Supporting Information

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SI Materials and Methods

Plasmids. The baits Diff1 [inositol hexakisphosphate kinase 1 (IP_6K1) aa 75–206] (*Mus musculus*) and Diff2 [inositol hexakisphosphate kinase 2 (IP_6K2) aa 68–202] (*Homo sapiens*) used in the yeast two-hybrid screen were cloned into the EcoRI and SalI sites of pGBKT7 (Clontech) by PCR from pCMV-IP_6K1 and pCMV-IP_6K2 (1) to generate pGBKT7-Diff1 and pGBKT7-Diff2. The pACT2-JMJD2C (847–1,056) construct was obtained from the yeast two-hybrid screen. Full-length mouse JMJD2C cDNA was obtained from Imagenes (Mm.209059, IRAVp968G1287D) and PCR cloned into pFlag-CMV-5.1 (Sigma). The catalytically inactive mutant IP_6K1 DF/AA was previous described (2) and recloned into pCMV and pEGFP-C1-IP_6K1 was previously generated (3). The PP-IP_5K1 clone was kindly provided by S. Shears (Laboratory of Signal Transduction, NIEHS, Research Triangle Park, NC) and cloned into SalI and NotI of pCMV-Myc (Clontech), whereas Myc-IP_6K1 (*M. musculus*) is described in ref. 4. For expression in *Escherichia coli*, several constructs were generated in pGex-4T-2 (GE Healthcare) or in pTRCHis (Invitrogen). The Genscript vector-based siRNA protocol was used with the pRNAT-H1.1 vector (Genscript Inc.). The target human IP_6K1 sequences were 5′-TGGAGAACCTCATCAGCATCA-3′ and 5′-CAACTTAGTGGCCTATCCTTA-3′. The mouse siRNA pool was acquired from Santa Cruz (sc-39070), whereas two human JMJD2C siRNA were purchased from Invitrogen (HSS118146 and HSS177158).

Yeast Strains and Yeast Two-Hybrid Screens. The yeast two-hybrid screen was performed using pGBKT7-Diff1 and pGBKT7-Diff2 as the baits and a human fetal brain MATCHMAKER cDNA library as a prey in the yeast strain AH109 (Clontech). The screening was performed according to manufacturer instructions (Clontech).

Antibodies. Rabbit polyclonal anti-IP_6K1 generated against the following peptide: DTPEREQPRRKHSRRS, was affinity purified and characterized in Fig. S2. The commercial antibodies used are as follows: anti-Flag (Sigma F3165); anti-Jumonji domain containing 2C (JMJD2C) (Abcam, ab85454); anti-Histone H3 (Millipore; Upstate 05–499); anti-trimethylhistone H3K9 (Millipore; Upstate 17–625); anti-acetylhistone H3K9 (Millipore; Upstate 07–352); anti-trimethylhistone H3K36 (Upstate 07–549; Abcam ab9050); anti-phosphohistone H3S10 (Abcam, ab5176 and Millipore, 06–570); anti-His (Roche 11922416001); anti-Myc (Clone 4A6; Millipore; Upstate); anti-GST (anti-GST; Sigma); anti-biotin (Sigma; B3640); and the fluorescent anti-mouse Alexa-fluor 568, Alexa-fluor 488, and the anti-rabbit Alexa-fluor 568 were all from Invitrogen.

Cross-Linking of Rabbit Anti-IP_6K1 Antibody to Protein A Sepharose Beads. A total of 500 μL of protein A Sepharose was equilibrated in 1 mL of binding buffer for 30 min (25 mM NaCl, 50 mM bicine, 100 mM sodium borate). After the 30 min, the beads were collected by mild centrifugation, the supernatant was removed, and 500 μL of fresh binding buffer was added together with 1 mg of rabbit anti-IP_6K1 antibody. After incubation at room temperature for 90 min, the beads were washed twice in 10 volumes of wash buffer, 100 mM sodium borate, pH 9.0. Beads were collected by centrifugation at 3,000 × *g* for 5 min, the supernatant was removed, the beads were resuspended in 10 volumes of wash buffer containing 0.4 mM of disuccinimidyl suberate (DSS), and incubated 30 min at room temperature. The reaction was quenched by the addition of quenching buffer (glycine pH 3.0 to a final concentration of 100 mM). Beads were washed twice for 1 h in quenching buffer to remove unbound antibody. Cross-linked IP_6K1 antibody was stored at 4 °C in storage buffer (PBS, 0.1% Tween, 0.02% sodium azide).

