A Histone H2A Deubiquitinase Complex Coordinating Histone Acetylation and H1 Dissociation in Transcriptional Regulation

Ping Zhu, Wenlai Zhou, Jianxun Wang, Janusz Puc, Kenneth A. Ohgi, Hediye Erdjument-Bromage, Paul Tempst, Christopher K. Glass, and Michael G. Rosenfeld

SUPPLEMENTAL DATA

Fig. S1

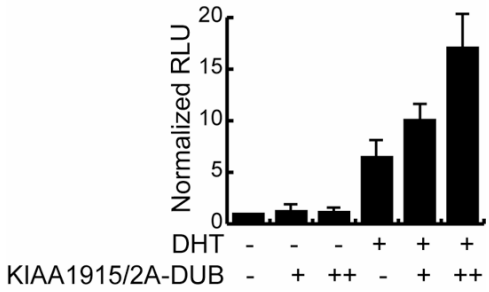


Fig. S1. 2A-DUB activates ARE-dependent Reporter. An ARE-driven luciferase reporter was used to evaluate the regulatory function of 2A-DUB in LNCaP prostate cancer cells.

Fig. S2

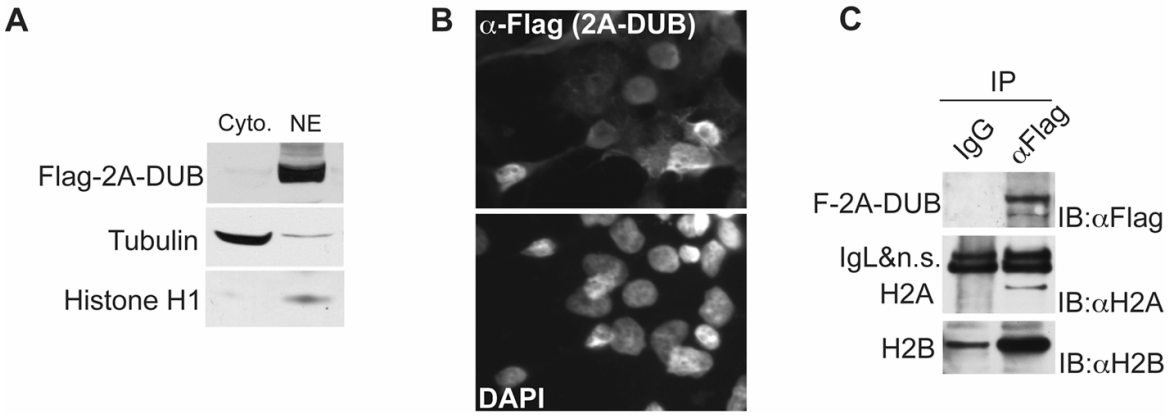


Fig. S2. Subcellular localization of 2A-DUB and interactions with histones. (A) Western blot analysis of the HEK293 cell fractions (Cyto.: cytosol; NE: nuclear extract). Tubulin and histone H1 were used as a cytoplasmic and a nuclear marker, respectively. **(B)** Immunostaining of Flag-2A-DUB using anti-Flag antibody in HEK293 cells. DAPI shows the nuclear DNA staining. **(C)** Coimmunoprecipitations of Flag-2A-DUB and histones H2A/H2B in soluble chromatin extracts from the tranfected HEK293T cells. IgL: IgG light chain; n.s.: non-specific.

Fig. S3

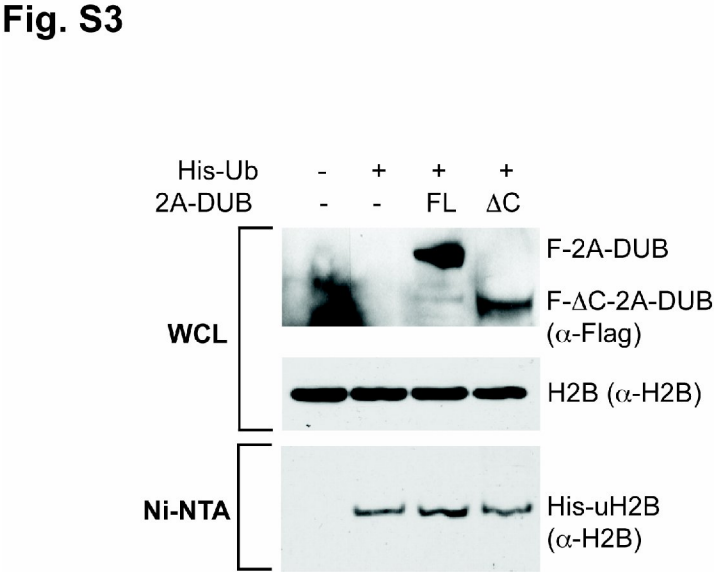


Fig. S3. *In vivo* ubiquitination assays for histone H2B. *In vivo* ubiquitination assay for H2B after overexpression of human full-length (FL) and C-terminal enzymatic JAMM/MPN+ domain-deleted mutant (ΔC) 2A-DUB (aa 17-531) in HEK293T cells. WCL represents whole cell lysates.

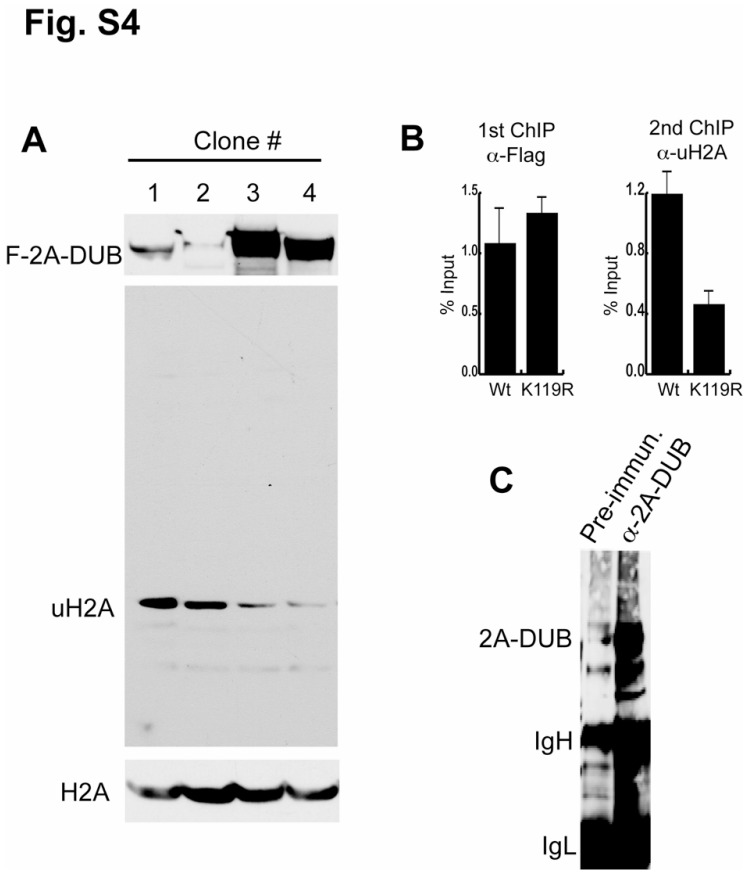


Fig. S4. Validation of the antibodies directed against uH2A and 2A-DUB. (A) uH2A levels were detected by Western blot analysis in HEK293 cell clones stably expressing Flag-tagged 2A-DUB. **(B)** A 2-step ChIP assay for *PSA* promoter using anti-Flag (1st ChIP) and anti-uH2A antibody (2nd ChIP) in HEK293 cells stably expressing wt (ubiquitinated) or K119R (non-ubiquitinated) Flag-H2A (see Fig. S5). Note that the 1st anti-Flag ChIP pulled down similar amount of *PSA* promoter DNA from the wt and K119R Flag-H2A-expressing cell lines, suggesting that both are incorporated into nucleosomes at a similar level. Importantly, 2nd ChIP using anti-uH2A pulled down a significantly higher amount of DNA from wt Flag-H2A, indicating the specificity and efficiency of anti-uH2A antibody in ChIP assay. **(C)** 2A-DUB protein was immunoprecipitated from LNCaP cell lysates using preimmune IgG and anti-2A-DUB IgG. Western blot analysis was used to evaluate the immunoprecipitates using anti-2A-DUB IgG.

