SUPPORTING INFORMATION

Regulation of estrogen receptor α by SMYD2-mediated protein methylation

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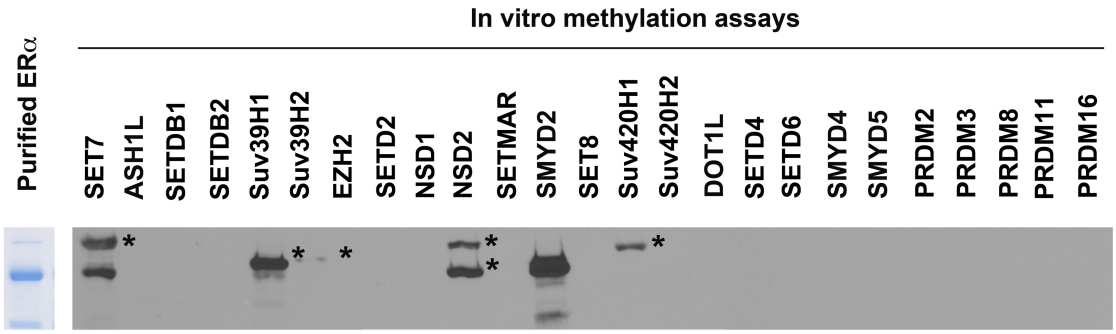


Fig. S1. SMYD2 methylates ERα *in vitro*.
Autoradiogram of *in vitro* methylation assays of recombinant full-length ERα incubated with the indicated recombinant SET domains. Left panel is GelCode Blue staining in the left panel showing purified ERα protein. Asterisks indicate automethylated SET domain proteins.

