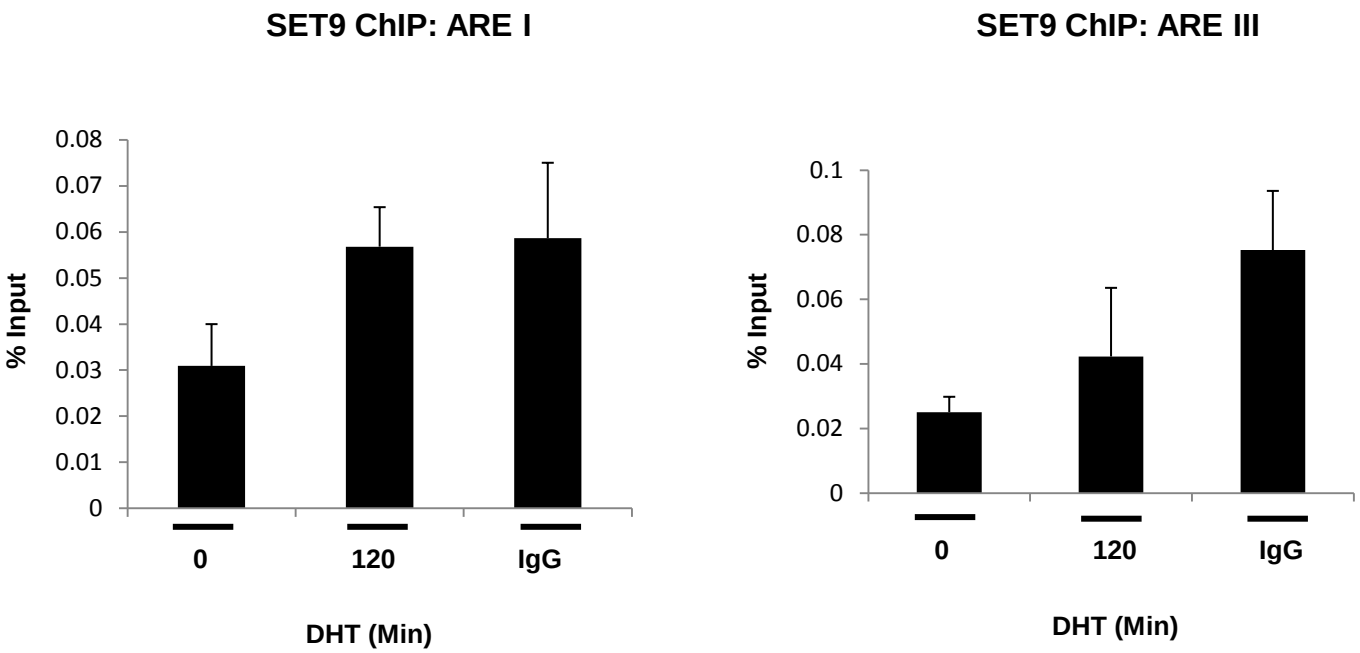
Supplementary Figure S3

LNCaP cells



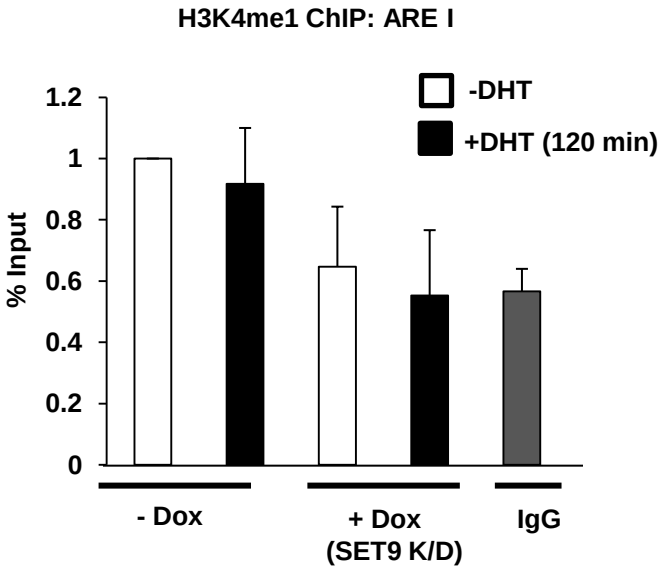
Supplementary Figure S4

PC3 cells



Supplementary Figure S5

A.