Fig. S5

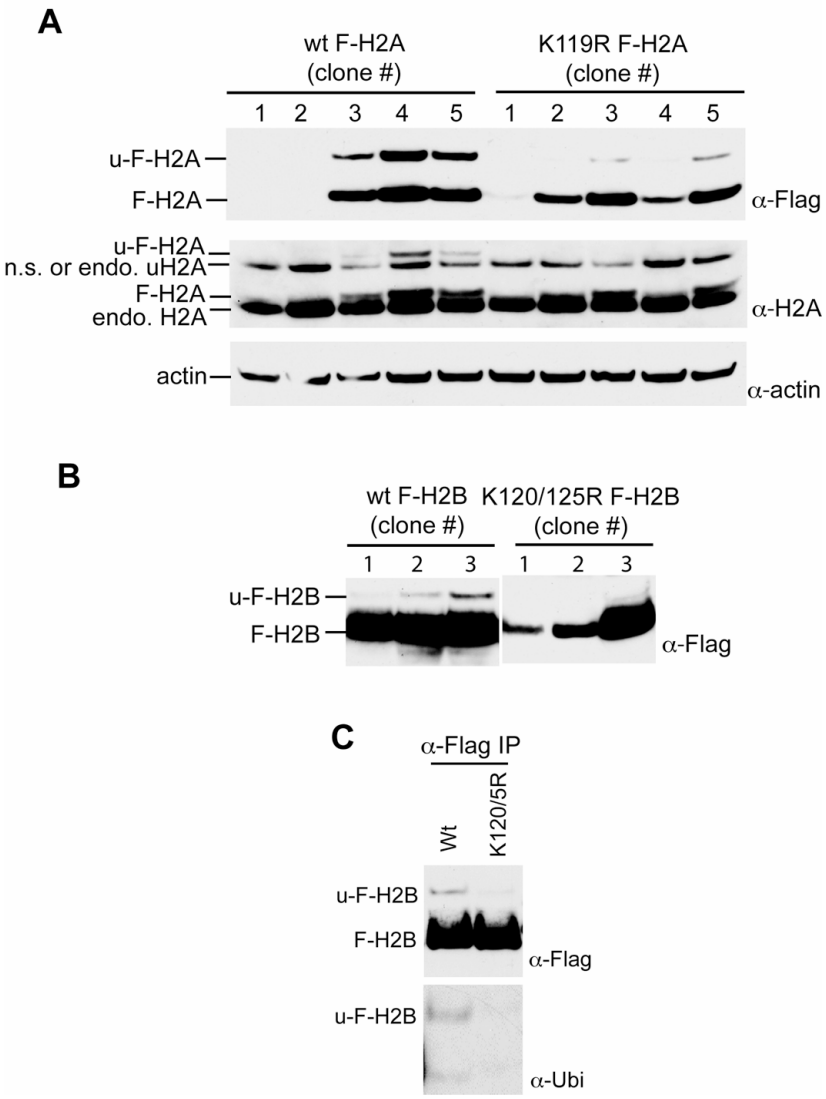


Fig. S5. Establishment of HEK293 cell lines stably expressing Flag-tagged wt H2A, K119R H2A, wt H2B and K120/125R H2B. (A) Western blot analysis of sonicated cell lysates from stable clones expressing Flag-H2A. **(B)** Western blot analysis of sonicated cell lysates from stable clones expressing Flag-H2B. **(C)** Western blot analysis of anti-Flag affinity-purified Flag-H2B-containing mononucleosomes.

Fig. S6

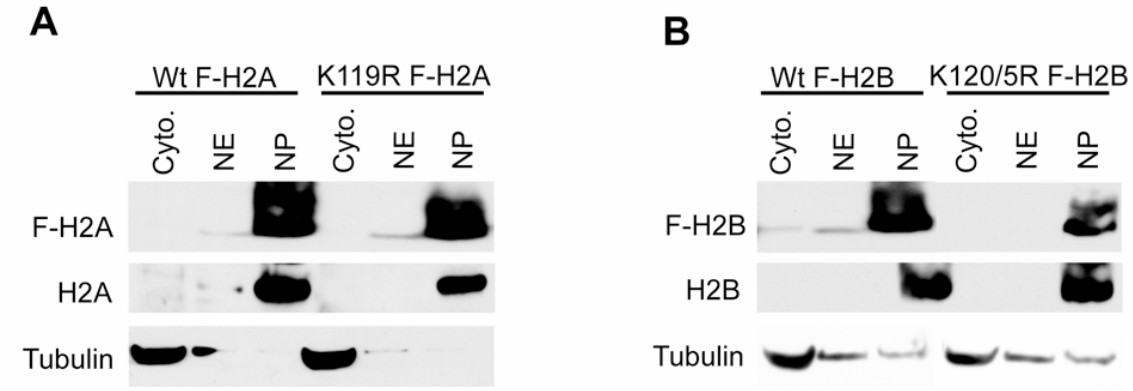


Fig. S6. Efficient incorporation of Flag-tagged H2A/H2B into chromatin in HEK293 stable cell lines (see Fig. S5). (A) From Flag-H2A-expressing HEK293 stable cells, cytoplasmic fractions (cyto.) were isolated using hypotonic buffer, and nuclear extracts (NE) were isolated by the nuclear extract buffer (hypotonic buffer plus 420 mM KCl). The nuclear pellets (NP) contain proteins incorporated into chromatin. All of the cell fractions were subject to Western blot analysis. Cytoplasmic and nuclear fractions were loaded at the same dilution and chromatin fractions (NP) were loaded at a 10-fold dilution. Endogenous H2A and tubulin serve as nucleosomal and cytoplasmic marks, respectively. **(B)** Same experiment carried out in Flag-H2B-expressing HEK293 stable cells.

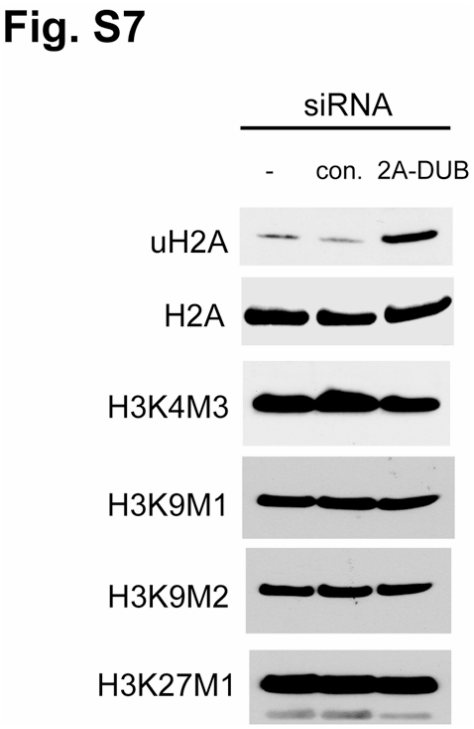


Fig. S7. Increasing uH2A levels by knock-down of 2A-DUB does not affect histone methylation marks. After knocking down 2A-DUB by specific siRNA in HEK293T cells, sonicated cell lysates were used for Western blot analysis using indicated antibodies.

Fig. S8

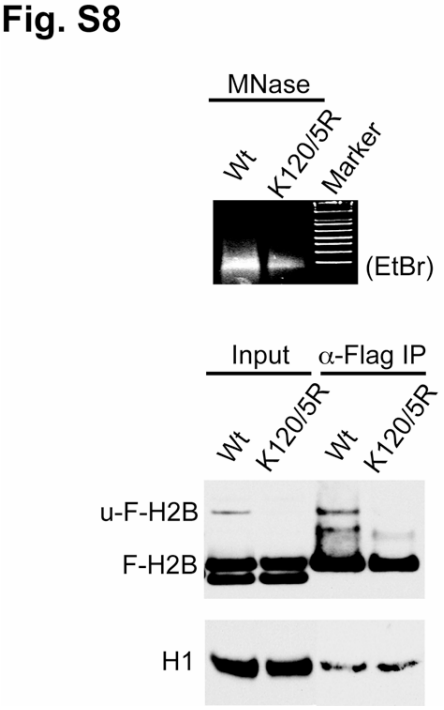


Fig. S8. Detection of composition of associated linker histone H1 in immuno-purified Flag-H2B-containing mononucleosomes. HEK293 cells stably expressing wt or K120/125R Flag-H2B were used to purify Flag-H2B-containing mononucleosomes. The associated histone H1 was detected by Western blot analysis.