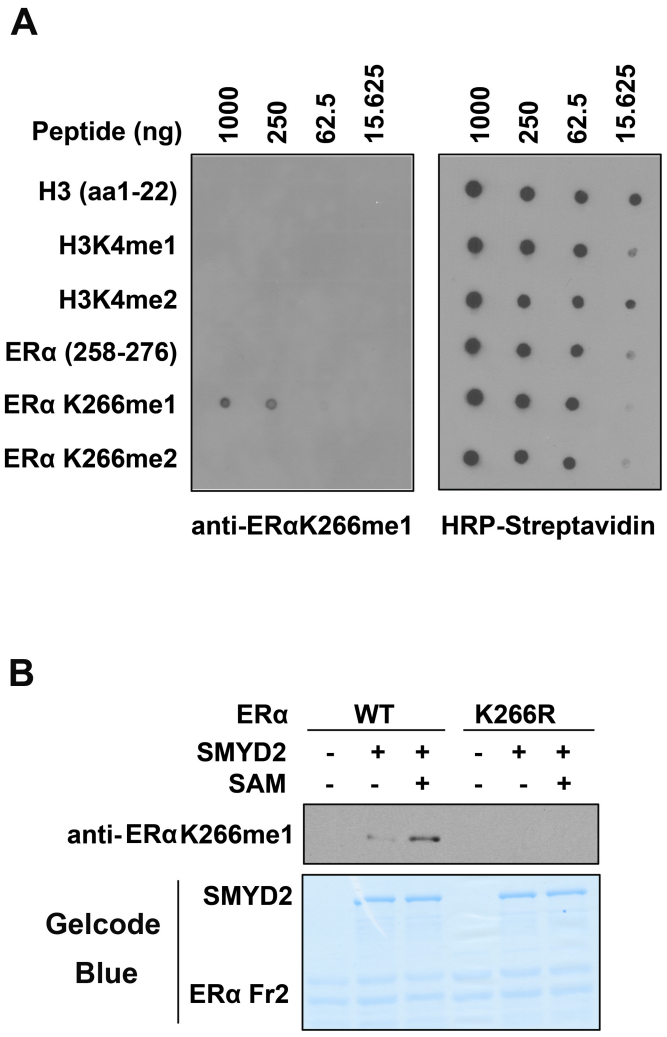
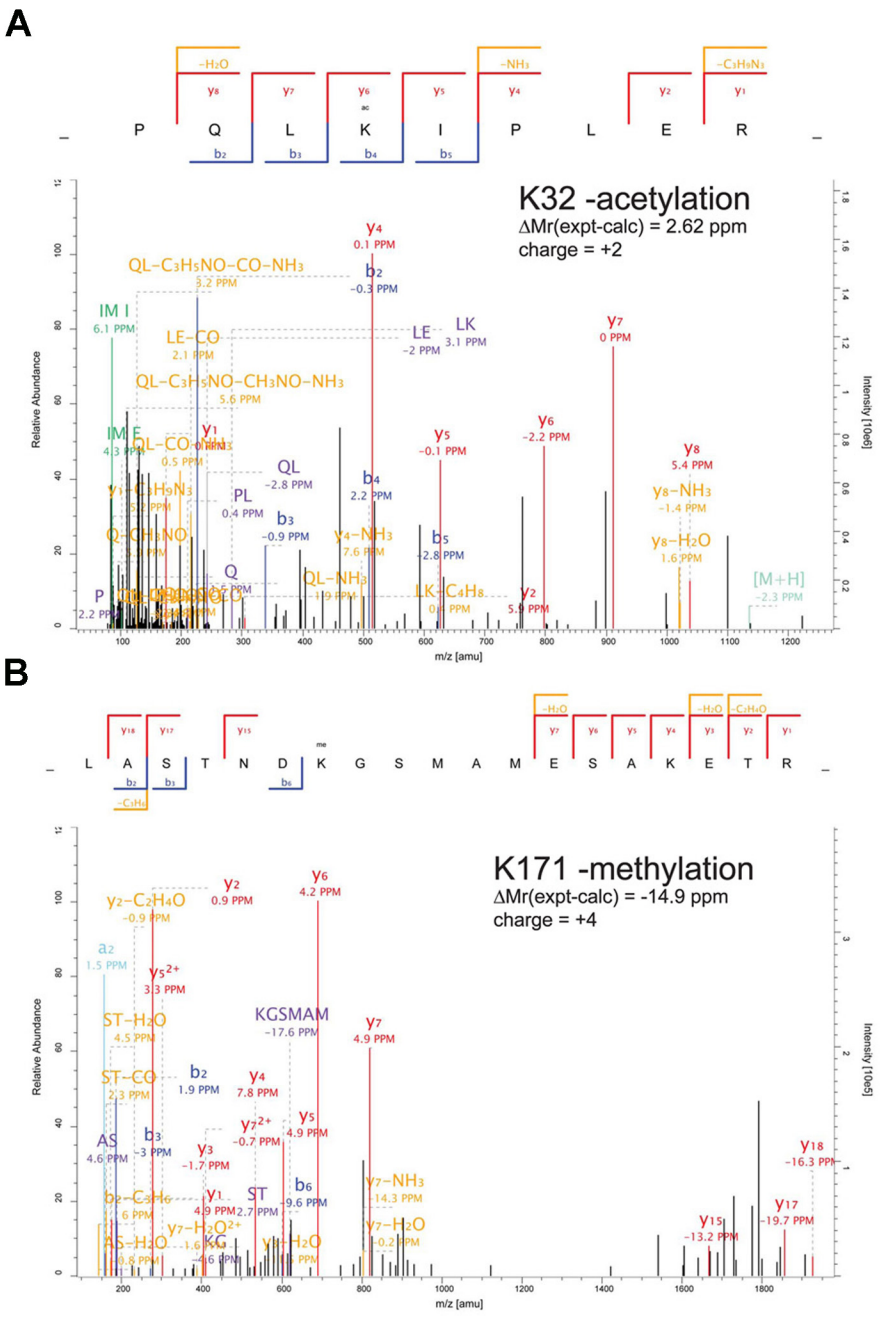
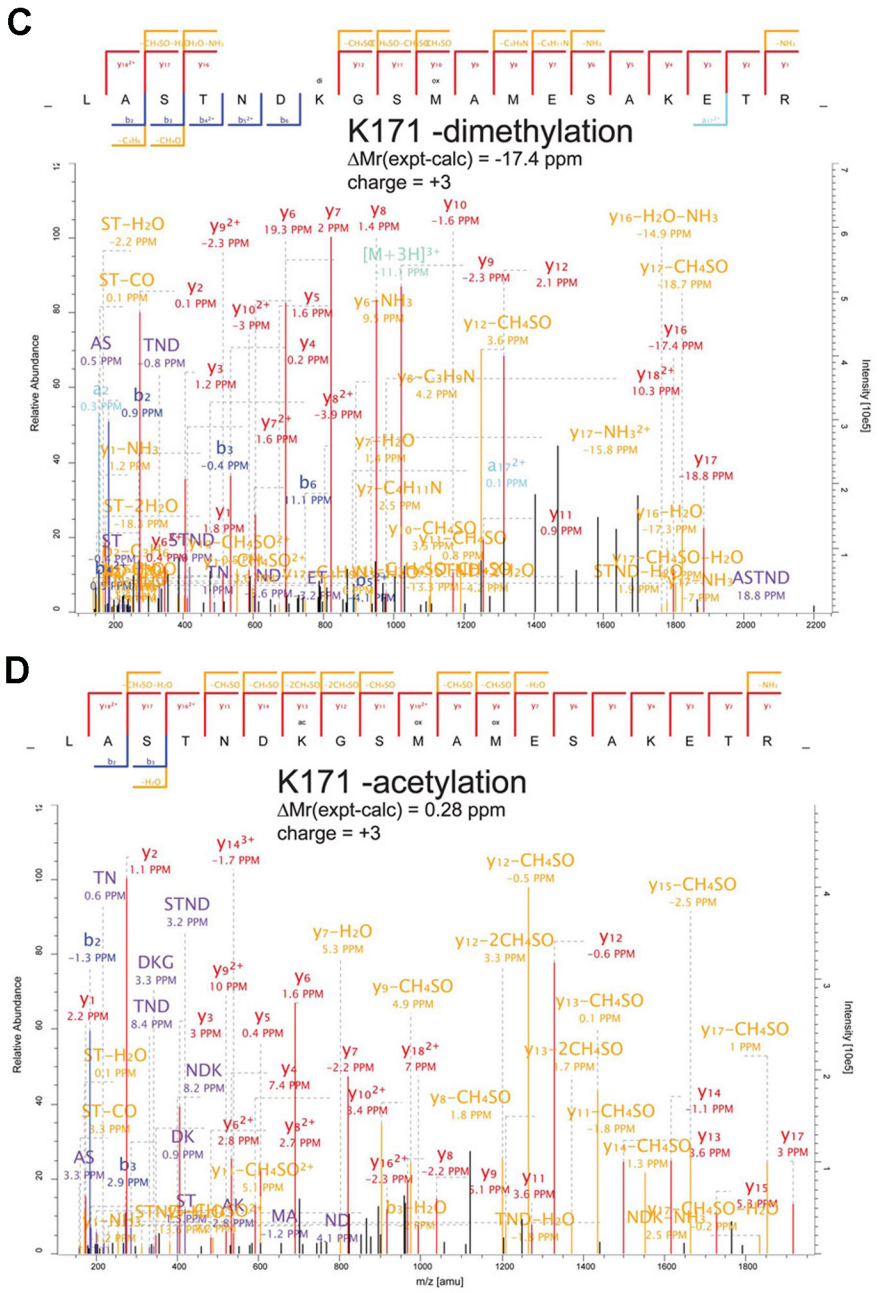
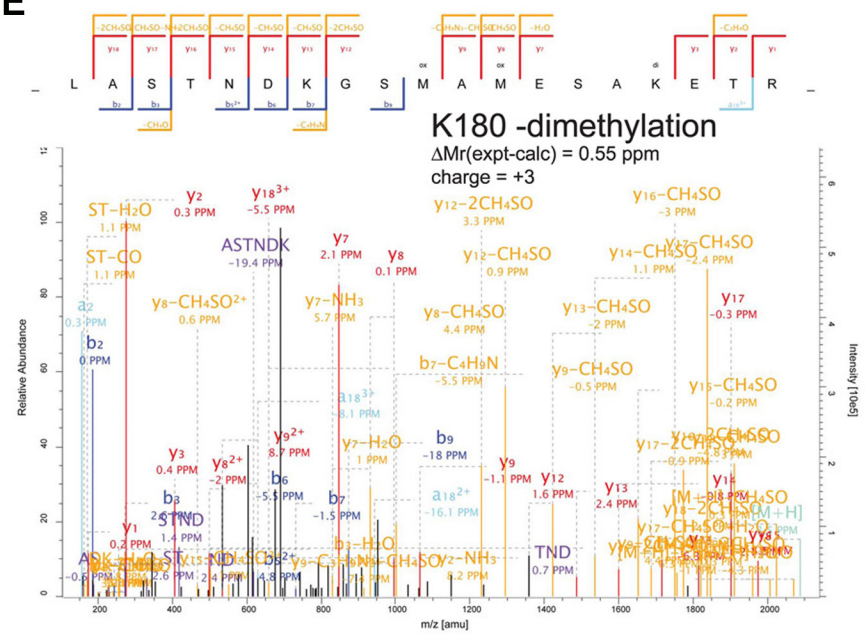