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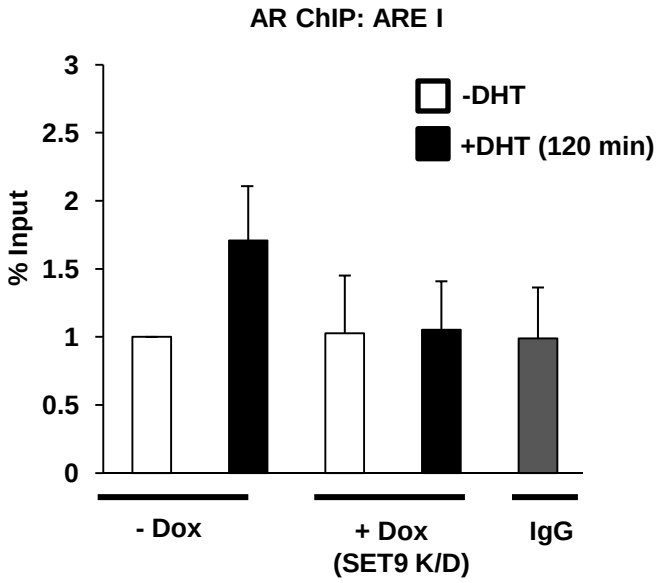
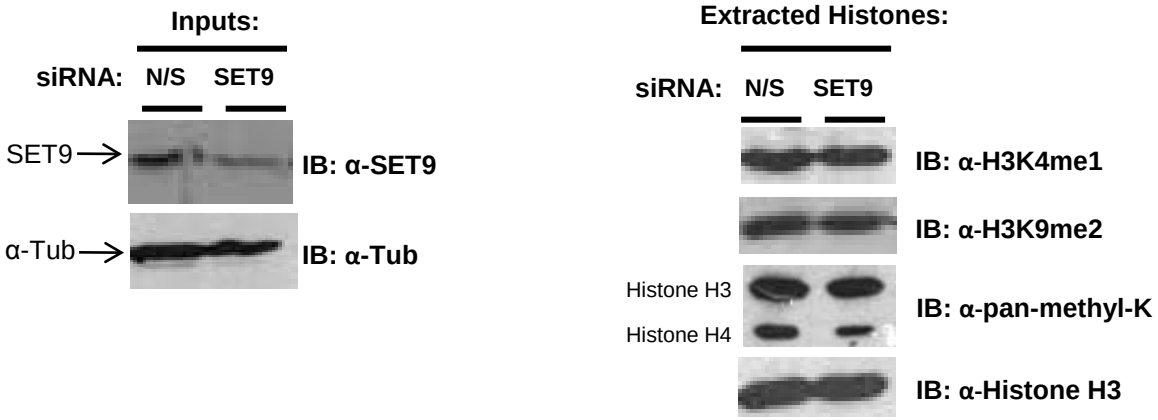
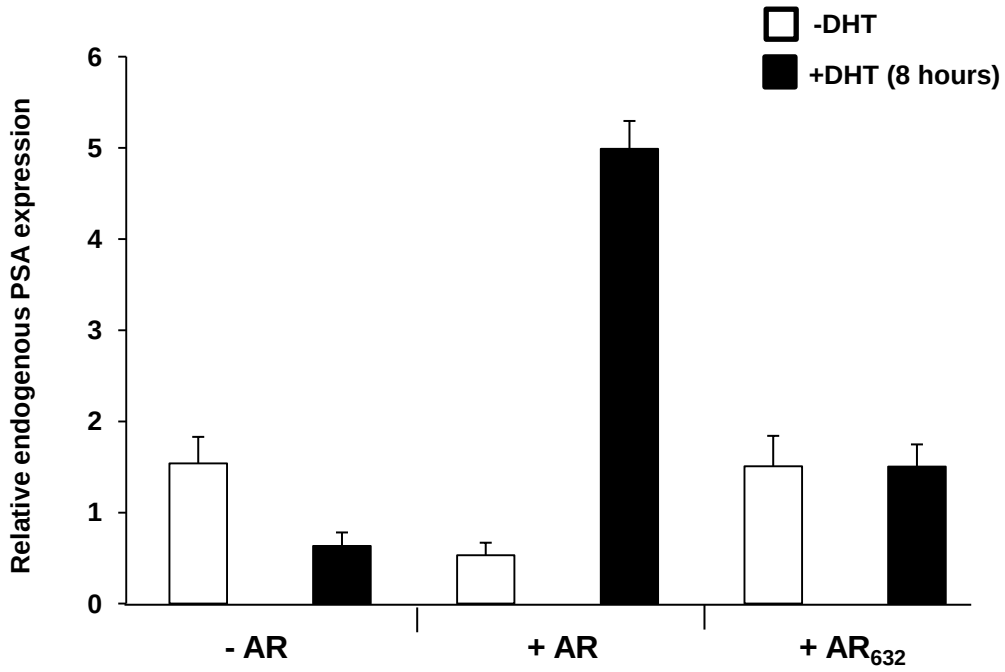