Fig. S9

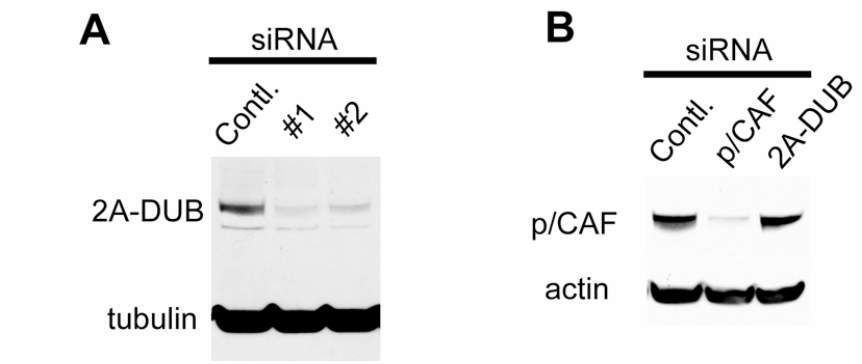


Fig. S9. Knocking down of 2A-DUB and p/CAF by specific siRNA in LNCaP cells. The knock-down efficiency of siRNAs against 2A-DUB (#1 and #2) **(A)** and p/CAF **(B)** were tested by Western blot analysis.

Fig. S10

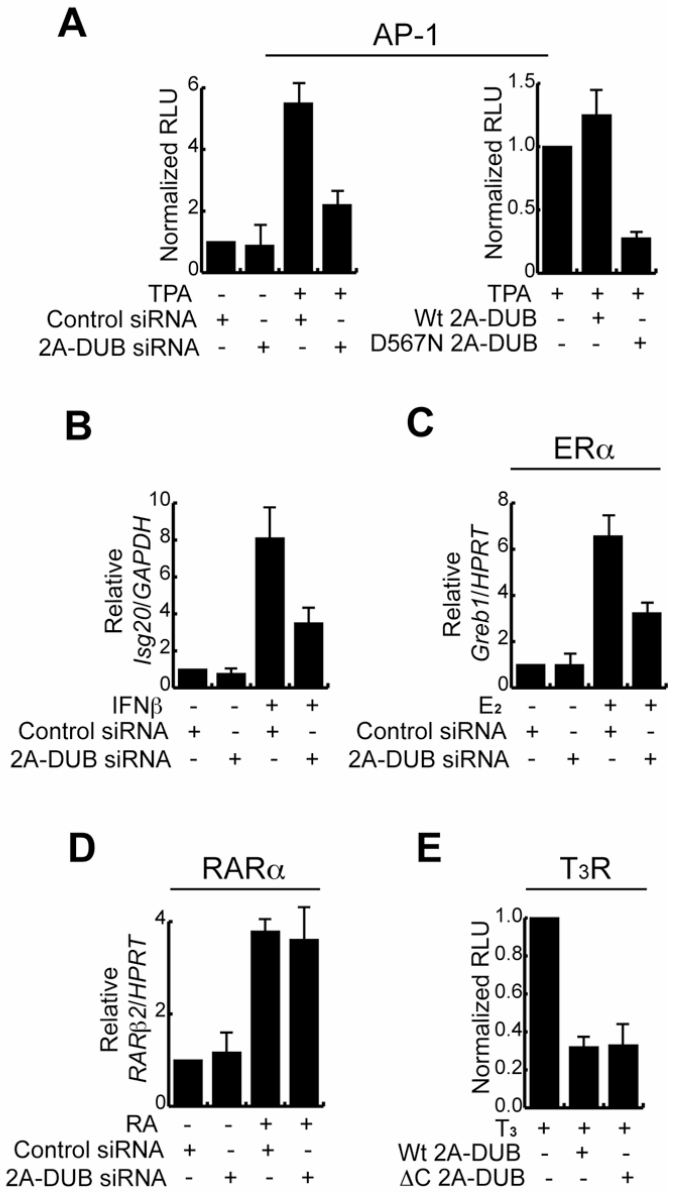


Fig. S10. The role of 2A-DUB in regulated transcription events. (A) Reporter assay using TRE-driven luciferase reporter (AP-1-dependent) in HeLa cells. Values are mean±SEM of three independent experiments. **(B)** Quantitative RT-PCR analysis of interferon β (IFNβ)-induced genes in RAW cells after treatment with siRNA and/or

ligands as indicated. Values (normalized to corresponding values of internal control gene *GAPDH*) are means±SEM of two independent experiments. **(C)** Quantitative RT-PCR analysis of ERα target gene *Greb1* in U2OS-ERα cells after treatment with siRNA and/or ligands as indicated. Values (normalized to corresponding values of internal control gene *HPRT*) are means±SEM of two independent experiments. **(D)** Quantitative RT-PCR analysis of RARα target gene *RARβ2* in HEK293T cells after treatment with siRNA and/or ligands as indicated. Values (normalized to corresponding values of internal control gene *HPRT*) are means±SEM of two independent experiments. **(E)** Reporter assay using a luciferase reporter driven by the promoter of *Dio1*, a T_3R target gene, in P19 cells treated with T_3.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cells, antibodies, siRNAs and other reagents

Human osteoblastoma cell line U2OS-ERα was a generous gift from Drs. D. Monroe and T. Spelsberg. Cell lines stably expressing tagged wild-type or mutant 2A-DUB and/or H2A/H2B were generated by G418 selection for 2 weeks after transfecting HEK293 cells. Expression levels of transfected proteins in selected single clones were confirmed by Western blot analysis, and then single clones were pooled for bulk culture if needed. The following commercially available antibodies were used: from Santa Cruz: anti-AR (441 and N-20), anti-p/CAF (E-8 and H-369), anti-CBP (C-20), anti-hnRNP H1 (N-16), anti-Trip5/KIF11/Eg5 (C-20), anti-H1 (AE-4), anti-His (H-15); from Upstate: anti-uH2A (05-678), anti-H2A (07-146), anti-H2B (07-371), anti-H1 (05-457), anti-phospho-H1 (05-805), anti-Ac-H3 (06-599), anti-H3K9Ac (07-352), anti-H3K14Ac (06-911), anti-H3K4M3 (07-473), anti-H3K9M1 (07-395), anti-H3K9M2 (07-521), anti-H3K27M1 (07-448); from Abcam: anti-H1 (ab7789) and anti-H3K36M3 (ab9050); from Covance: anti-phospho-Ser-2 CTD of polII (H5), anti-phospho-Ser-5 CTD of polII (H14), and anti-ubiquitin (P4D1); and anti-Flag (M2) and anti-actin (A-3853) were from Sigma. Anti-2A-DUB antibodies were generated by immunizing Guinea Pigs by a recombinant 2A-DUB fragment (aa 1-280), and verified by Western blotting, immunoprecipitation and ChIP. Anti-Flag M2 resins and 3xFlag peptides were purchased from Sigma. Dihydrotestosterone (DHT), 17-β-Estradiol (E_2), all-trans retinoic acid (RA), thyroid hormone T_3, trichostatin A (TSA), 12-O-Tetradecanoylphorbol 13-acetate (TPA) and calf thymus histone extracts were purchased from Sigma. Other compounds and reagents used in this work were: The concentrations of treatment were: DHT (20 nM), E_2 (10-20 nM), RA (1 mM), T_3 (50 nM), TPA (0.1 nM), and IFNβ (1000 unit/ml) unless otherwise indicated. Lipofectamine 2000 (Invitrogen) was used for transfection experiments following the manufacturer's manuals. The siRNAs are as follows: human 2A-DUB (Mysm1) #1 (Dharmacon, SMARTpool), Human 2A-DUB #2 (Qiagen), human p/CAF (Qiagen), human RING2/Ring1B (Dharmacon, SMARTpool). Control siRNA were purchased from Dharmacon and Qiagen. Sequence information is available upon request.

Affinity purification of 2A-DUB protein complex

In brief, nuclear extracts were prepared from $5x10^9$ HEK293 cells transfected with empty plasmid or cells stably expressing Flag-tagged 2A-DUB. After incubating with M2 anti-Flag resin (Sigma) at 4C overnight, resin were packed in columns and washed sequentially with TBS (20 bed volumes), TBS with 420 mM KCl (3 bed volumes), and TBS until OD280 of the flow-through is <0.02 over that of TBS. Then the Flag-2A-DUB complex was eluted by 1 bed volume of 3xFlag peptide (250 ng/ml, Sigma) for three times sequentially. The eluates were combined and concentrated for SDS-PAGE and Commassie staining. Visible protein bands were excised from the gel and subject to mass spectrometric analysis.

