Supplemental Data

JHDM2A, a JmjC Domain-Containing H3K9 Demethylase, Facilitates Transcriptional Activation by Androgen Receptor

Kenichi Yamane, Charalambos Toumazou, Yu-ichi Tsukada, Hediye Erdjument-Bromage, Paul Tempst, Jiemin Wong, and Yi Zhang

Supplemental Experimental Procedures

Histone Demethylase Assay

The histone demethylase assay was performed essentially as previously described (Tsukada et al., 2006). Briefly, various ^{3}H-labeled histone substrates or histone peptides were incubated with protein fractions or recombinant JHDM2A in histone demethylation buffer [50 mM HEPES-KOH (pH 7.5), 70 μM $Fe(NH_4)_2(SO_4)_2$. 1 mM α-ketoglutarate, 2 mM ascorbate] at 37^0C for 1-3 hr. Demethylation was analyzed by the NASH method and mass spectrometry. For detection of ^{3}H-labeled formaldehyde, a modified NASH method (Kleeberg and Klinger, 1982) was used. After TCA precipitation, an equal volume of NASH reagent (3.89 M ammonium acetate, 0.1 M acetic acid, 0.2% 2,4-pentanedione) was added into the supernatant and the mixtures were incubated at 37^0C for 50 min before extraction with equal volume of 1-pentanol. The extracted radioactivity was measured by scintillation counting. For detection of demethylation with peptide substrates, peptides in the reaction mixture were desalted on a RP micro-tip and analyzed by MALDI-TOF.

Purification of the Native and Recombinant JHDM2A

The procedure for conventional purification of JHDM2A is outlined in Figure 2A. Preparation and fractionation of HeLa cell nuclear extracts on P11 phosphocellulose column was carried out as described previously (Wang et al., 2003). The P11 fraction eluted with BC300 was dialyzed into buffer D (40mM HEPES-KOH pH 7.9, 0.2mM EDTA, 1mM DTT, 0.2mM PMSF, and 10% glycerol) containing 50mM ammonium sulfate (BD50) and loaded to a 45ml DE5PW column (TosoHaas). The bound proteins were eluted with 12 column volume (cv) linear gradient from BD50 to BD450. The flow-through containing the HDM activity was adjusted to 700mM ammonium sulfate before it was loaded onto a 22ml Phenyl Sepharose column (Pharmacia). The bound proteins were eluted with 10cv linear gradient from BD700 to BD0. The active fractions, which eluted from BD150-BD50, were combined and concentrated to 5ml before they were loaded onto a 120ml Sephacyl S300 gel filtration column (Pharmacia). The active fractions, which elute around 300 kDa, were pooled and loaded onto a 1ml Mono S column (Pharmacia) equilibrated with BC50. Bound proteins were eluted with 20 cv linear gradient from BC50 to BC400. The active fractions eluted from BC100 to BC150. The proteins in the active fractions were pooled and resolved in a 6.5-12% gradient SDS-PAGE. After Coomassie staining, candidate polypeptides were excised for protein identification.

Generation of baculovirus expressing Flag-JHDM2A and purification of Flag-JHDM2A from infected Sf9 cells were performed as described (Cao and Zhang, 2004). For purification of wild-type and deletion mutant Flag-JHDM2A from COS-7 cells, plasmids were transfected with FuGENE 6 following the manufacture's instructions. Two days after transfection, cells were washed with phosphate-buffered saline (PBS) and lysed with lysis buffer (20 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 20 mM β-Glycerophosphate, 1 mM EGTA, and 0.5% NP-40) containing protease inhibitor cocktail (Roche) and 1 mM phenylmetyl sulfonate fluoride. The cell lysates were incubated with M2 α-Flag agarose (Sigma) for 3 h at 4^0C. The beads were washed with lysis buffer three times before being used in a demethylase assay or Western blot.