Fig. S2. The anti-ERαK266me1 antibody specifically recognizes ERα methylated by SMYD2 *in vitro*.
(A) Dot blot analysis of the polyclonal anti-ERαK266me1 antibody on biotin-labeled ERα peptides (aa 258-276) and histone H3K4 peptides (aa 1-22). HRP-streptavidin was used as a loading control.
(B) Western blot analysis of the anti-ERα-K266me1 antibody on recombinant ERα and ERα-K266R proteins methylated by SMYD2. GelCode Blue staining shows equal amounts of ERα proteins and SMYD2 protein were used in the *in vitro* methylation assay.


A
K32 -acetylation
ΔMr(expt-calc) = 2.62 ppm
charge = +2
Relative Abundance
m/z [amu]
Intensity [10e6]
B
K171 -methylation
ΔMr(expt-calc) = -14.9 ppm
charge = +4
Relative Abundance
m/z [amu]
Intensity [10e5]


C
L A S T N D K G S M A M E S A K E T R
K171 -dimethylation
ΔMr(expt-calc) = -17.4 ppm
charge = +3
Relative Abundance
Intensity [10e5]
D
L A S T N D K G S M A M E S A K E T R
K171 -acetylation
ΔMr(expt-calc) = 0.28 ppm
charge = +3
Relative Abundance
m/z [amu]

E


L A S T N D K G S M A M E S A K E T R
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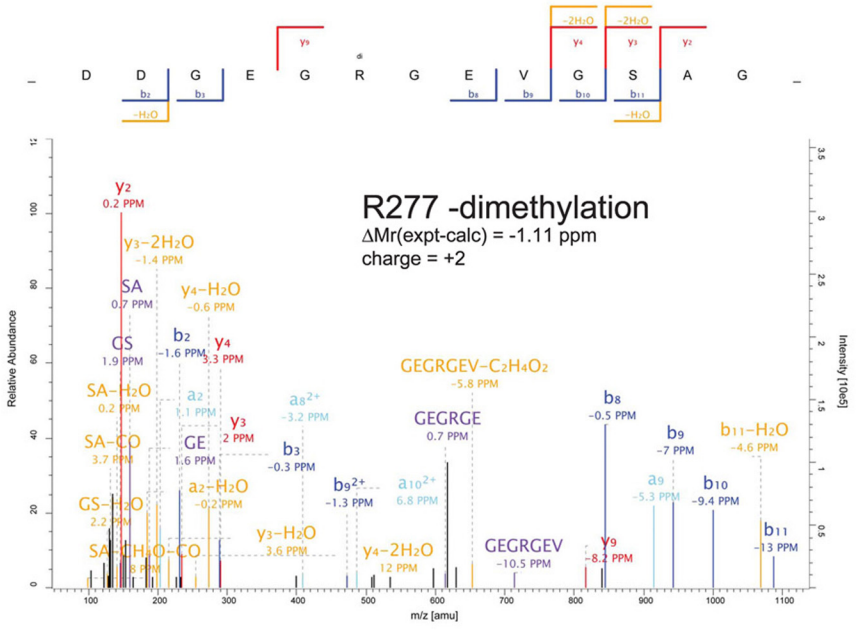
Relative Abundance
Intensity [10e5]
m/z [amu]

F


D D G E G R G E V G S A G
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charge = +2
Relative Abundance
Intensity [10e5]
m/z [amu]

