SUPPLEMENTARY DATA

Title.

Identifying dysregulated epigenetic enzyme activity in castrate-resistant prostate cancer development

Authors

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Western blot analysis of prostate cancer cell lines

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Supplementary Materials and Methods

Antibodies and other materials used in immunoblot and immunohistochemistry

Each cell extracts were normalized against the total protein contents as estimated by Bradford assay. Total cell extracts were separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 2% BSA in TBST for 1 h at room temperature, the membranes were incubated with each primary antibody diluted to the manufacture's recommendation. The following antibodies were purchased from Cell Signaling Technology (in brackets are the catalog numbers), anti-acetyl-p300/anti-acetyl-CBP (cst4771S), anti-p300 (cst61402), anti-CBP (cst7389), anti-Gcn5L2 (cst3305), anti-PCAF (cst3378), anti-Sirt1 (cst2310), anti-Sirt2 (cst1267), anti-Sirt3 (cst2627), anti-Sirt6 (cst12486), anti-Sirt7 (cst5360), anti-rabbit IgG, HRP-linked antibody (cst7074), anti-mouse IgG, HRP-linked antibody (cst7076), and anti-α-tubulin (cst3874); from abcam, anti-Sirt2 (ab51023), anti-histone H3 acetyl-Lys14, (H3K14ac, ab82501), anti-histone H3 acetyl-Lys18 (H3K18ac, ab1191), and anti-Sirt4; from Active Motif, anti-histone H3 (#61476), anti-histone H3 acetyl-Lys9 (H3K9ac, #39917); and from, anti-VDAC1 (#75-204, Antibodies Incorporated).

For a tissue microarray immunohistochemistry analysis, TMA sections were subjected to primary antibodies of p300 (#NB500-161, Novus Biologicals; Littleton, CO), Sirt2 (#SC-28298, Santa Cruz Biotechnology; Santa Cruz, CA), and H3K18ac (#Ab1191, Abcam; Cambridge, UK) primary antibodies. E-Cadherin (#790-4497, Ventana; Tucson, AZ) antibody was used to define the epithelial compartment for automated tissue segmentation.

Filter-binding HAT assay using radiolabeled acetyl-CoA

In a typical filter-binding assay, 50-500 μM histone peptide or protein substrate was incubated with 50 μM ^{3}H-acetyl CoA (2.5 μCi, Moravek Biochemicals), 0.5-2 μM purified

recombinant HATs in an assay buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT). The assay was carried out in a 30 °C water bath, and for each time point, 10 μL of the reaction was removed and spotted evenly on a 2.5 cm phosphocellulose (P81) filter disc (Whatman). The discs were allowed to dry completely, and the unbound small molecule reagents including acetyl CoA were washed off in 500 mL of 50 mM sodium bicarbonate (pH 9.0). The discs were transferred into 20 mL glass scintillation vials containing 5 mL LSC ScintiSafe Econo II cocktail (Fisher Scientific) for counting. Incorporation of radiolabeled ^{3}H-acetyl group by HATs was quantified on a Tri-carb 2910 TR Liquid Scintillation Analyzer (PerkinElmer), using Tritium detection protocol. For histone deacetylation condition, 50 mM Tris (pH 8.0), 1 mM DTT, 1 mM NAD^+ were added. The HDAC reaction was quenched with 50% TFA. Deacetylation of histone H3K9ac (1-20) was monitored by reverse-phase HPLC on Kinetex C18 column (100Å, 100 × 4.6 mm, 2.6 μm, Phenomenex) by monitoring the formation of deacetylated product at 214 nm, as described (1). For deacetylation of p300, nuclear extract from each cell was incubated in 50 mM Tris-HCl (pH 8.0) in presence of 1 mM NAD^+ and 2 μM recombinant SIRT2. The reaction was incubated up to 12 h at 30°C before quenched by 5 × SDS sample loading buffer.

Preparation of cell extracts and cell fractionation

Prostate carcinoma (LNCaP and C4-2) cells were cultured in a 100-mm plates, harvested, flash-frozen, and stored until used. Before the enzyme assay, the cell pellets were thawed on ice and resuspended in 300 μL lysis buffer; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, DNase I (Roche), protease inhibitor cocktail (Promega), and NaF and incubated at room temperature for 10 min with gentle rotation. The cell suspension was passed through 27-gauge needle 10 times, and the lysate was centrifuged at 14,000 g for 5 min at 4°C.