In Vitro Binding Assay. Recombinant His-IP_6K1(FL) or His-Diff1 were incubated with GST-JMJD2C purified on glutathione-Sepharose beads or biotinylated H3 peptides (Millipore) in 300 μL of 20 mM Tris·HCl pH 7.6, 120 mM NaCl, 10% (vol/vol) glycerol, 2 mM EDTA, 1 mM DTT for 2 h at 4 °C with rotation. The beads were washed three times for 10 min in the same buffer and 10 μL of 4× NuPAGE lithium dodecyl sulphate (LDS) sample buffer (Invitrogen) was added to the samples. The samples were boiled for 10 min before loading 4–12% (vol/vol) Bis-Tris NuPAGE gels, Western blotted, and probed with anti-GST or anti-biotin antibody. All binding assays were repeated at least three times.

Histone H3 binding assays were performed by incubating purified H3.1 (New England Biolabs) with of purified GST-IP_6K1 or GST in binding buffer (20 mM Hepes pH 7.5, 200 mM NaCl, 0.1% (wt/vol) BSA, 5 mM 2-mercaptoethanol, 0.01% Nonidet P-40, and 10% glycerol) for 30 min at 4 °C. Beads were washed three times in 1 mL of wash buffer 500 (20 mM Hepes pH 7.5, 500 mM NaCl, 0.1% (wt/vol) BSA, 5 mM 2-mercaptoethanol, 0.1% Nonidet P-40, and 10% glycerol) and three times in Wash Buffer 200 (20 mM Hepes pH 7.5, 200 mM NaCl, 0.1% (wt/vol) BSA, 5 mM 2-mercaptoethanol, 0.1% Nonidet P-40, and 10% glycerol). All supernatant was removed and beads were boiled for 5 min in 15 μL of 2× LDS sample buffer. Samples were run on 4–12% Invitrogen precast gel with Mes buffer and Western blotted accordingly with anti-H3 antibody.

His-Tag and GST-Tag Proteins Purification. Recombinant protein purifications were performed with standard procedures, as previously described (5).

Immunofluorescence Microscopy. Transfected HeLa cells were fixed between 14 and 20 h posttransfection in 4% (wt/vol) paraformaldehyde for 20 min at room temperature. Fixed cells were permeabilized/blocked in 0.1% (wt/vol) Triton X-100/3% (wt/vol) BSA in PBS (blocking buffer, BB) for 1 h. The permeabilized cells were incubated with the appropriate antibody diluted in BB for 1 h followed by Alexa-fluor–conjugated secondary antibody diluted in BB for 1 h. Nuclei were stained with DAPI at 1 μg/mL. Confocal images were acquired using a Leica DM2500 laser-scanning confocal microscope and data processing was performed with LAS AF software (Leica). For quantification of fluorescence intensity, DAPI-stained nuclei were selected and the average fluorescence intensity of trimethyl-H3K9 staining within the selected regions was recorded. For counting the number of trimethyl-H3K9 foci, the images were subjected to uniform background subtraction and the number of spots remaining per nuclei were counted. All immunofluorescence microscopy experiments were repeated a minimum of three times.

Protein Kinase Assay. Protein kinase reactions were performed in reaction buffer (25 mM Hepes pH 6.8, 100 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 1 μCi [^{32}P]γATP (3,000 Ci/mM). In a 10-μL reaction, His-IP_6K1 (100 ng) was incubated for 60 min at 37 °C with 100 ng of histone H3.1 and recombinant JMJD2C (650–1,054) in absence or presence of IP_6 0.5 mM. To visualize the synthesis of ^{32}P-IP_7 1 μL of the reaction was spotted

on a TLC plate, developed as previous described (1). The remaining reactions were loaded on 4–12% Bis-Tris NuPAGE gel after adding NuPAGE LDS sample buffer and run in MES SDS buffer (Invitrogen). The gels were silver stained, dried, and exposed.

Mammalian Cell Culture and Transfections. HEK293T, HeLa, and MEF cells were cultured in DMEM (Gibco) supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin (P/S). Transfections were performed using either Lipofectamine (Invitrogen) for HEK293T or HeLa cells or Fugene (Roche) for MEF cell lines, according to the manufacturer's instructions. For the [^{3}H]-inositol labeling experiment, cells were incubated for 4 d in presence of 20 $\mu Ci \cdot mL^{-1}$ of [^{3}H]-inositol before transfection and processed as described (6). Embryonic stem (ES) cells were cultured using high glucose (4.5 g/L) DMEM, supplemented with 15% FCS, 100 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, and 1,000 units/mL leukemia inhibitory factor (Gibco). ES cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer's instructions.