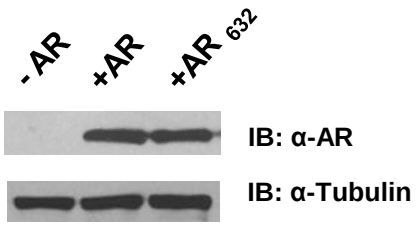
Supplementary Figure S6



Supplementary Figure S7

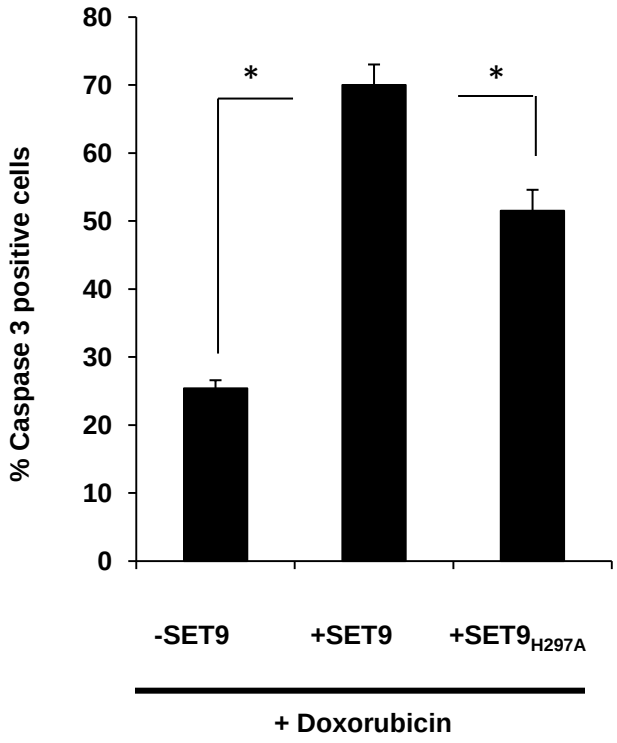
PC3 cells: Endogenous PSA





Supplementary Figure S8

A.



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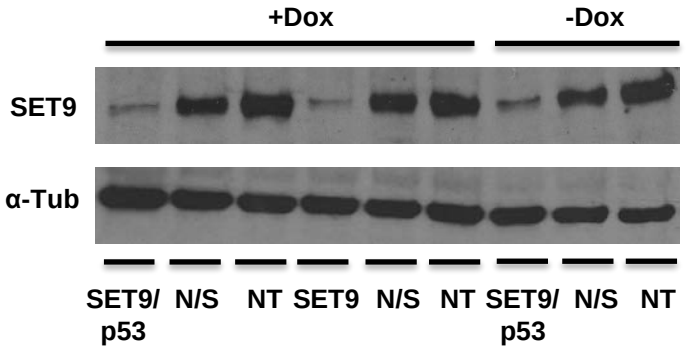

Supplementary Figure S9