For fractionation of different cell compartments, the following procedure was followed. For nuclear fraction, the nuclear pellet was resuspended in 400 μL chilled nuclear extraction buffer;

20mM HEPES pH 7.5, 1.5mM MgCl2. 0.42 mM NaCl, 1mM DTT, 25% glycerol, DNase I (Roche), protease inhibitor cocktail (Promega), and NaF and gently shaken for 15 min at 4°C. Nuclear lysate were spun at 21,000 g for 15 min at 4°C to pellet tightly packed histones and DNA. Supernatant of chromatin associated nuclear proteins was kept for downstream histone peptide microarray analysis.

Microarray HAT/Sirtuin assay

All the microarray assays were performed with a dual-chamber simplex gasket, which allows two reaction conditions for each array. Each array was blocked with 2% bovine serum albumin (BSA) to eliminate non-specific protein binding to the nitrocellulose surface. Following blocking, each chamber was filled with each reaction buffers containing co-factors, inhibitos, and cell lysate. In the HAT specific assay, acetyl-CoA, TSA (HDAC inhibitor), and nicotinamide (sirtuin inhibitor) were supplementedto promote HAT activity and inhibit both HDAC I and II as well as sirtuin family HDACs. In the Sirtuin-specific assay, NAD^+, TSA, and anarcardic acid (HAT inhibitor) were added to promote sirtuin activity and inhibit HAT activity in the lysate. LNCaP and C4-2 were run in parallel on a single microarray for each HAT assay and Sirtuin assay for downstream analysis. The reaction was stopped by removing the assay components by aspiration. The array was washed with TBST before incubated with rabbit-derived *pan*-K_{ac} antibody. Subsequently, the slide was incubated with *anti*-rabbit IgG conjugated to Alexa647. The slide was washed with TBST before scanned on an Axon GenePix 4000B microarray fluorescence scanner (Molecular Devices) at 5-μm pixel resolution and 33% laser power. Slide images were collected at 532-nm/635-nm dual channel, and analyzed using GenePix Pro 6.1 software (Molecular Devices). Data Analysis was performed by utilizing the average total intensity at 635nm of each peptide triplicate spot for a 200 μm diameter circle. The ratio of the two cell lines were calculated within a single microarray slide to minimize differences from histone peptide microarray printing. The $\log_2$ fold change was then calculated for each slide and then the 3 biological replicates were averaged to

obtain the average $\log_2$ fold change in enzymatic activity between C4-2 and LNCaP cell lines. Statistical significance was determined for each microarray using a student's t-test on each microarray, comparing the technical printed triplicates amongst the two wells on the individual microarray slides to minimize error from printing differences.

Primers used for mRNA quantitation:

AR F	TGTACACGTGGTCAAGTGGG
AR R	ATGACAGCCATCTGGTCGTC
PSA F	AGTCTGCGGCGGTGTTCT
PSA R	CAGGATGAAACAGGCTGTGC
Sirt2 F	CATCCACCGGCCTCTATGAC
Sirt2 R	CGAAGAAGGGTTCCGGATGT
P300 F	CCTGGGTCCTATGCCAACAG
P300 R	TCCGGCGTAGGAAATATGGC
GAPDH F	CTGACTTCAACAGCGACACC
GAPDH R	TGCTGTAGCCAAATTCGTTG