Protein Extracts and GST Pull-Down from Mammalian Cells. Protein lysates were prepared from transfected HEK293T or MEFs using the following lysis buffer: 20 mM Tris·HCl pH 7.6, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 0.2% Nonidet P-40, 5 mM DTT, 1:500 protease inhibitor mixture, 1:1,000 phosphatase inhibitor mixture (Sigma). The samples were sonicated and cleared by centrifugation at 16,000 × *g* for 10 min. The supernatant was added to 20 μL preequilibrated glutathione-agarose beads at a concentration of 1.5 μg/μL. The samples were rotated for 2 h at 4 °C and washed three times for 10 min in lysis buffer. The supernatant was removed, 10 μL 4× NuPAGE LDS sample buffer was added, and the samples were then boiled for 10 min at 100 °C before loading on 4–12% Bis-Tris NuPAGE gels in MES SDS running buffer (Invitrogen), then transferred to PVDF membranes, and Western blots were performed using standard procedures. All GST–pull-down experiments were repeated a minimum of three times.

Coimmunoprecipitation from Mammalian Cells. Protein lysates were prepared from subconfluent cells in a buffer containing 150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris·Cl pH 8.0. After centrifugation at 16,000 × *g* for 10 min at 4 °C, protein concentration of the supernatant was determined by bichinchoninic assay (BCA) assay (Pierce) according to manufacturer instructions. Lysates were precleared by incubation for 1 h at 4 °C with protein A-Sepharose preequilibrated in lysis buffer. For immunoprecipitation, rabbit polyclonal anti-IP_6K1 antibody was cross-linked to protein A-Sepharose as described followed by incubation with 450 μg of precleared proteins overnight at 4 °C. Beads were then washed four times with lysis buffer and immunocomplexes were eluted by boiling in 2× Laemmli buffer. Eluted proteins were then processed for Western blotting using standard procedures. All coimmunoprecipitation experiments were repeated at least three times.

Cellular Fractionation. Cellular fractionation was performed using an adaptation of the high-salt chromatin extraction method as described in ref. 7. Cell pellets were resuspended in 10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, plus protease inhibitors, and incubated for 10 min at 4 °C before centrifugation at 10,000 × *g* for 10 min at 4 °C. Nuclear pellets were then resuspended in 3 mM EDTA, 0.2 mM EGTA, plus protease inhibitors, and incubated for 30 min with rotation at 4 °C. Chromatin fractions were then isolated by centrifugation at 15,000 × *g* for 10 min and resuspended in 50 mM Tris·HCl pH 8.0, 100 mM NaCl by sonication for 3 × 5 s at setting 1.5 on a Branson Sonifier 450 (Branson Ultrasonics Co.) and finally cleared by centrifugation at 15,000 × *g* for 10 min. All fractionation experiments were repeated a minimum of three times.

Demethylase Assays. Demethylase assays were performed using the formaldehyde release assay as described in ref. 8. The catalytic domain of JMJD2C (12–349) was recombinantly expressed and purified. A total of 5 μg of His-JMJD2C (12–349) was incubated with histones purified and dialyzed from HEK293T cells by the high-salt extraction method in 50 μL demethylation buffer (50 mM Tris pH 8.0, 50 mM KCl, 1 mM α-ketoglutarate, 40 μM $FeSO_4$, 2 mM ascorbic acid, 2 mM NAD^+ and 0.2 units formaldehyde dehydrogenase; Sigma) at 37 °C and the absorbance at 340 nm was measured at indicated times.

IP_7 Phosphorylation Assays. 5β[^{32}P]IP_7 was produced and purified and IP_7 pyrophosphorylation analysis was performed as described (9).