Real-Time PCR and ChIP Assays

Real-time PCR was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) and the ABI prism 7900 sequence detection system (Applied Biosystems). Quantitative PCR reactions were performed under conditions standardized for each primer. Standard curves were generated using 10-fold dilutions of standard

plasmids. To compare the relative amount of target in different samples, all values were normalized to the appropriately quantified 36B4 control. The primers used in quantitative PCR were as follows: mJhdm2a-F, 5’-TGAGTACACCAGGCGAGATG -3’ and mJhdm2a-R, 5’- GGTCCCATATTTCCGATCCT -3’; 36B4-F, 5’-CTGATGGGCAAGAAAACCAT -3’ and 36B4-R, 5’- GTGAGGTCCTCCTTGGTGAA-3’; Nanog-F, 5’-AAGCAGAAGATGCGGACTGT -3’ and Nanog-R and 5’- ATCTGCTGGAGGCTGAGGTA -3’; Oct4-F, 5’-CCAATCAGCTTGGGCTAGAG -3’ and Oct4-R, 5’- CCTGGGAAAGGTGTCCTGTA -3’; Sox2-F, 5’-GAACGCCTTCATGGTATGGT -3’ and Sox2-R, 5’- TTGCTGATCTCCGAGTTGTG -3’; LamininB1-F, 5’-GTTCGAGGGAACTGCTTCTG -3’ and LamininB1-R, 5’- GTTCAGGCCTTTGGTGTTGT -3’; Hoxa1-F, 5’-GCCCTGGCCACGTATAATAA -3’ and Hoxa1-R, 5’- TCCAACTTTCCCTGTTTTGG -3’; Stra6-F, 5’-GTTCAGGTCTGGCAGAAAGC -3’, Stra6-R, 5’- CAGGAATCCAAGACCCAGAA -3’.

For ChIP assays in F9 cells, 90% confluent F9 cells in 150 mm dishes were first treated with DMEM containing 1% formaldehyde for 10 min. The crosslinking was stopped by the addition of 0.125 M glycine for 5 min. After washing twice with PBS, the cells were resuspended in 1ml of cell lysis buffer (10mM HEPES [pH 7.9], 0.5% NP-40, 1.5mM $MgCl_2$, 10 mM KCl, 0.5mM DTT) by pipetting and kept on ice for 10 min. After centrifugation at 4,000 rpm for 5 min, the cell pellets were resuspended in nuclear lysis buffer (20mM HEPES [pH 7.9], 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM , 0.2mM EDTA) containing protease inhibitors to extract nuclear proteins at 4 ^{o}C for 20 min and then the chromatin were sonicated into fragments with an average length of 1 kb. After centrifugation at 13,000 rpm for 10 min, the supernatants were diluted in equal volume of dilution buffer containing 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 7.9], 50 mM NaCl, and protease inhibitors. ChIP assays were then performed with anti-JHDM2A, anti-dimethyl-K9, and anti-trimethyl-K4. For all ChIP experiments, quantitative PCR analyses were performed in real-time using the ABI PRISM 7900 sequence detection system and SYBR Green master mix. Quantity of DNA was determined following the algebraic formula of 2^{-Ct} (where Ct is the cycle threshold number). The relative amount of immunoprecipitated DNA to input DNA was calculated. Primer pairs were as follows: LamininB1-F, 5’- CTTTTCTCCCCGCTACCTCT -3’ and LamininB1-R, 5’-CTAGGACACCAAAGGCGAAC -3’; Stra6-F, 5’- TGGAAGAGGAGGGTCTCTGA -3’ and Stra6-R, 5’-CTCCTGCCATGGAGTCTCTC -3’; Hoxa1-F, 5’- ACTGCCAAGGATGGGGTATT -3’and 5’-CTTCGCAGGATCCAATCACT -3’.

For ChIP assay in LNCaP cells, the cells were cultured in charcoal-stripped serum medium for three days before treatment with R1881 (50 nM) for 1 h. The ChIP assay was performed essentially as described (Yoon et al., 2005). Primers for qRT-PCR analysis of the PSA, NKX3.1 and TMPRSS2 mRNAs are as follow: PSA, 5-GGAAATGACCAGGCCAAGAC-3 and 5’-CAACCCTGGACCTCACACCTA-3’; NKX3.1, 5-TGAAGGCGCAGGCTTACTG-3 and 5’-TAGGCTGCCTTCTTTTCCATGT-3’; TMPRSS2, 5-TGGCAGGGCGCCAA-3 and 5’-TCAATTTCCAGTGACTAGCAG-3’. The 18s RNA was used as internal control and all samples were first normalized to 18s RNA. The SMART pools of siRNA for JHDM2A and LSD1 were ordered from Dhmarcom. To knockdown JHDM2A or LSD1 in LNCaP cells, LNCaP cells were transfected with 40 nM siJHDM2A or 80 nM siLSD1 for three days. A scramble siRNA (siCon) serves as a control. The transfected cells were treated with 50 nM R1881 for 8 hours before collected for quantitative RT-PCR analysis or 1 h for ChIP assays. The primers for ChIP analysis of PSA enhancer and NKX3.1 were as described (Yoon and Wong, 2005).