Supplementary Figures

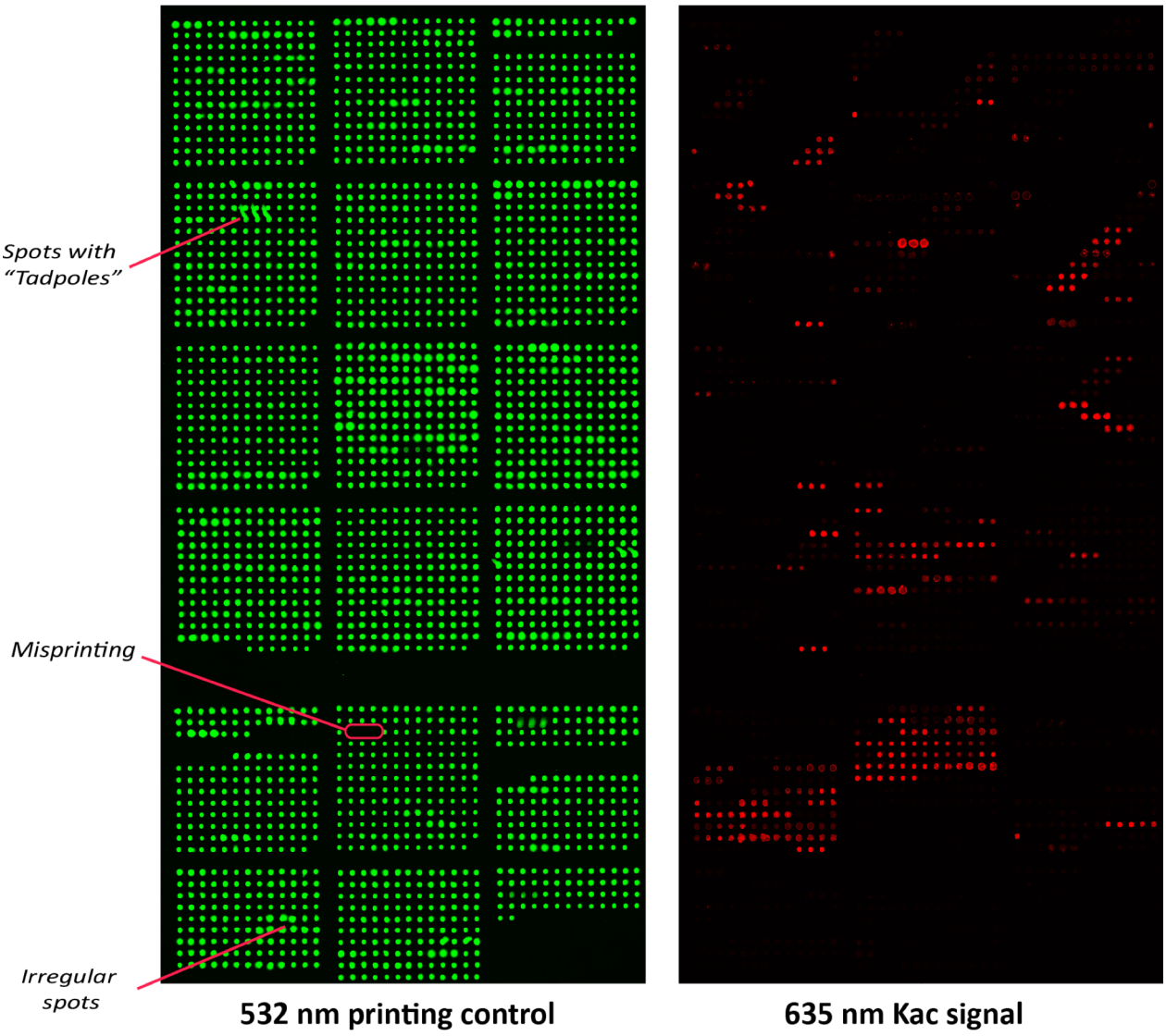
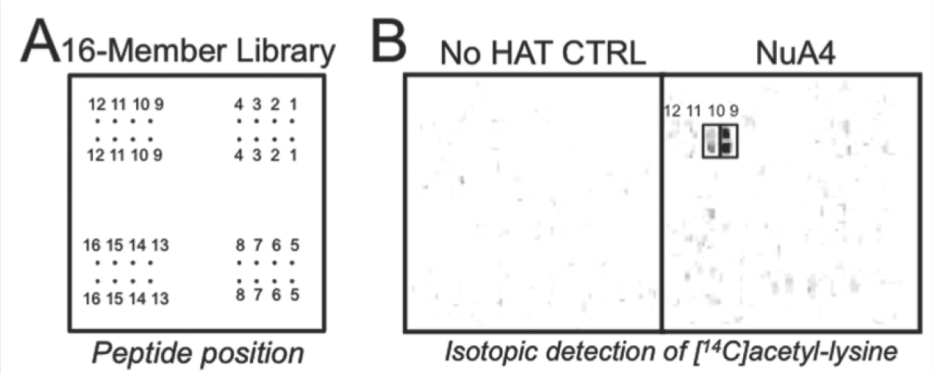
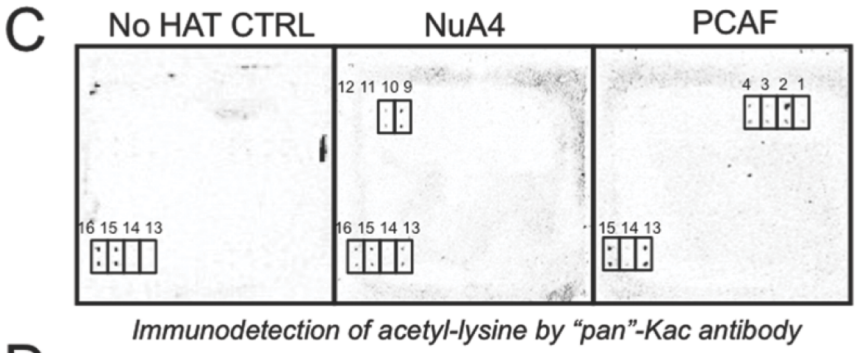