Mass spectrometric analysis

Gel-resolved proteins were digested with trypsin, batch purified on a reversed-phase micro-tip, and resulting peptide pools individually analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS) (*UltraFlex TOF/TOF*; BRUKER; Bremen, Germany) for peptide mass fingerprinting, as described (Erdjument-Bromage et al., 1998; Winkler et al., 2002). Selected peptide ions (*m/z*) were taken to search the human segment of a "non-redundant" protein database (NR; 4,626,804 entries on 16th February, 2007; National Center for Biotechnology Information, Bethesda, MD), utilizing the PeptideSearch algorithm (Matthias Mann, personal communication; an updated version of this program is available as 'PepSea' from MDS-Denmark). A molecular mass range up to twice the apparent molecular weight (as estimated from electrophoretic relative mobility) was covered, with a mass accuracy restriction of less than 40 ppm, and maximum one missed cleavage site allowed per peptide. To confirm PMF results with scores ≤40, mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples, using the UltraFlex instrument in 'LIFT' mode. Fragment ion spectra were taken to search NR using the MASCOT MS/MS Ion Search program (Perkins et al., 1999), version 2.2. for Windows (Matrix Science Ltd., London, UK). Any tentative confirmation (Mascot score ≥ 30) of a PMF result thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

Western blot, immunoprecipitation (IP) and chromatin IP (ChIP)

Western blot, IP and ChIP were performed as described previously (Zhu et al., 2006). For monoclonal IgM antibodies, protein L conjugated beads (Santa Cruz) were used for precipitation. Primer pairs used for ChIP assays are: *PSA* enhacer: 5'-AGATCCAGGCTTGCTTACTGTCCT-3' and 5'-ACCTGCTCAGCCTTTGTCTCTGAT-3'; *PSA* promoter: 5'-TGGGTCTTGGAGTGCAAAGGATCT-3' and 5'-AGACACGCCCAGGATGAAACAGAA-3'; *PSA* promoter for anti-H1 ChIP: 5'-GTC TTGGAGTGCAAAGGATC-3' and 5'- TTCATCTAGGGGACAAAGGC-3'; and *PSA* exon 4: 5'-GCACAACTCATCTGTTCCTGCGTT-3' and 5'-TTCTGTGTTGTGGTCCCTGTTCCT-3'.

***In vivo* ubiquitination assay**

Cells were co-transfected with plasmids encoding His_6-tagged ubiquitin and full-length 2A-DUB (or ΔC 2A-DUB), and harvested 40 h after transfection of cells. The cells were washed twice in ice-cold PBS and 7.5 x 10^6 cells (from 6-mm dish) were lysed in 6 ml of guanidinium lysis buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 [pH 8], 0.01 M Tris [pH 8], 5 mM imidazole (fresh-add), 10 mM ß-mercaptoethanol). Then 75 µl of Ni^{2+}-nitrilotriacetic acid (NTA)-agarose beads (Qiagen) were added to the lysates, followed by incubation/rotation overnight. The beads were successively washed with the following buffers: guanidinium buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 [pH 8], 0.01 M Tris-HCl [pH 8], 10 mM ß-mercaptoethanol), urea buffer (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4 [pH 8], 0.01 M Tris-HCl [pH 8], 10 mM ß-mercaptoethanol), buffer A (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4 [pH 6.3], 0.01 M Tris-HCl [pH 6.3], 10 mM ß-mercaptoethanol), and buffer A plus 0.1% Triton X-100. Elution was carried out with 200 mM imidazole in 0.15 M Tris-HCl (pH 6.7) supplied with 5% SDS, 30% glycerol and 0.72 M ß-mercaptoethanol. The eluates were diluted 1:1 with 2x SDS-PAGE sample buffer and subjected to SDS-PAGE, and ubiquitinated proteins probed with specific antibodies by Western blot analysis.

***In vitro* deubiquitination assay**

Briefly, to purify mono/oligonucleosomal substrates, nuclear pellets from Flag-H2A/H2B-expressing HEK293 cells were prepared by lysing cells sequentially in hypotonic buffer (25 mM Tris-HCl [pH 8], 10 mM KCl, 1 mM MgCl2, 0.3% NP40 and 1x protease inhibitors) and nuclear extract buffer (hypotonic buffer plus 420 mM NaCl). After washing in MNase digestion buffer (50 mM Tris-HCl [pH 8], 10 mM CaCl2, 2 mM MgCl2), pellets were digested with MNase (Sigma, used at 1 unit/mg DNA) for 5-30 min at 25C, and the reactions were stopped by adding 2 mM (f.c.) EDTA. Following brief sonication and checking the size of digested DNA fragments, lysates were centrifuged and the supernatants were subjected to chromatography with M2 anti-Flag resin (Sigma). Using about 3 μg of affinity-purified 2A-DUB complex and 2 μg of purified Flag-H2A (or H2B)-containing mono/oligonucleosomes, deubiquitination assay was carried out in the reaction buffer (125 mM Tris-HCl [pH 7.4], 50 mM NaCl, 25 mM MgCl2, 10 mM ATP, 1x ATP regeneration system, and 10% glycerol) for 1 hrs at 37C. Samples were then subject to Western blot analysis for monoubiquitinated Flag-tagged H2A or H2B.

***In vitro* HAT assay**

Calf thymus histones (Sigma) or purified oligonucleosomes (about 10 μg) were incubated with affinity-purified Flag-tagged 2A-DUB protein complex (about 2 μg) or recombinant p/CAF HAT domain (0.2 μg, Biomol) in the HAT assay buffer (100 mM Tris-HCl [pH 8], 50 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 400 nM TSA, 1x protease inhibitor cocktail [Roche], 10% glycerol, and 25 nCi [^{3}H] acetyl-coA [GE healthcare]). After 90 min incubation at 37C, samples were subjected to SDS-PAGE, stained by Commassie blue, and incubated with Amplify solution (GE healthcare).

Reporter assay and quantitative RT-PCR

Reporter assay and quantitative RT-PCR were performed as described previously (Zhu et al., 2006). Primer pairs used for quantitative RT-PCR were: human *PSA*, 5'-ACTTCAGTGTGTGGACCTCCATGT-3' and 5'-AGCACACAGCATGAACTTGGTCAC-3'; human *Nkx3.1*: 5'-TGAAGGCGCAGGCTTACTG-3' and 5'-TAGGCTGCCTTCTTTTGGATGT-3'; human *2A-DUB*: 5'-GCTGTTTGAACAAGGGCTGGCTAA-3' and 5'-

GGCCGGTCTTCTGATTTGGTGTTT-3'; human *β-actin*, 5'-GGCACCCAGCACAATGAAGATCAA-3' and 5'-ACTCGTCATACTCCTGCTTGCTGA-3'; human *HPRT*: 5'-TGGAGTCCTATTGACATCGCCAGT-3' and 5'-AACAACAATCCGCCCAAAGGGAAC-3'; human *Greb1*: 5'-TGCCAGATGACAATGGCCACAATG-3' and 5'-TCTGCTTCTTGGGTTGAGTGGTCA-3'; human *RARβ2*: 5'-TCTGTCAGTGAGTCCTGGGCAAAT-3' and 5'-TCAGAGCTGGTGCTCTGTGTTTCA-3'; mouse *Isg20*: 5'-AGAAGCTGCCACTCCTCAAGTTCT-3' and 5'-TTTACACATTGCGGTGGTTGGTGG-3'; mouse *GAPDH*: 5'-TCAACAGCAACTCCCACTCTTCCA-3' and 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'.

Immunostaining of Prostate TMA

Prostate tissue microarrays (Cat# A302) was purchased from ISU Abxis (Seoul, South Korea) containing prostate cancer tissues with corresponding normal tissues. For detecting uH2A levels in prostate tissues, anti-uH2A antibody (Upstate, 1:50 dilution) and Rhodamine-conjugated anti-mouse IgM secondary antibodies (Jackson ImmunoResearch) were used for staining. Staining was performed as previously described (Zhu et al., 2006). Almost no fluorescence and no specific patterns were observed in control slides prepared without primary antibodies. Staining was scored in a 4-grade scale independently for uH2A with fixed instrument settings. Grade one was designated as no detectable staining above background. Score two corresponded to weak nuclear staining over the background or weak-stained cells less than 10% out of total cells. Grade three was strong staining, and grade four was considered as very strong staining that reached saturation in the chosen sensitivity setting for screen presentation. Data sets were combined after completion of scoring, and average grades of tumor tissue and corresponding normal tissues was calculated and analyzed by the paired two-tail Student's *t* test for statistical significance.