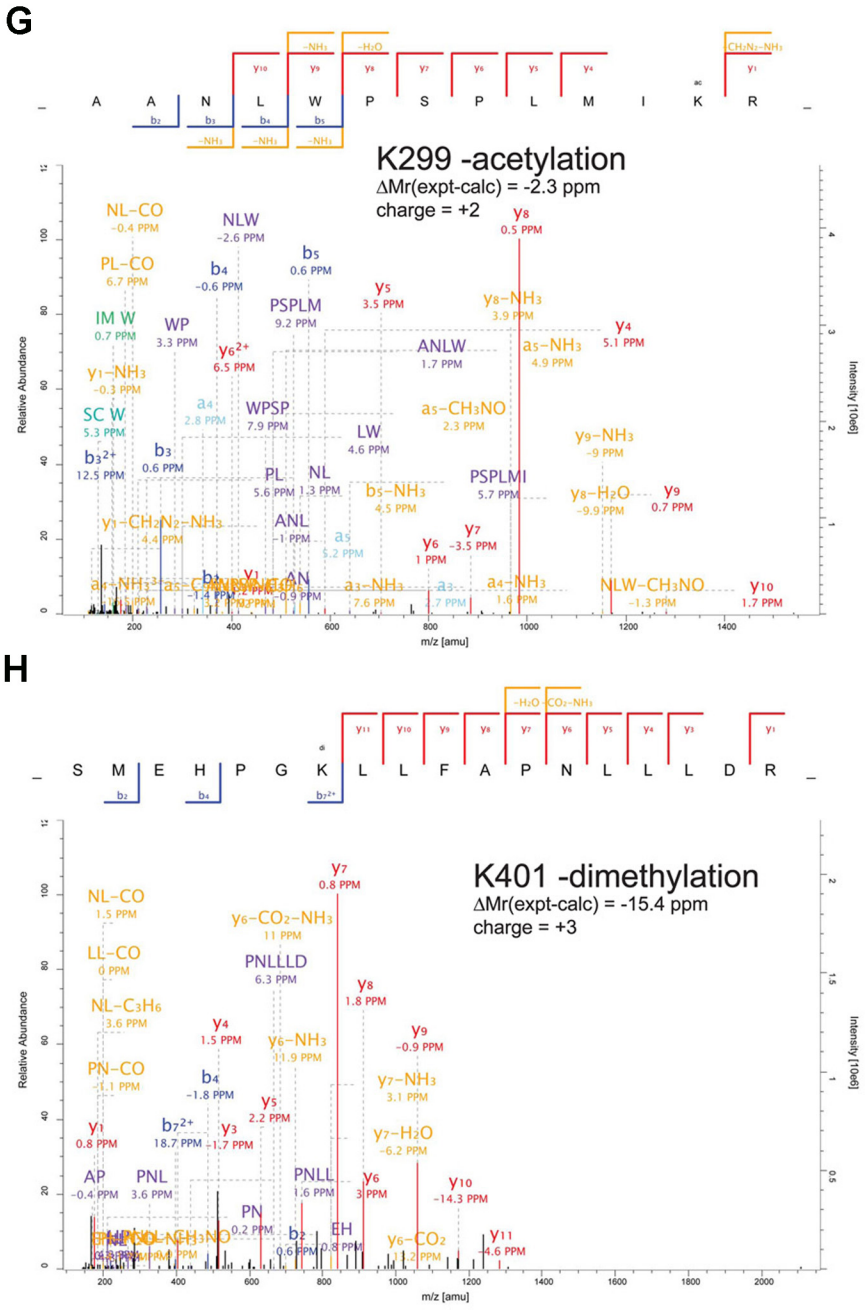

Fig. S3. Endogenous ERα are methylated and acetylated at multiple sites.
(A-H) MS/MS spectra of identified peptides with modification of monomethylation (me), dimethylation (di) or acetylation (ac). Ions of a, b, y and internal fragmentation are annotated and differences in molecular weight (DMr) or m/z between the experimental and calculated are indicated in parts per million (ppm) for precursor and fragmented ions.