Figure S1. Typical microarray images scanned at 532 nm and 635 nm after microarray assay followed by *anti*-pan-acetyllysine immunodetection. The image at 532 nm provides a printing control (green spots) for any signs of artifacts in the array. Misprinted or irregular spots as well as spots with "tadpoles" are taken into consideration during spot area quantification. Also, the 532nm spots are used to guide the spot positions. Image taken at 635 nm shows positive signals (red spots) for acetylated lysine (K_{ac}).





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No.	Amino acid residue sequence	Position in histone	Lysine (K) position	Site-specific acetylation
1	ARTKQTARKSTGG	H3(1-13)	H3 K4K9	PCAF-dependent
2	TKQTARKSTGGKA	H3(3-15)	H3 K4K9K14	PCAF-dependent
3	RKSTGGKAPRKQL	H3(8-20)	H3 K9K14K18	PCAF-dependent
4	GGKAPRKQLATKA	H3(12-24)	H3 K14K18K23	PCAF-dependent
5	RKQLATKAARKSA	H3(17-29)	H3 K18K23K27	
6	ATKAARKSAPATG	H3(21-33)	H3 K23K27	
7	PATGGVKKPHRYR	H3(30-42)	H3 K36K37	
8	EIRRYQKSTELLI	H3(50-62)	H3 K56	
9	SGRGKGGKGLGKG	H4(1-13)	H4 K5K8K12	NuA4-dependent
10	GGKGLGKGGAKRH	H4(6-18)	H4 K8K12K16	NuA4-dependent
11	LGKGGAKRHRKVL	H4(10-22)	H4 K12K16K20	NuA4-dependent
12	GAKRHRKVLRDNI	H4(14-26)	H4 K16K20	NuA4-dependent
13	DVVYALKRQGRTL	H4(85-97)	H4 K91	
14	TAQTARASTGGAA	H3(3-15)	H3 no Lys Ctrl	No Lys (-) CTRL
15	TK$_{ac}$QTARK$_{ac}$STGGK$_{ac}$A	H3(3-15)	H3 K4$_{ac}$K9$_{ac}$K14$_{ac}$	Kac (+) CTRL
16	EIRRYQK$_{ac}$STELLI	H3(50-62)	H3 K56$_{ac}$	Kac (+) CTRL

Figure S2. HAT assays on histone peptide microarray. A) Arrangement of 16-member mini-library showing duplicate spots of each peptide. **B**) Isotopic detection of NuA4-dependent HAT assay show positive signal with peptides H4(1-13) and H4(6-18). **C**) Fluorescence immunodetection of HAT assay displaying acetylation specificity towards peptides, which were previously identified as substrates. **D**) Numbering of peptides; *N*-terminal of histone H3 (peptide 1-4), globular region of H3 (peptide 5-8), *N*-terminal H4 (peptide 9-12), globular region of H4 (peptide 13), a peptide without lysine (negative control, peptide 14), and peptides with pre-acetylated lysine (positive control, peptide 15, 16).