SUPPLEMENTAL REFERENCES

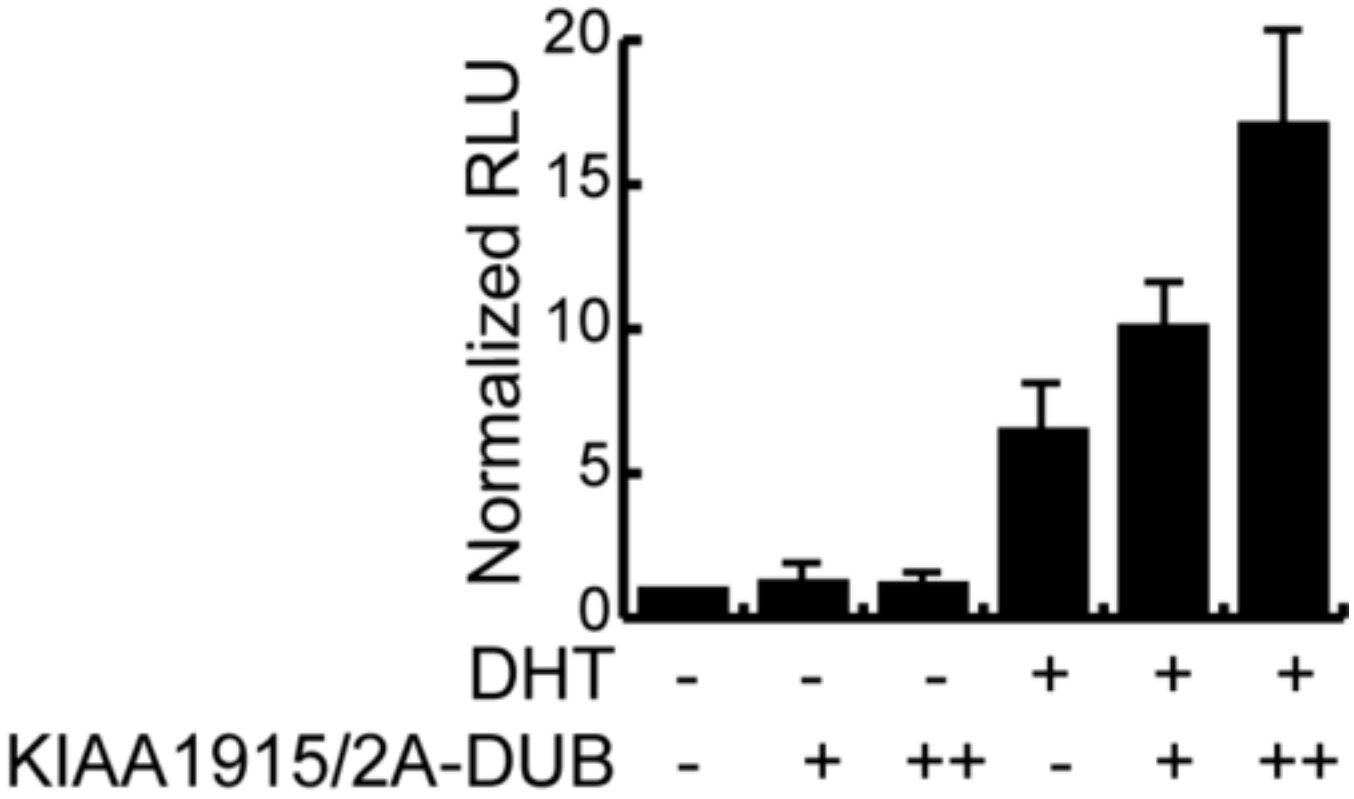
Erdjument-Bromage, H., Lui, M., Lacomis, L., Grewal, A., Annan, R. S., MacNulty D. E., Carr, S. A., and Tempst, P. (1998). Micro-tip reversed-phase liquid chromatographic extraction of peptide pools for mass spectrometric analysis. *J. Chromatogr. A* 826: 167-181.

Perkins, DN, Pappin, DJ, Creasy, DM and Cotrell, JS. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3551-3567.

Winkler, G S., Lacomis, L., Philip, J., Erdjument-Bromage, H. Svejstrup, J. Q., and Tempst. P. (2002). Isolation and mass spectrometry of transcription factor complexes. *Methods* 26, 260-269.

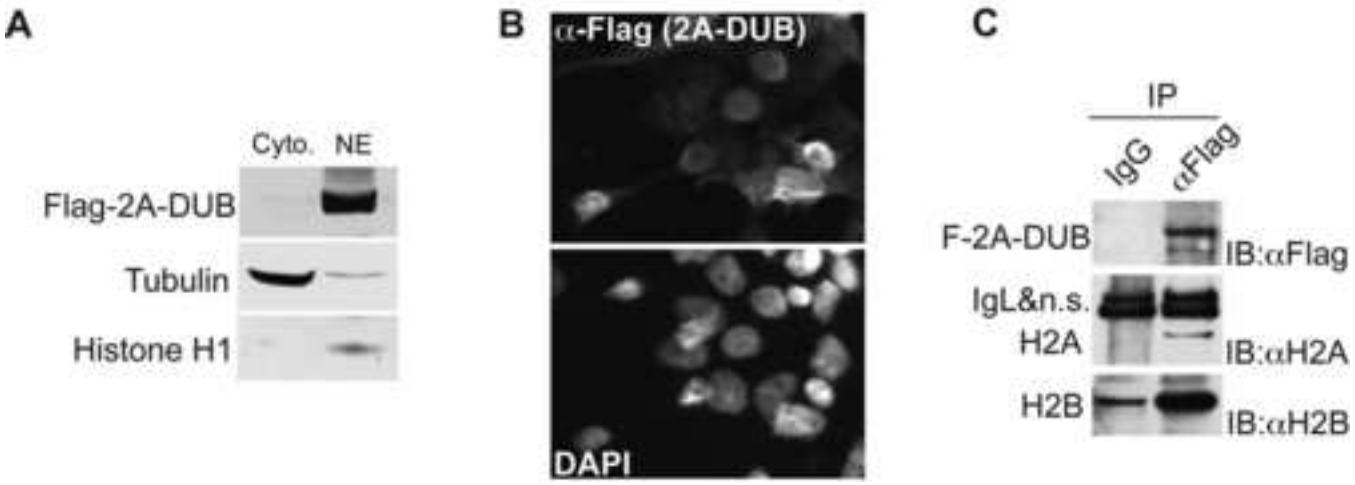
Zhu P, Baek SH, Bourk EM, Ohgi KA, Garcia-Bassets I, Sanjo H, Akira S, Kotol PF, Glass CK, Rosenfeld MG, Rose DW. (2006). Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. *Cell* 124(3): 615-629.

Fig. S1



F) Supplemental Text and Figures

Fig. S2



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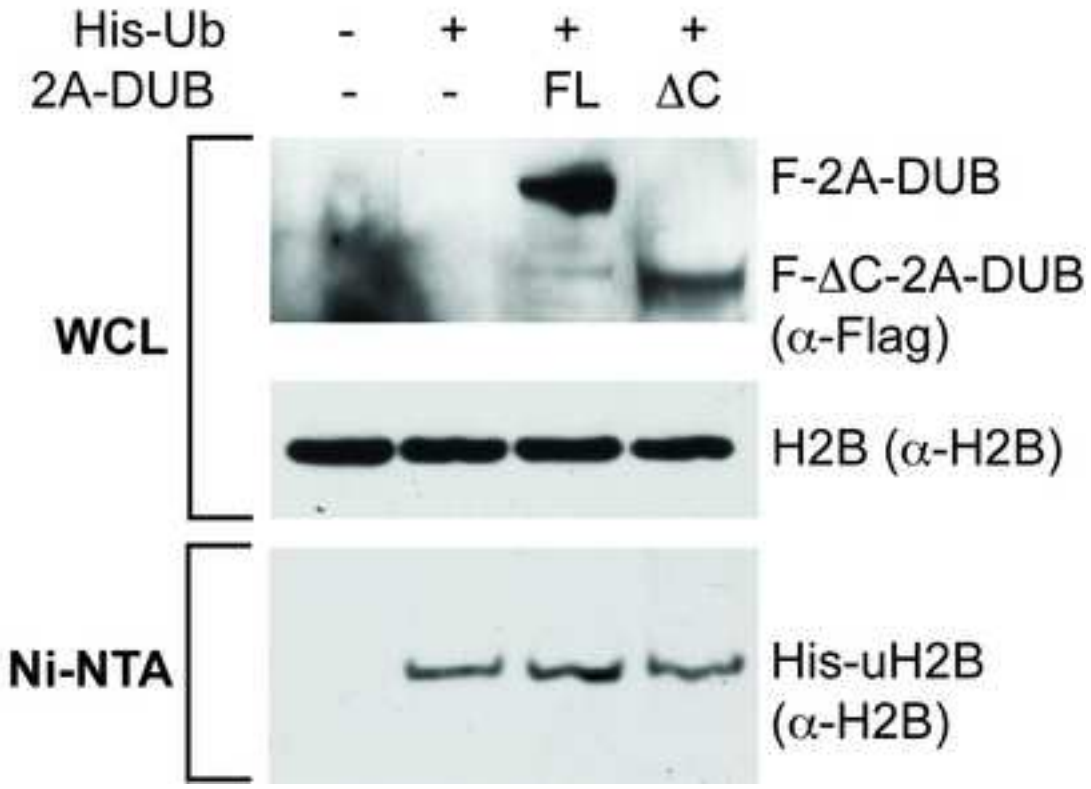
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Fig. S4