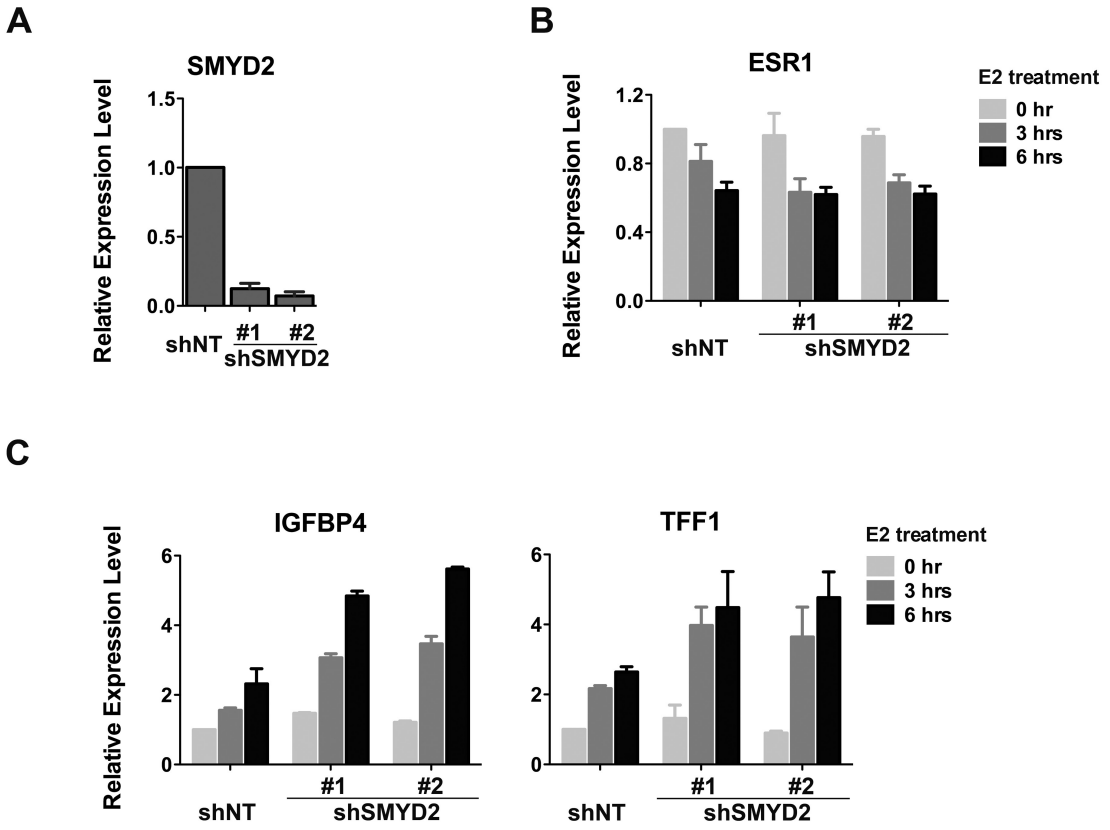

Fig. S4. Depletion of SMYD2 enhances the expression of ERα target genes.
(A) qPCR analysis of *SMYD2* gene expression in control (shNT) and SMYD2-knockdown (shSMYD2) MCF7 cells.
(B-C) qPCR analysis of the expression of (B) *ESR1* gene and (C) ERα target genes *IGFBP1* and *TFF1* in control (shNT) and SMYD2-knockdown (shSMYD2) MCF7 cells after 0, 3, and 6 h of E2 treatment.

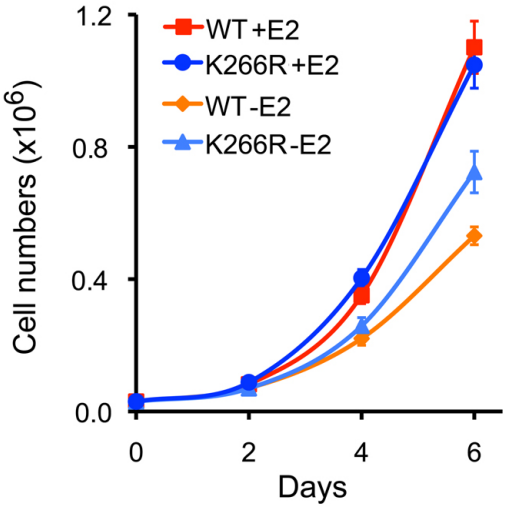

Fig. S5. MDA-MB 231 cells stably expressing the ERα-K266R mutant displays a higher growth rate then the WT ERα-expressing cells. Cells were grown without or without E2 and counted for 6 days after seeding. Error bars indicate SEM of six repeats.

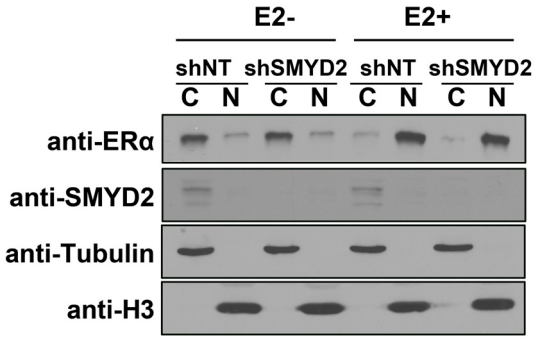

Fig. S6. SMYD2 does not affect the cellular distribution of ERα. Western blot analysis of cell fractionation assays of control (shNT) and SMYD2 knockdown (shSMYD2) MCF7 cells. Tubulin and histone H3 were used as markers for cytoplasmic and nuclear fraction, respectively.