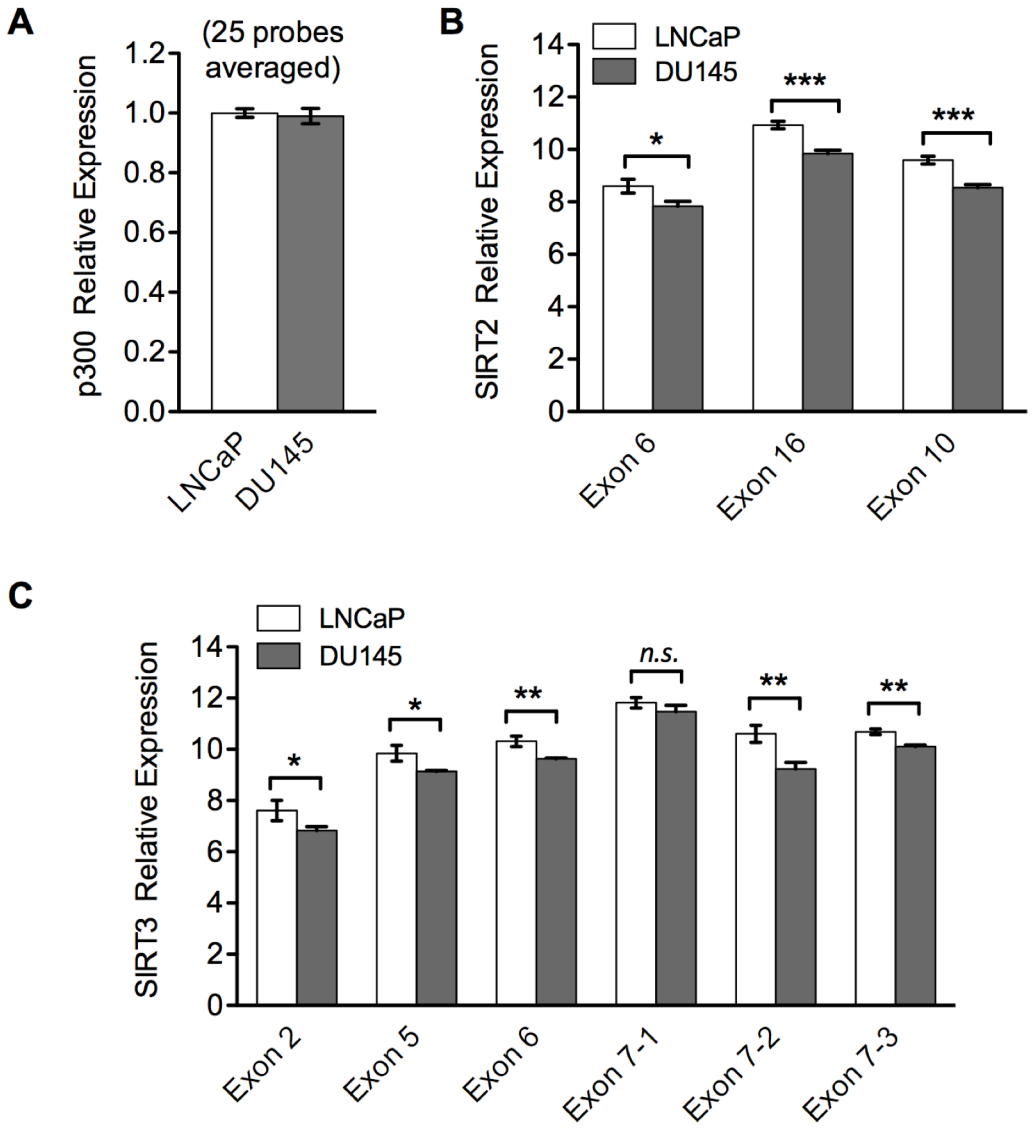

Figure S3. Gene expression of Sirt2, Sirt3, and EP300 in LNCaP and DU145 prostate cancer cell lines. Gene expression microarray data was obtained from NCBI Gene Expression Omnibus (GEO) for prostate cancer cell lines DU145 and LNCaP (GSE63196). Microarray data was queried for probes targeting SIRT2, SIRT3, and EP300. **A)** The average expression, normalized to LNCaP, was calculated for 25 probes targeting P300 gene expression. No significant change was seen in EP300 expression between DU145 and LNCaP. **B)** SIRT2 expression was significantly lower across all probes in the castrate-resistant cell line DU145 compared to hormone-sensitive LNCaP. **C)** DU145 showed significantly lower Sirt3 expression in 5 of 6 probes.
(Statistical significance is *, $p < 0.05$; **, $0.001 \leq p < 0.01$)