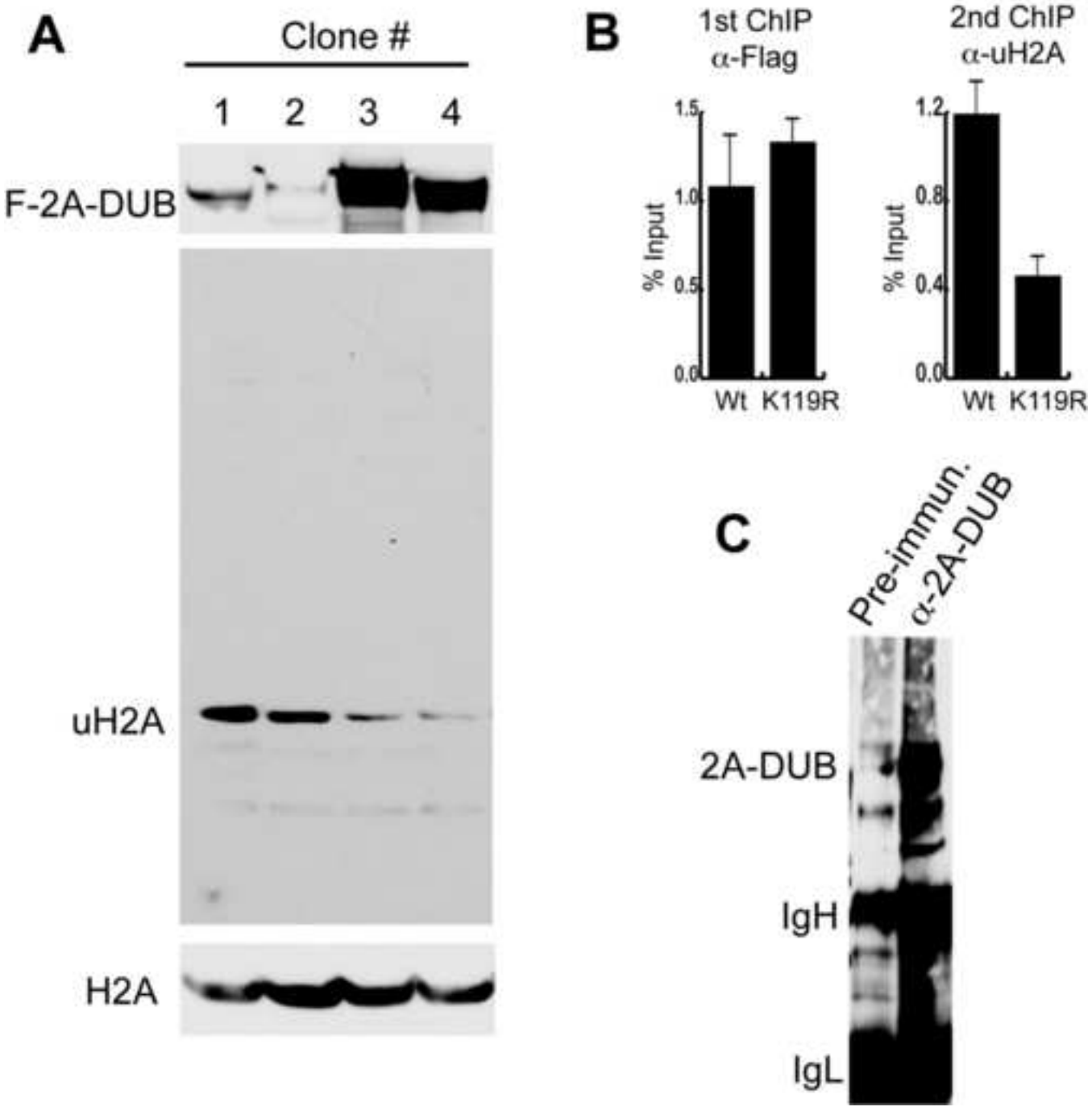
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Fig. S5

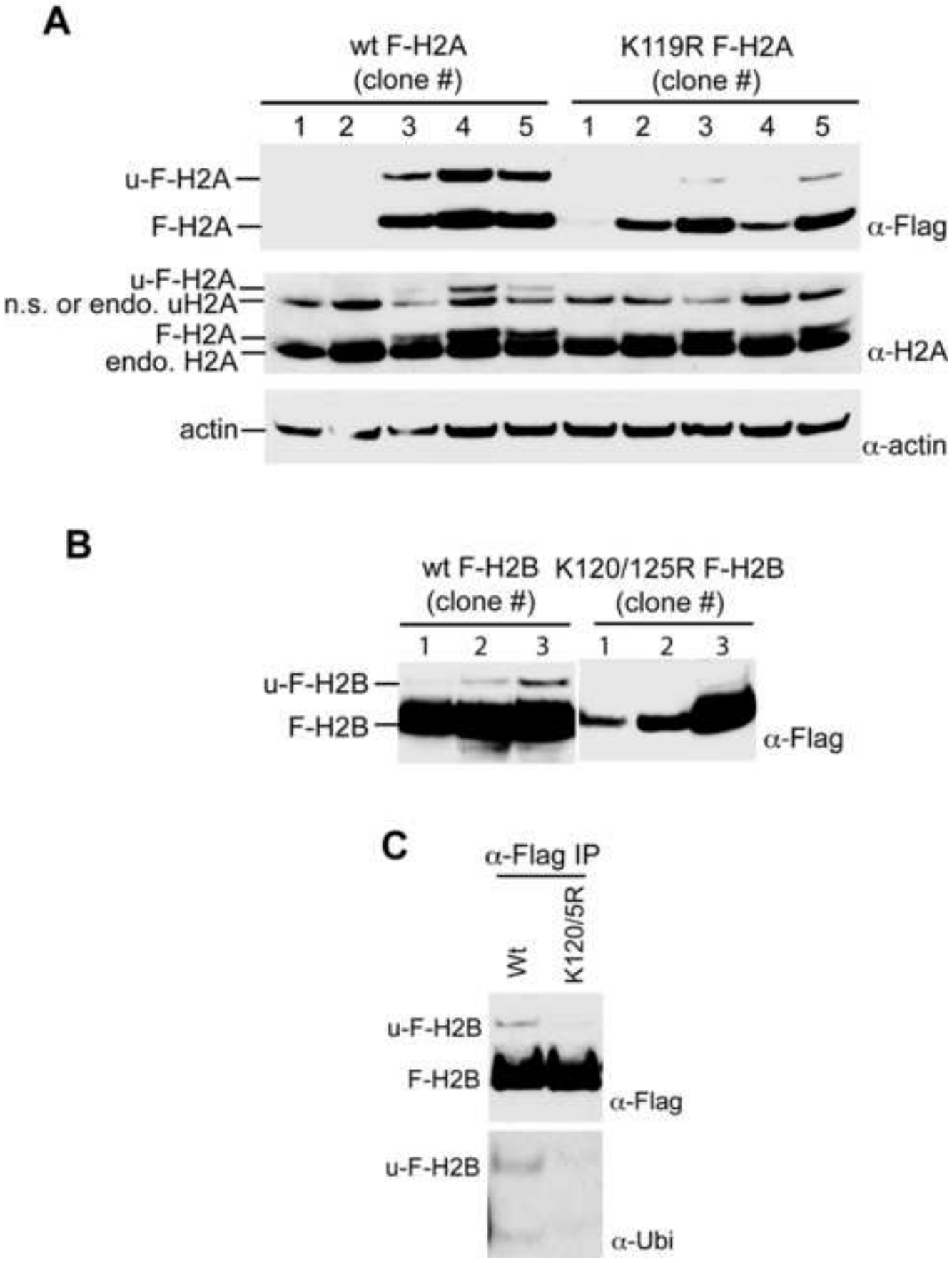
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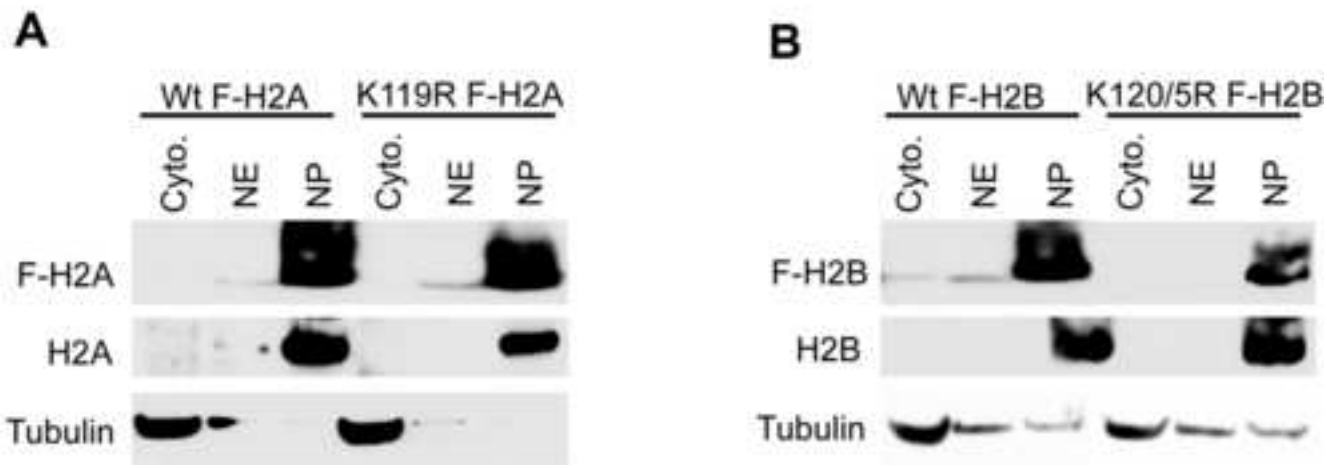
Fig. S6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Tubulin