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2. Onnebo SM, Saiardi A (2009) Inositol pyrophosphates modulate hydrogen peroxide signalling. *Biochem J* 423(1):109–118.
3. Saiardi A, Nagata E, Luo HR, Snowman AM, Snyder SH (2001) Identification and characterization of a novel inositol hexakisphosphate kinase. *J Biol Chem* 276(42): 39179–39185.
4. Illies C, et al. (2007) Requirement of inositol pyrophosphates for full exocytotic capacity in pancreatic beta cells. *Science* 318(5854):1299–1302.
5. Azevedo C, Burton A, Bennett M, Onnebo SM, Saiardi A (2010) Synthesis of InsP7 by the Inositol Hexakisphosphate Kinase 1 (IP6K1). *Methods Mol Biol* 645:73–85.
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7. Shechter D, Dormann HL, Allis CD, Hake SB (2007) Extraction, purification and analysis of histones. *Nat Protoc* 2(6):1445–1457.
8. Cloos PA, et al. (2006) The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* 442(7100):307–311.
9. Saiardi A, Bhandari R, Resnick AC, Snowman AM, Snyder SH (2004) Phosphorylation of proteins by inositol pyrophosphates. *Science* 306(5704):2101–2105.

Fig. S1. Alignment of the amino acid sequences of IP6Ks 1, 2, and 3. The sequences of *Homo sapiens* IP6Ks 1, 2, and 3 are shown. The regions highlighted are encoded by exons 3 and 4 of each gene, showing a relatively low level of sequence homology, and were called the "Diff" regions. Accession numbers are as follows; IP6K1 NP_695005.1, IP6K2 NP_057375.2, and IP6K3 NP_001136355.1.

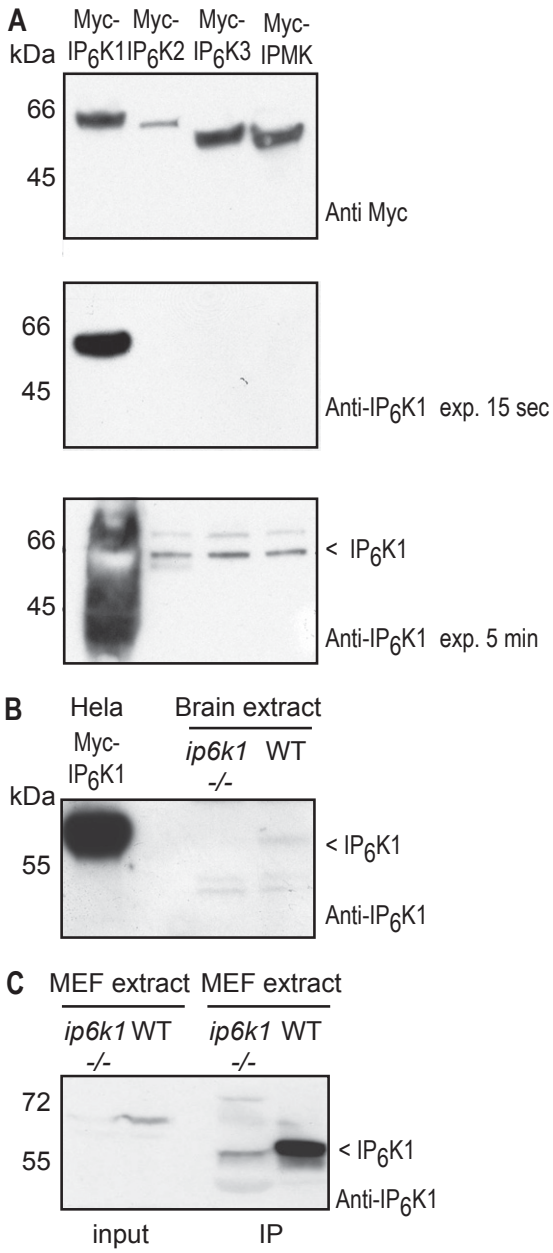

Fig. S2. Characterization of the anti-IP6K1 antibody. (*A*) Myc-tagged IP6K1–3 and inositol polyphosphate multikinase (IPMK) were transfected into HeLa cells and Western blotting was performed on lysates separated by SDS/PAGE with anti-IP6K1 or anti-Myc antibodies. (*B*) Brain extracts from wild-type (WT) or *ip6k1*$^{-/-}$ mice were resolved by SDS/PAGE and immunoblotted with anti-IP6K1, revealing a unique band in wild-type brain extract, which runs at the same size as Myc-IP6K1. (*C*) Endogenous IP6K1 was immunoprecipitated from extracts of WT or *ip6k1*$^{-/-}$ MEFs using anti-IP6K1–Sepharose cross-linked antibody revealing a band corresponding to the molecular weight of IP6K1 only from wild-type extracts, even if the IP6K1 expression is below detection in MEF extract by simple Western blotting.