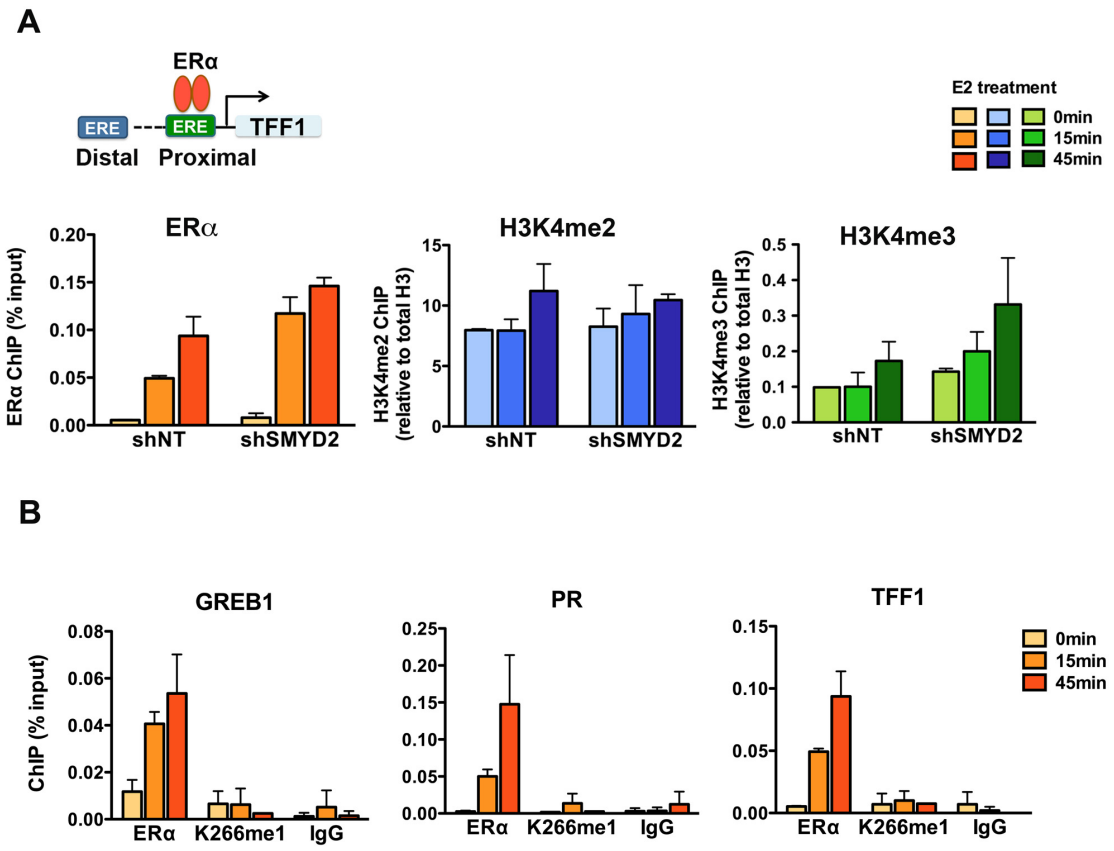

Fig. S7. SMYD2 attenuates ERα chromatin recruitment.
(A) Depletion of SMYD2 enhances ERα chromatin recruitment, but does not decrease enhancer H3K4me2 levels and promoter H3K4me3 levels of *TFF1* gene. qPCR analysis of ERαm H3K4me2 and H3K4me3 ChIP in control (shNT) and SMYD2 knockdown (shSMYD2) MCF7 cells after 0, 15 and 45 min of E2 treatment.
(B) Methylated ERα does not bind to ERE sites of *GREB1, PR* and *TFF1* genes. qPCR analysis of ChIP using the anti-ERαK266me1 antibody in MCF7 cells after 0, 15, and 45 min of E2 treatment. ChIP using the anti-ERα antibody and IgG were used as a positive and a negative control, respectively. All error bars indicate SEM of two to three independent experiments.

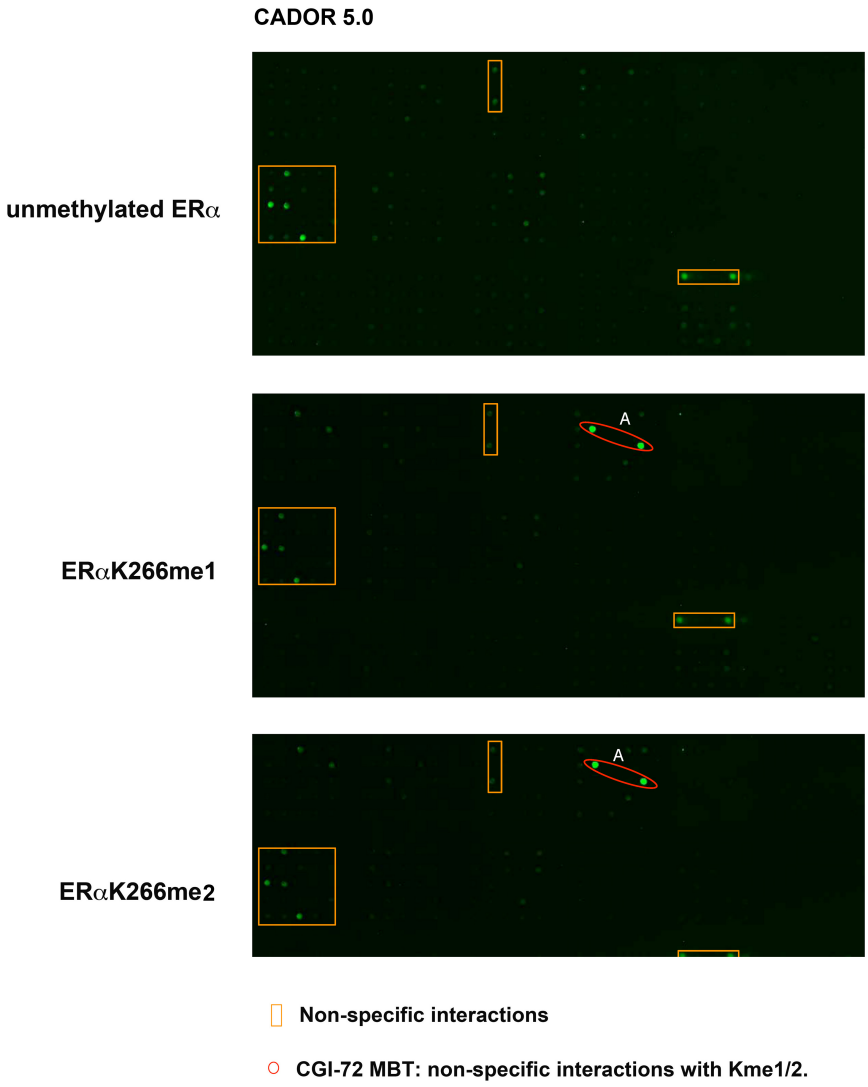

Fig. S8. ERαK266me1/2 are not recognized by methyl-readers present in the Chromatin-associated domain array (CADOR). Shown are CADOR arrays probed with ERαK266me1, ERαK266me2 and control unmethylated ERα peptides. Boxes highlight non-specific interactions and ovals indicate the CGI-72 MBT domain that binds to mono- and di-methylated lysine (Kme1/2) non-specifically.

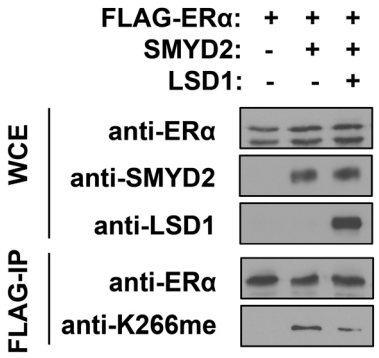

Fig. S9. LSD1 demethylates SMYD2-mediated ERαK266 methylation.
Western blot analysis with indicated antibodies of WCE and Flag-IP of 293T cells cotransfected with Flag-ERα and SMYD2 with and without LSD1. Note that overexpression of LSD1 leads to decreased ERαK266 methylation.