Fig. S7

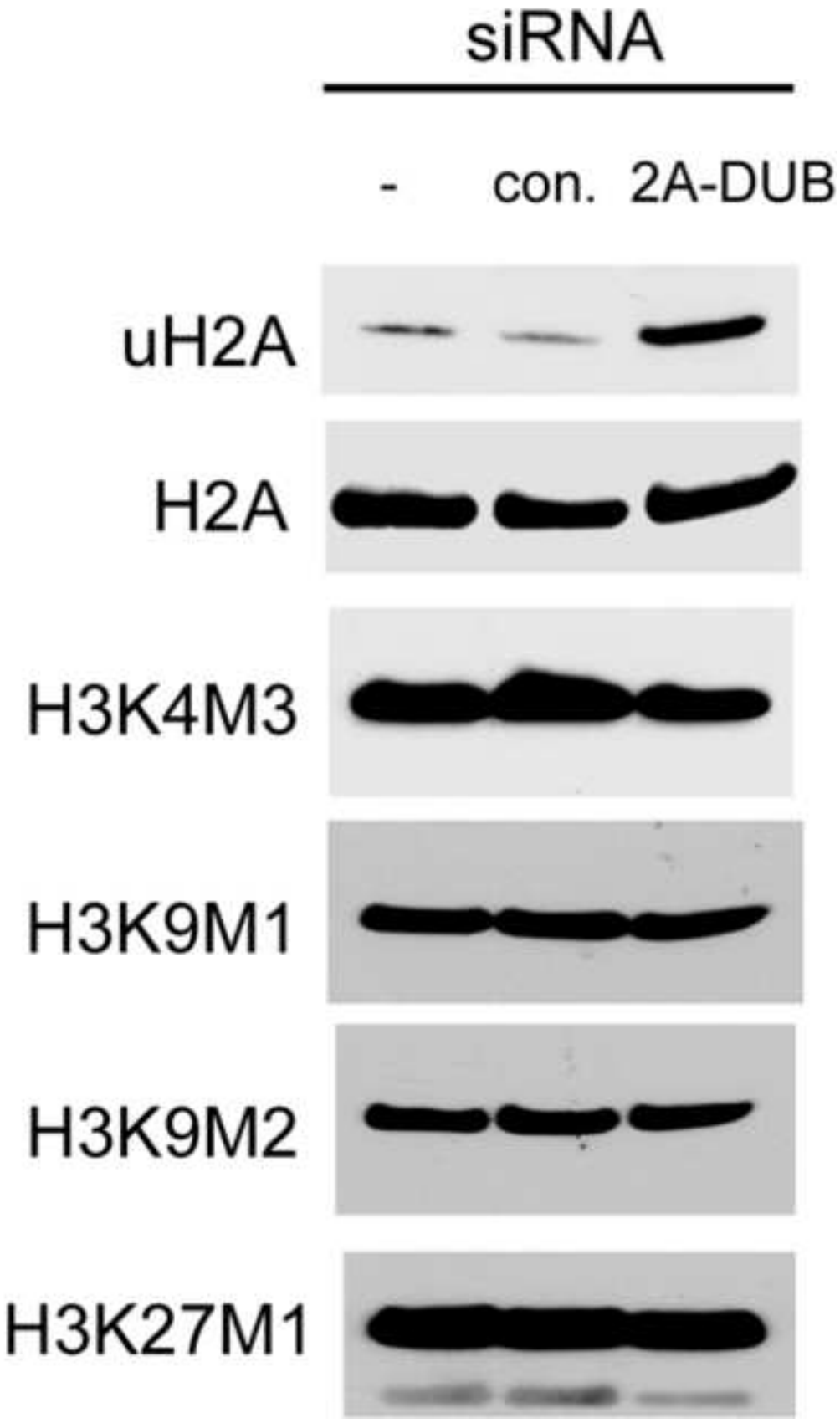
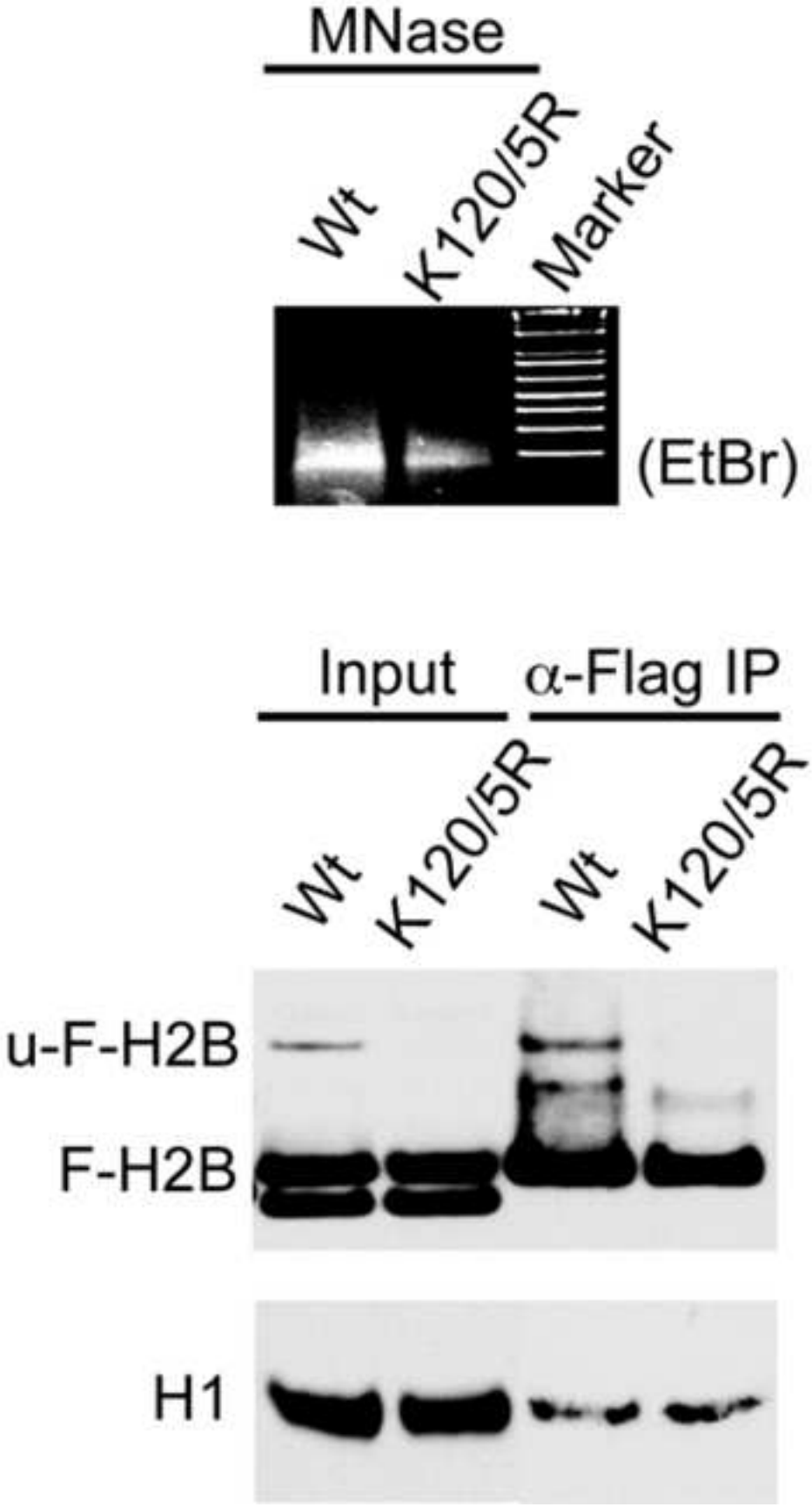