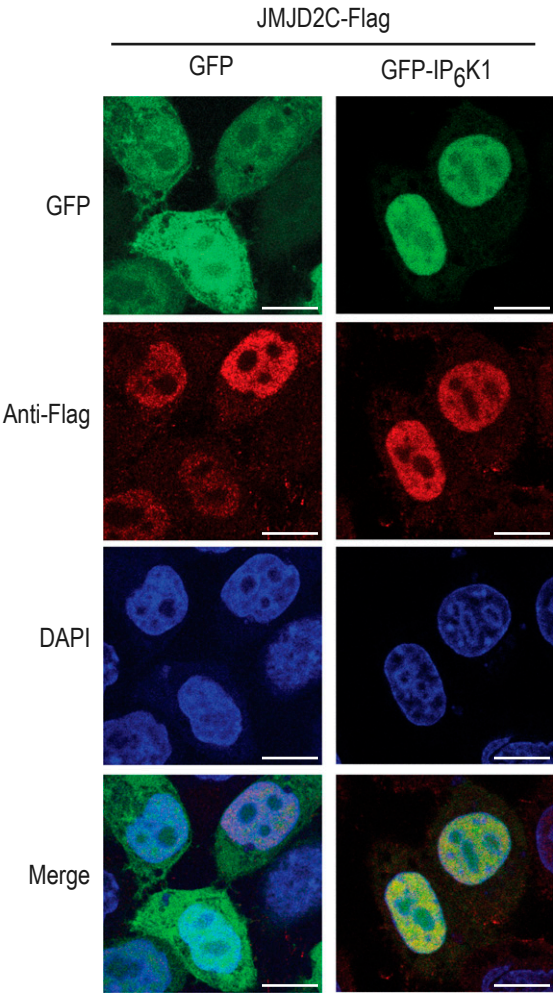

Fig. S3. Nuclear localization of IP6K1 and JMJD2C. HeLa cells were transfected with EGFP empty vector or EGFP-IP6K1 and JMJD2C-Flag. Cells were fixed 14 h posttransfection, immunostained with anti-Flag antibody, and the nuclei stained with DAPI. (Scale bar, 10 μm.)

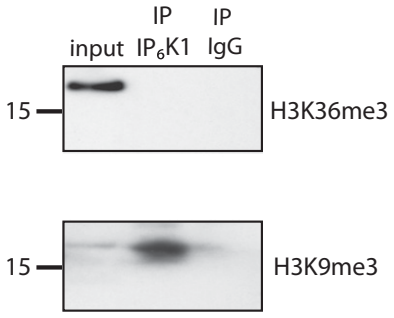

Fig. S4. IP6K1 associates with H3K9me3 but not H3K36me3-containing chromatin. Coimmunoprecipitation with an anti-IP6K1 antibody was performed on DNase-treated HEK293T lysates. Pull-downs and 5% inputs were separated by SDS/PAGE and membranes were blotted with anti-H3K9me3 and anti-H3K36me3 antibodies.

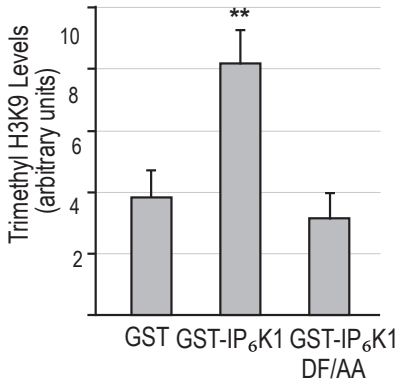

Fig. S5. Quantification of the effect of the overexpression of IP6K1 and catalytically inactive IP6K1 (DF/AA) on the levels of H3K9me3. Quantification of the average histone H3K9me3 levels normalized to total histone H3 ±SD of four independent experiments. The Mann–Whitney *u* test was used to determine statistical significance.