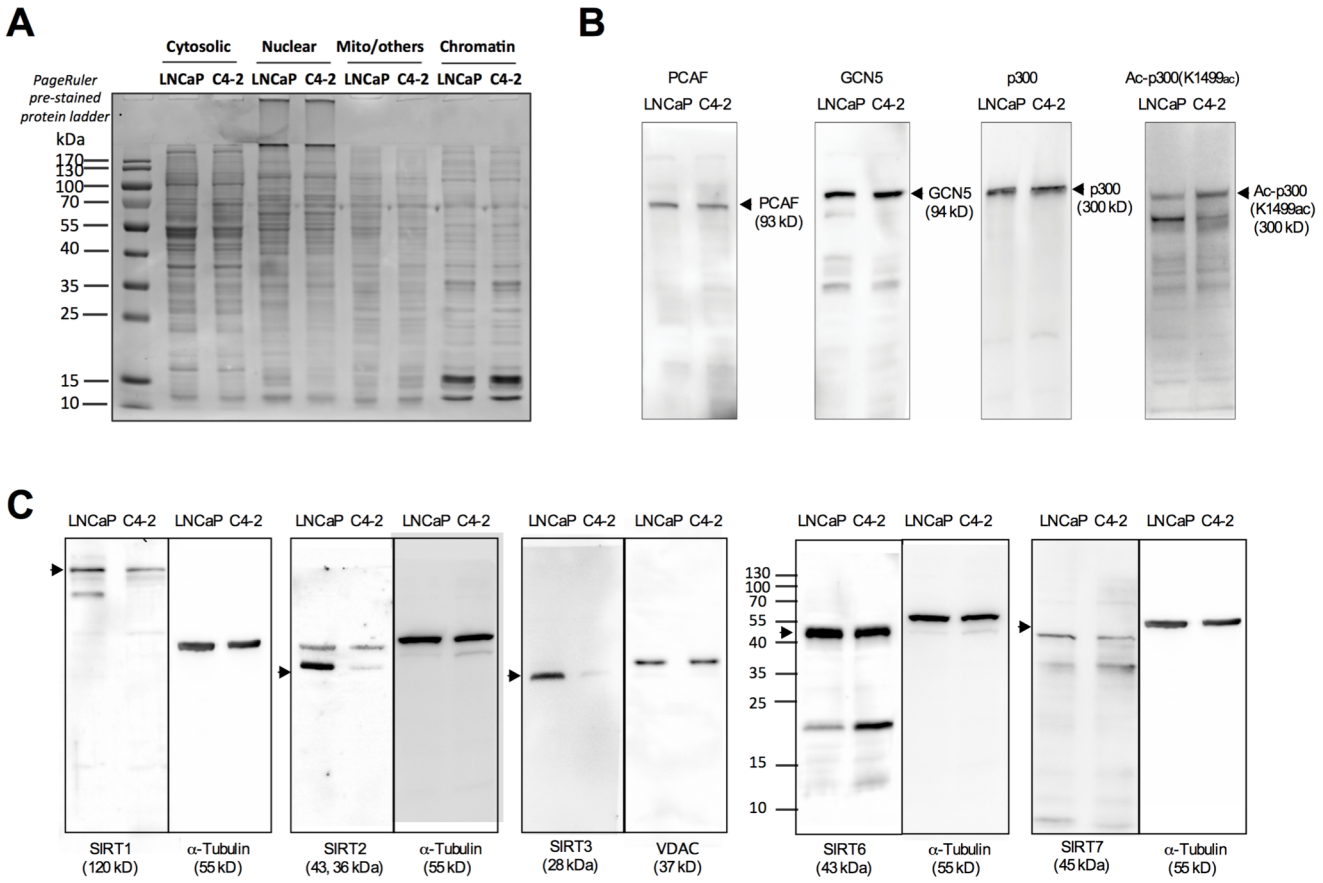

Figure S4. Quantitative western blots for histone-modifying enzymes. A) LNCaP and C4-2 cell lines each representing androgen-dependent/sensitive prostate cancer (PCa) cell line, and androgen-independent PCa cell line was fractionated. Each cell fractions in both cell lines was stained by Coomassie brilliant blue. **B**) Full blots showing bands corresponding to HATs analyzed in this study. Nuclear fractions (for PCAF and GCN5) and chromatin fractions (for p300 and Ac-p300) were blotted against each antibody. **C)** Full blots showing bands corresponding to sirtuins discussed in the main text.