Table S1 Unique peptides with monomethylation, dimethylation or acetylation modifications identified in this study.

Modification	Peptide sequence	Number of peptides (unmodified/ modified)	Mascot score
K32-acetyl	R.PQL**K**IPLER.P	38/2	34
K171-methyl	R.LASTND**K**GSMAMESAKETR.Y	58/3	20
K171-dimethyl	R.LASTND**K**GSMAMESAKETR.Y	58/47	81
K171-acetyl	R.LASTND**K**GS**M**AMESAKETR.Y	58/7	48
K180-dimetyl	R.LASTNDKGS**M**A**M**ESA**K**ETR.Y	58/13	47
R277-dimetyl	R.DDGEG**R**GEVGSAG.D	12/4	33
K299-acetyl	R.AANLWPSPLMI**K**R.S	31/2	22
K401-dimetyl	R.SMEHPG**K**LLFAPNLLLDR.N	5/4	53

Modified Lys or Arg are highlighted in bold red. Oxidized Met residues are indicated in bold.

Table S2. ChIP primer sequences used in this study.

Primer	Sequence
GREB1-distal-ERE-F	TCACCGATGTTCACATAGCTAATTC
GREB1-distal-ERE-R	CCCGAAGCTGAACACTCTTTG
GREB1-proximal-ERE-F	GCCTGAAGTGACCAGCTTTTTG
GREB1-proximal-ERE-R	GCAGGTGCTCGCTTGCA
PR-distal-ERE-F	AATTTTGCAATGGCTCTGCAT
PR-distal-ERE-R	CGGATCTCCTGGAAAATGTCA
PR-proximal-ERE-F	TTGGTTCTGCTTCGGAATCTG
PR-proximal-ERE-R	CCTCCTCTCCTCACTCTTGG
TFF1-distal-ERE-F	CTGGGTGACAGGAAAGAAGC
TFF1-distal-ERE-R	CATTCTGGAAGGGACACACA
TFF1-proximal-ERE-F	GCTTAGGCCTAGACGGAATGGGC
TFF1-proximal-ERE-R	CCAGGTCCTACTCATATCTGAGAG

Supplementary Materials and Methods

Reagents and Plasmids

Complementary DNA encoding human SMYD2, ERα and ERα truncation mutants were cloned into pENTR3C and subsequently cloned into pBABE-FLAG, p3FLAG, pCAG-Myc, and pGEX destination vectors using Gateway techniques (Invitrogen). Point mutations were generated using site-direct mutagenesis (Stratagene). ShRNA constructs were purchased from Sigma. The SMYD2-targeting shRNA sequences used in this study were 5'- CGATATTTCCTGATGTTGCAT and 5'- GCTGTGAAGGAGTTTGAATCA. Polyclonal anti-monomethylated ERαK266 rabbit sera were affinity-purified with ERαK266me1 peptide and depleted against the corresponding unmethylated peptide. ERα and histone peptides were synthesized at the W.M. Keck Facility at Yale University (1, 2). Anti-ERα K266/K288ac antibody was a gift from Dr. William Lee Kraus at The University of Texas Southwestern Medical Center. Anti-SMYD2 and anti-MYC antibodies were purchased from Cell Signaling; anti-ERα, anti-LSD1 and anti-GST antibodies were from Santa Cruz; anti-FLAG (M2) and anti-tubulin antibodies were from Sigma; anti-histone antibodies including anti-H3, anti-H3K4me2, anti-H3K4me3 antibodies were obtained from Abcam.

In Vitro Methylation Assays

Recombinant GST-SMYD2 (2 μg) was incubated with various amounts of ERα proteins or peptides in methylation assay buffer (50mM Tris-HCl, pH 8.0, 10% glycerol, 20mM KCl, 5 mM MgCl2, 1 mM DTT, 1 mM PMSF and 0.1 mM SAM or ^{3}H-labeled SAM [GE

Health Care]) at 30°C for 4 h. Reactions were stopped by adding SDS-PAGE sample buffer, and the methylation status was measured by Western blotting, autoradiography, or mass spectrometry analysis. Mass spectrometry analysis of the methylated peptides was performed at the Mass Spectrometry Core at the University of Texas Medical Branch.

In-Gel Digestion and Mass Spectrometry

Endogenous ERα was affinity-purified from MCF7 cells and separated by SDS-PAGE. In-gel digestion and peptide extraction were performed as previously described (3). Briefly, Coomassie G-250-stained ERα proteins were excised from the gel and washed with 50% acetonitrile in 25 mM ammonium bicarbonate. The proteins were then digested at 37 °C overnight with sequencing grade Arg-C and Asp-N proteases (Promega), separately or together in 2 mM calcium acetate, 6 mM DTT, 50 mM NH_4HCO_3 after reduction with 50 mM DTT in 50 mM NH_4HCO_3. Digested peptide mixtures were extracted, dried in a speed vacuum concentrator and subjected to LC-MS/MS analysis.