Fig. S8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F-H2B
H1

Fig. S9

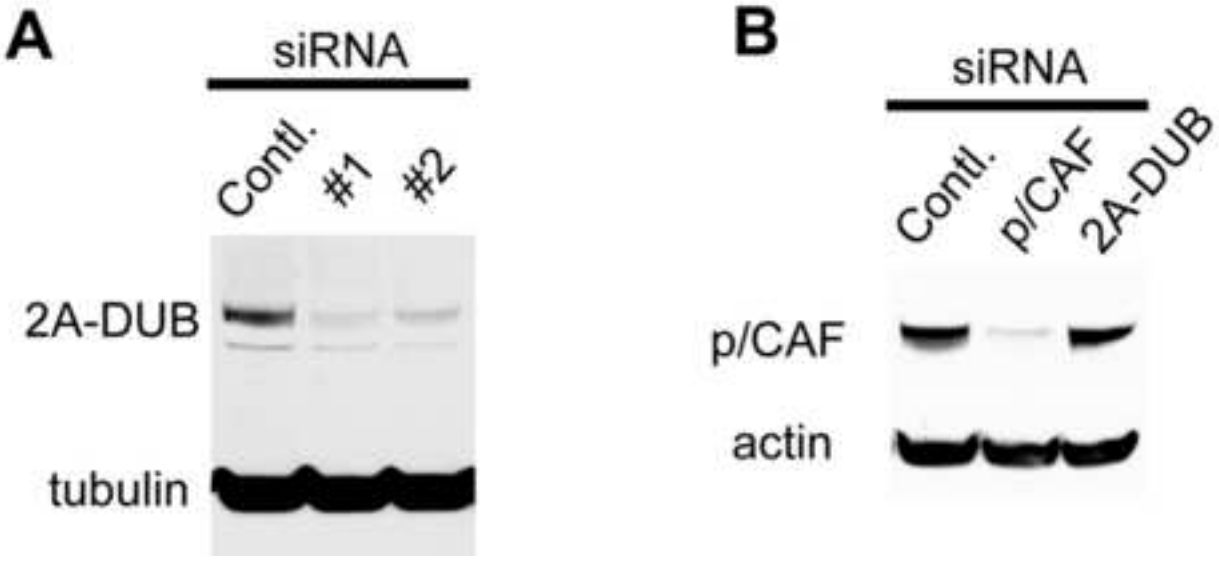
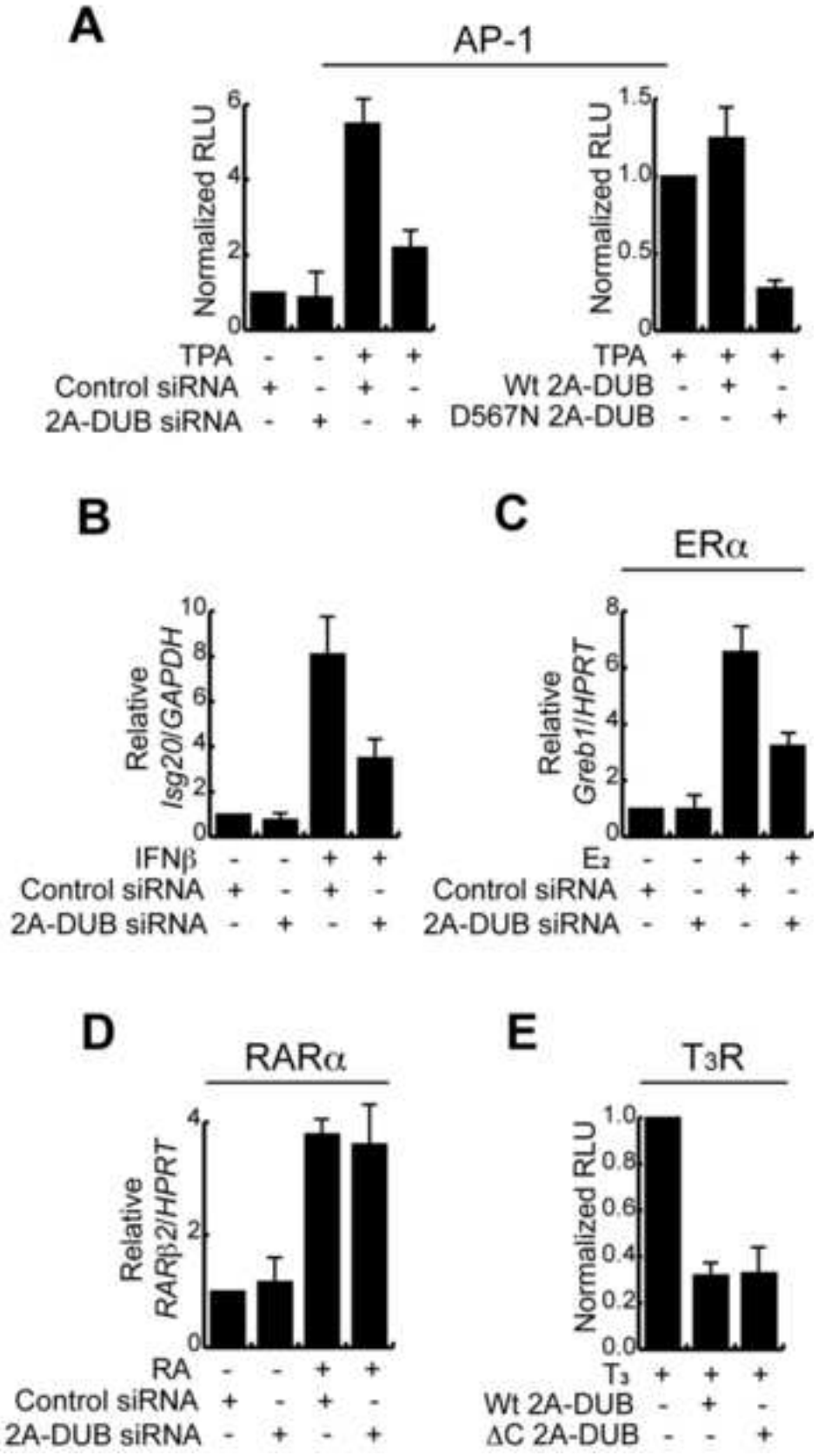

A
siRNA
Contl.
#1
#2
2A-DUB
tubulin
B
siRNA
Contl.
p/CAF
2A-DUB
p/CAF
actin

Fig. S10


A
AP-1
Normalized RLU
6
4
2
0
TPA - - + +
Control siRNA + - + -
2A-DUB siRNA - + - +
Normalized RLU
1.5
1.0
0.5
0
TPA + + +
Wt 2A-DUB - + -
D567N 2A-DUB - - +
B
Relative Isg20/GAPDH
10
8
6
4
2
0
IFNβ - - + +
Control siRNA + - + -
2A-DUB siRNA - + - +
C
ERα
Relative Greb1/HPRT
8
6
4
2
0
E2 - - + +
Control siRNA + - + -
2A-DUB siRNA - + - +
D
RARα
Relative RARβ2/HPRT
4
2
0
RA - - + +
Control siRNA + - + -
2A-DUB siRNA - + - +
E
T3R
Normalized RLU
1.0
0.8
0.6
0.4
0.2
0.0
T3 + + +
Wt 2A-DUB - + -
ΔC 2A-DUB - - +