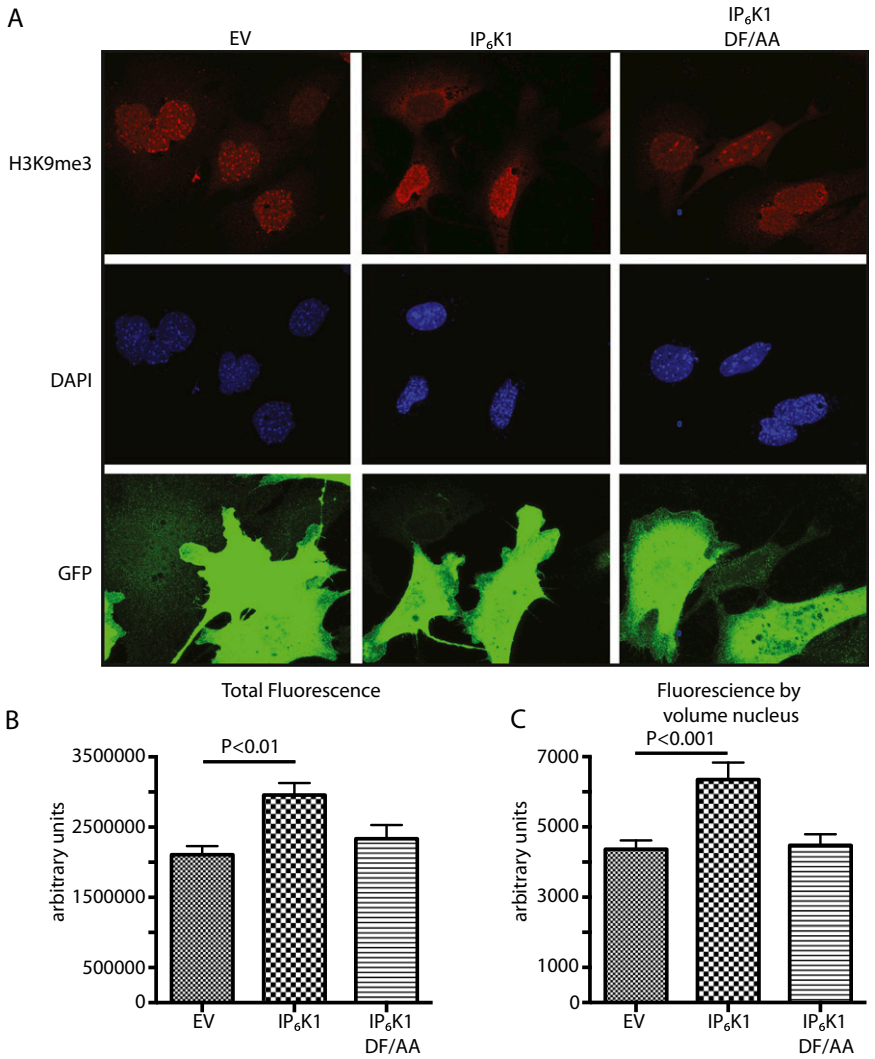

Fig. S6. IP6K1 catalytic activity is required to enhance the level of H3K9me3 in *ip6k1*$^{-/-}$ MEFs. MEFs from *ip6k*$^{-/-}$ mouse were transfected with either pMyc empty vector (EV), pMyc-IP6K1, or the kinase dead mutant pMyc-IP6K1 DF/AA, in plates containing glass coverslips. Forty-eight hours later, cells were detached and transfected again. After an additional 48 h, cells were fixed with 3% PFA in PBS for 20 min at room temperature, rinsed in PBS, and permeabilized with 0.5% BSA, 0.1% Triton. The coverslips were incubated with the following antibodies: Overnight at 4C: chicken anti GFP (Abcam ab 13970) 1:1,000, rabbit anti-H3K9m3 (Millipore 17–625) 1:100. After washing in PBS, samples were incubated with anti-chicken Dynalight 488 (Jackson Laboratories; 103-485-155), anti-rabbit Alexa 555 (Life Technologies; A-21428), and DAPI 1 μg/mL in blocking solutions. After washing, coverslips were mounted with ProlongGold Life Technologies. Samples were imaged using a Leica SP5 confocal and fluorescence was quantified using Volocity Analysis software. (*A*) Representative images of transfected MEFs with the indicated vectors. Note the increase in intensity of H3K9me3 staining in pMyc-IP6K1 transfected nuclei, whereas no effect is observable in kinase dead transfected MEFs. (*B* and *C*) Statistical analysis of the data shown in *A* expressed as total fluorescence (*B*) and fluorescence normalized by nuclear volume (*C*). Data are mean ±SEM of two independent experiments performed in duplicate; ∼50 transfected cells were analyzed for each conditions. Statistics was performed using one-way ANOVA Tukey's post hoc test.