Supplemental References

Cao, R., and Zhang, Y. (2004). SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell *15*, 57-67.

Kleeberg, U., and Klinger, W. (1982). Sensitive formaldehyde determination with Nash's reagent and a 'tryptophan reaction'. J Pharmacol Methods *8*, 19-31.

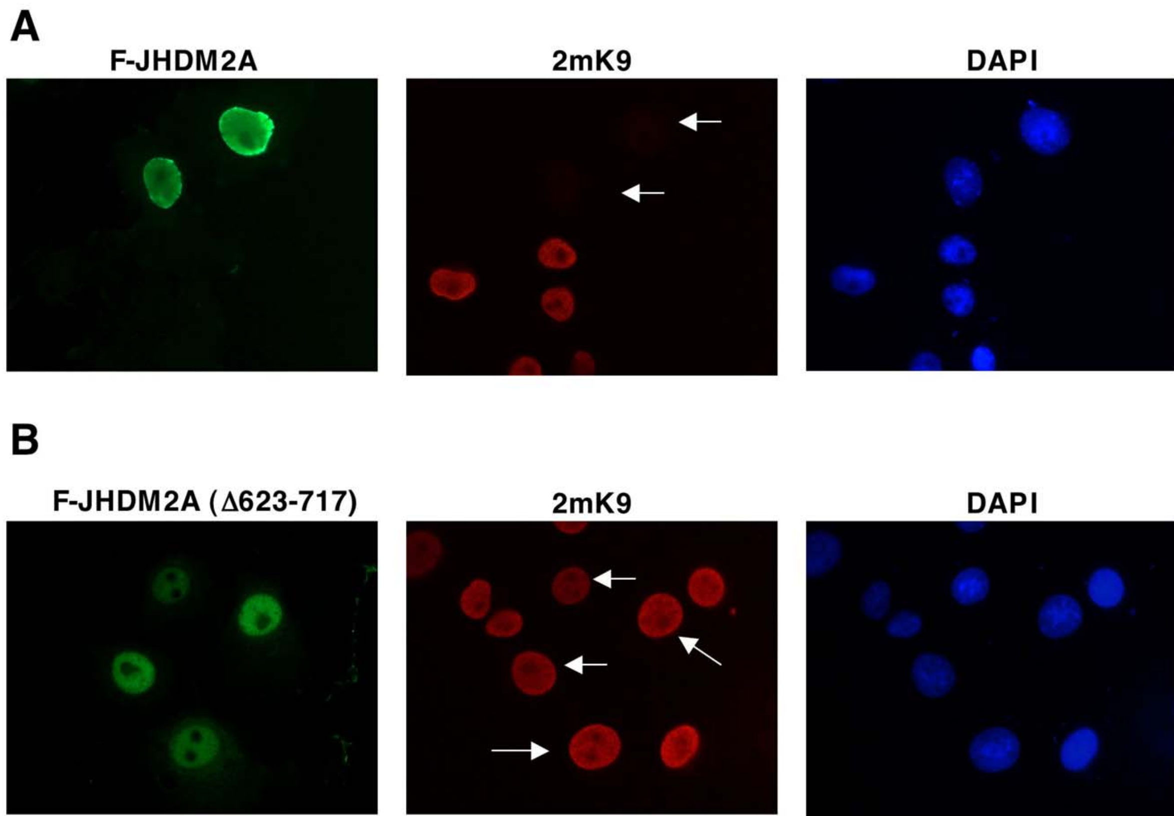
Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M. E., Borchers, C. H., Tempst, P., and Zhang, Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. Nature *439*, 811-816.

Wang, H., An, W., Cao, R., Xia, L., Erdjument-Bromage, H., Chatton, B., Tempst, P., Roeder, R. G., and Zhang, Y. (2003). mAM facilitates conversion by ESET of dimethyl to trimethyl lysine 9 of histone H3 to cause transcriptional repression. Mol Cell *12*, 475-487.

Yoon, H. G., Choi, Y., Cole, P. A., and Wong, J. (2005). Reading and function of a histone code involved in targeting corepressor complexes for repression. Mol Cell Biol *25*, 324-335.