LC-MS/MS analysis was performed using a standard top 15 method on a Q-Exactive Orbitrap mass spectrometer in conjunction with a Proxeon Easy-nLC II HPLC (Thermo Scientific) and Proxeon nanospray source. The digested peptides were reconstituted in 2% acetonitrile/0.1% trifluoroacetic acid and loaded onto a 100 μm x 20 mm Magic C18 reverse-phased trap cartridge where they were desalted online and separated using a 75 μm x 150 mm Magic C18 reverse-phased column. MS/MS data were collected using higher-energy collisional dissociation (HCD). Unassigned charge states and charge states greater than +7 were excluded for MS/MS selection. Peptides were eluted using a flow

rate of 300nL/min and a gradient of 0.1% formic acid (A) and 100% acetonitrile (B). A 90-min gradient was run with 5% to 35% B over 70 min, 35% to 80% B over 10 min, 80% B for 1 min, 80% to 5% B over 1 min, and finally held at 5% B for 8 min. MS/MS spectra were interpreted using the Mascot v.2.4 (Matrix Science) by searching against Swiss-Prot protein sequence database. Database searches were pferformed with a peptide mass tolerance of 20 ppm and MS/MS tolerance of 50 mmu, allowing one missed cleavage site. Peptide ions with a Mascot ion score of < 20 were validated manually. Each MS/MS spectrum exhibiting possible methylation or acetylation was validated manually. Annotated MS/MS spectra were generated with Expert System (4).

Cell Culture, Estrogen Treatment, Viral Transduction, and RNA Interference

Human MCF7, MDA-MB-231, U2OS and HEK 293T cells (ATCC) were maintained in DMEM (Cellgro) supplemented with 10% fetal bovine serum (Sigma). For estrogen treatment experiments, MCF-7 or engineered MDA-MB-231 cells were cultured under normal growth conditions until cell density reached 25% confluence and the media were then replaced to estrogen-free medium (phenol-red-free DMEM supplemented with 10% charcoal-stripped FBS [Sigma]). After 3-4 days of culture in the estrogen-depleted medium, cells were treated with 10 nM 17β-estradiol (E2) (Sigma) or ethanol control for various times and then collected for the designed experiments.

Retroviral transduction was performed as described previously (2). Briefly, 293T cells were co-transfected with pVPack-VSV-G, pVPack-GP (Stratagene), and pBABE-FLAG-ERα (WT and mutants) or a control vector, and viral supernatants were harvested after 48

hours. For lentiviral shRNA packaging, 293T cells were co-transfected with pMD2.G and pPAX2 (Addgene) as well as pLKO-shRNA constructs. For infections, the cells were incubated with viral supernatants in the presence of 8 ug/ml polybrene; after 48 h, the infected cells were selected with puromycin (2 μg/ml).

Chromatin immunoprecipitation

ChIP analysis was performed essentially as previously described (2). Briefly, cells were crosslinked with 1% formaldehyde for 10 min at room temperature, and the reaction was stopped with 125 mM glycine. Nuclei were isolated by resuspending the cells in swelling buffer containing 5 mM PIPES pH 8.0, 85 mM KCl, 1% NP-40 and a complete protease inhibitor for 20 min at 4 °C. The isolated nuclei were resuspend in nuclei lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) and sonicated using a Bioruptor Sonicator (Diagenode). Samples were IPed with the appropriate antibodies overnight at 4°C. Immunoprecipitates were washed twice with dialysis buffer (50 mM Tris pH 8.0, 2 mM EDTA, 0.2% Sarkosyl) and four times with IP wash buffer (100 mM Tris pH 8.0, 500 mM LiCl, 1% NP-40, and 1% deoxycholic acid sodium salt). After reverse crosslinking was performed, DNA was eluted and purified using PCR purification kit (Qiagene).

Reverse transcription PCR (RT-PCR) and Real-Time PCR

RT-PCR and real-time PCR and were performed as previously described (2). mRNA was prepared using the RNeasy Plus kit (Qiagen) and reverse-transcribed using the First Strand Synthesis kit (Invitrogen). Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI 7500-FAST Sequence Detection System using the Power SYBR

Green PCR Master Mix (Applied Biosystems). Gene expressions were calculated following normalization to GAPDH levels using the comparative Ct (cycle threshold) method. The primer sequences used for ChIP analyses are listed in Table S2.

Cell Fractionation, Immunoprecipitation (IP) and co-IP

Cell fractionation experiments were performed as described previously with slight modifications (2). Briefly, cells were collected from one 100-mm dish and resuspended in buffer A containing 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34M sucrose, 10% glycerol, 1 mM DTT, and a complete protease inhibitor tablet (Roche). Trition X-100 was added to a final concentration of 0.1%. The cells were incubated for 8 minutes, and nuclei were collected using centrifugation at 1,300 x g at 40°C for 5 min. The supernatant was clarified using centrifugation at 20,000 x g at 40°C for 5 min. The nuclear pellet from low-speed centrifugation was washed three times and resuspended in buffer A. The suspended cells were then sonicated using Branson digital sonifier. All fractions were boiled in SDS sample buffer and analyzed by Western blotting.

For IP and co-IP experiments, cells were lysed in cell lysis buffer containing 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM DTT, and a complete protease inhibitor tablet (Roche). ERα antibodies conjugated with Protein A/G beads (Millipore) or anti-FLAG M2-conjugated agarose beads (Sigma) were incubated with lysates overnight at 4°C. The beads were then washed 3-6 times with cell lysis buffer, and the bound proteins were eluted in SDS buffer and analyzed by Western blot.

References

1. Shi X*, et al.* (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* 442(7098):96-99.
2. Wen H*, et al.* (2010) Recognition of histone H3K4 trimethylation by the plant homeodomain of PHF2 modulates histone demethylation. *J Biol Chem* 285(13):9322-9326.
3. Yan J*, et al.* (2008) Profiling the phospho-status of the BKCa channel alpha subunit in rat brain reveals unexpected patterns and complexity. *Mol Cell Proteomics* 7(11):2188-2198.
4. Neuhauser N, Michalski A, Cox J, & Mann M (2012) Expert system for computer-assisted annotation of MS/MS spectra. *Mol Cell Proteomics* 11(11):1500-1509.