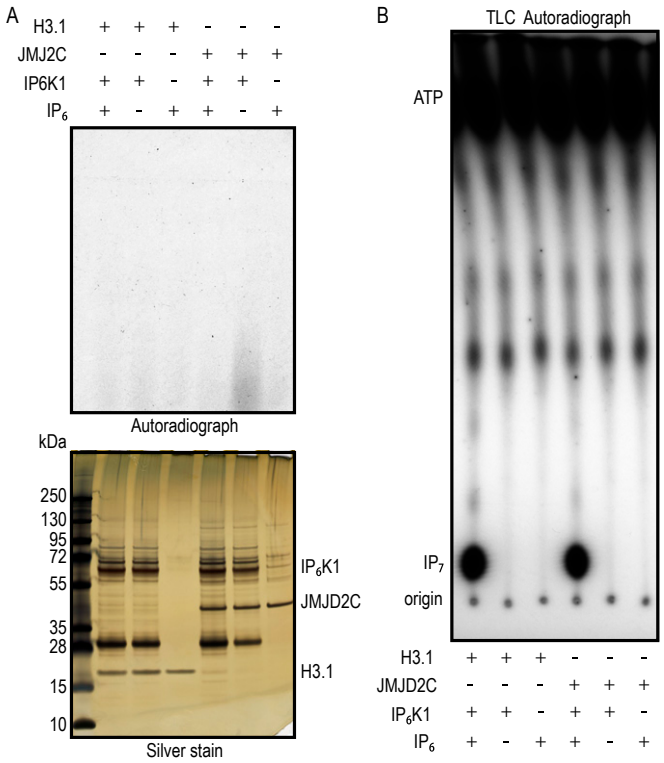

Fig. S7. The inositol hexakisphosphate kinase IP6K1 does not possess protein kinase activity. Recombinant his-IP6K1 was incubated with a tracer amount of [^{32}P]γATP in the presence of histone H3.1 or JMJD2C (650–1,056), in reactions with or without its natural substrate IP_6. (*A*) Ninety percent of the reactions were resolved on a NuPage; after silver stain (*Lower*) the dried gel was exposed for 48 h (*Upper*); the autoradiogram fails to reveal any ability of IP6K1 to phosphorylate the two protein substrates. (*B*) Ten percent of the reactions were spotted on a TLC plate and developed as previously described (2); the dried TLC plate was exposed for 24 h, revealing the synthesis of IP_7 in the lanes in which IP6K1 was incubated with the inositol substrate IP_6.