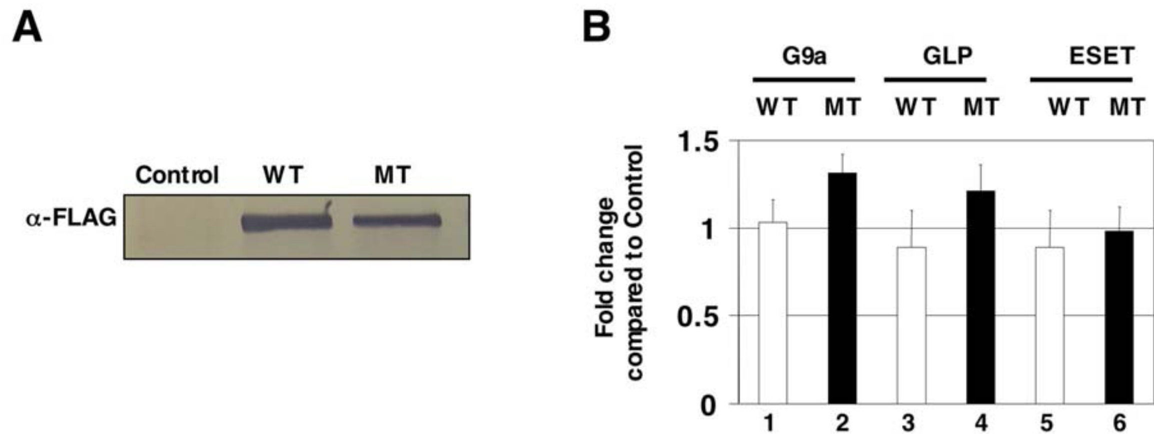
Yoon, H. G., and Wong, J. (2005). The corepressors SMRT and N-CoR are involved in agonist- and antagonist-regulated transcription by androgen receptor. Mol Endocrinol.

Figure S1. Deletion of the Zinc Finger Domain Abolishes JHDM2A Demethylase Activity In Vivo



COS7 cells were transfected with wild-type (A) or the zinc-finger deletion mutant (B) Flag-JHDM2A. Cells were co-stained with Flag antibody and dimethyl-H3K9 antibodies as indicated in the figure. Arrows point to the transfected cells.

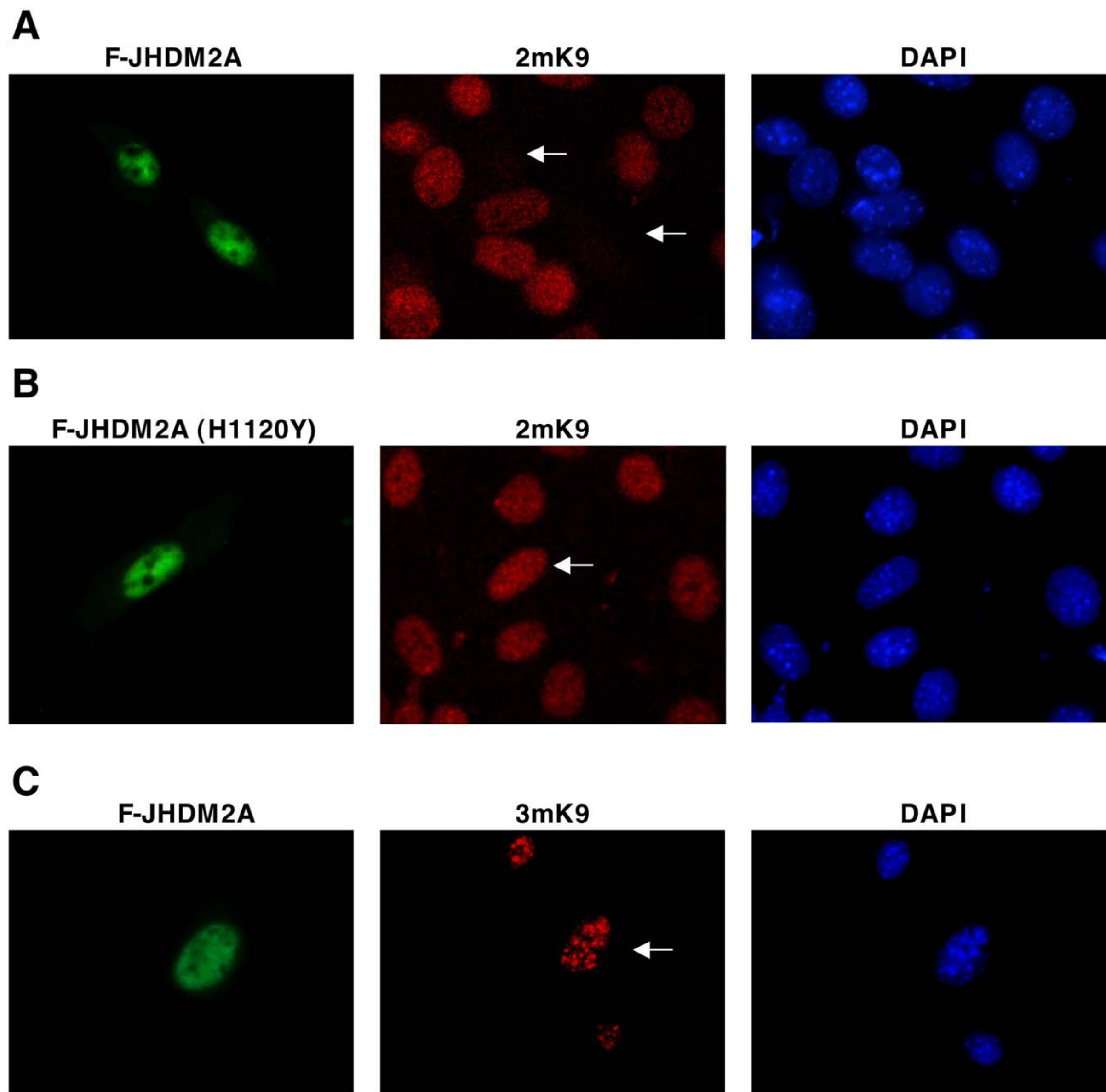
Figure S2. Overexpression of JHDM2A Does Not Affect the Expression Levels of Euchromatin H3K9 Histone Methyltransferases



(A) Wild-type and mutant Flag-JHDM2A proteins were expressed in HEK293 cells, and immunoprecipitated samples were subjected to Western blotting.

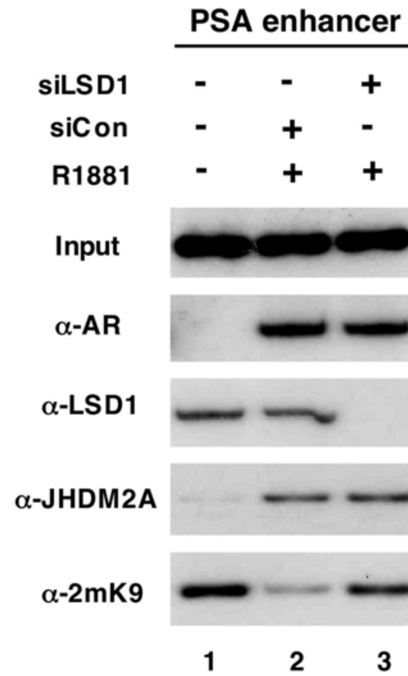
(B) Q-PCR analysis of the changes at the RNA level of several euchromatic H3K9 histone methyltransferase genes in response to overexpression of wild-type or mutant Flag-JHDM2A in HEK293 cells. The changes are expressed as the ratio of the expression levels in JHDM2A transfected to that of the empty vector transfected cells. Data represent average of three independent experiments with error bars.

Figure S3. Overexpression of JHDM2A Does Not Affect Trimethyl-K9 Levels



NIH3T3 cells were transfected with plasmids encoding wild-type (A and C) or mutant (B) Flag-JHDM2A. Cells were co-stained with Flag antibody and different methyl-H3K9 antibodies as indicated in the figure. Arrows point to the transfected cells. Note the trimethyl-H3K9 levels of pericentric heterochromatin are not affected by over expression of JHDM2A.

Figure S4. Knockdown of LSD1 Does Not Affect the Hormone-Dependent Recruitment of JHDM2A but Does Impair the Hormone-Induced Reduction of Dimethyl-H3K9 on PSA Enhancer



LNCaP cells were treated with or without control siRNA (scramble) or siLSD1 for three days and then treated with 50 nM R1881 for 1 h followed by ChIP assay using antibodies as indicated. The effectiveness of siLSD1 treatment was confirmed by the loss of ChIP signal for LSD1 in the siLSD1-treated sample.