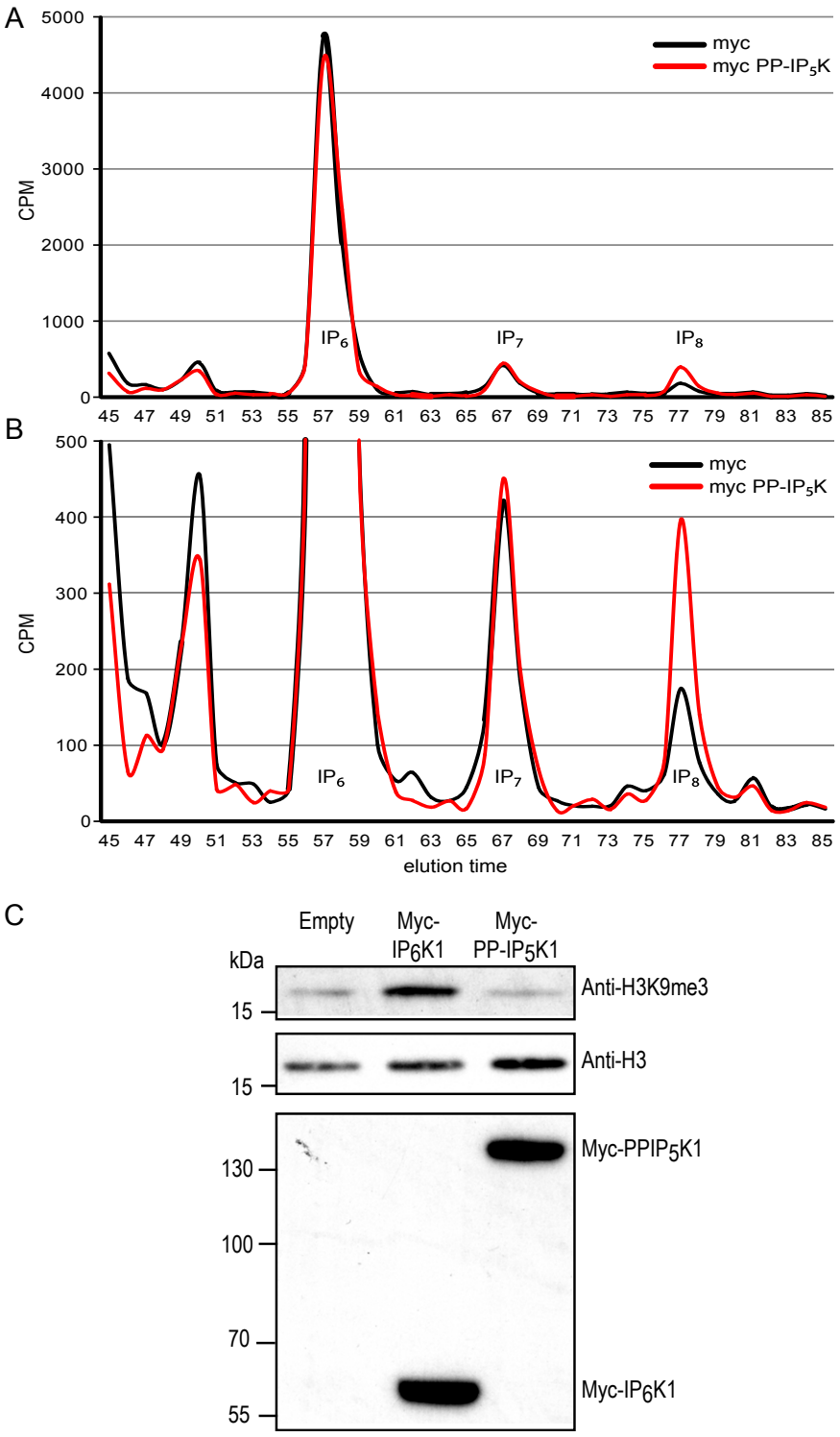

Fig. S8. PP-IP5 kinase does not affect H3K9me3 levels. Cells grown in a six-well plate were labeled with [^{3}H]-inositol for 4 d before transfection with empty vector or myc-PP-IP5K1. Acidic extraction of soluble inositol polyphosphate was performed 24–36 h posttransfection, and inositol polyphosphates were analyzed by strong anion exchange-HPLC. (*A*) HPLC elution profile revealing that the IP levels are unaffected by PP-IP K expression. (*B*) Magnification of the same HPLC elution profile revealing the unchanged level of IP_7 and the increase level of IP_8 upon PP-IP5K overexpression. (*C*) HEK293T cells were transfected with Myc-IP6K1 or Myc-PP-IP5K1 (Vip1a) and extracts were prepared 16 h later, resolved by SDS/PAGE, and Western blotting was performed with anti-H3K9me3, anti-Myc, and anti-histone H3 antibodies.

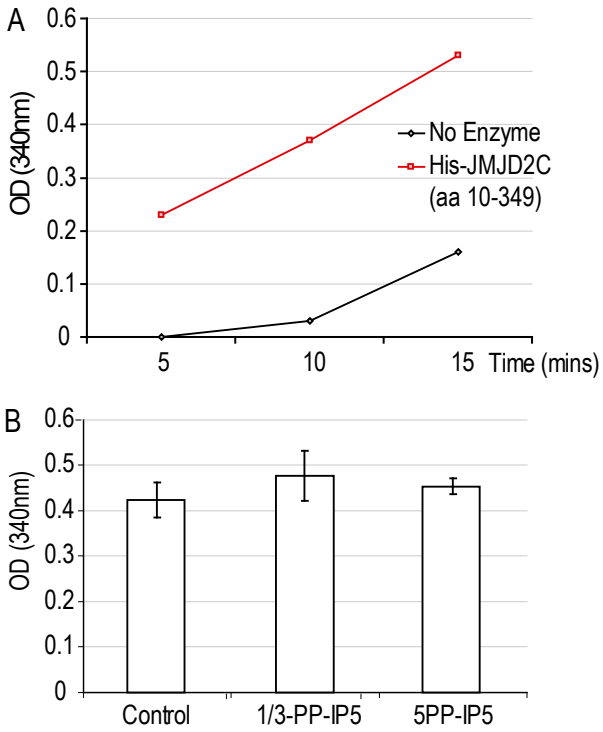

Fig. S9. IP_7 does not directly inhibit demethylase activity of JMJD2C. (*A*) Recombinant catalytic domain (amino acids 0–349) of His-JMJD2C was incubated at 37 °C with histone extracts and demethylase activity was monitored by performing formaldehyde release assay (1) at the indicated time points. The background demethylase activity is likely to be caused by demethylases copurified with the histone extracts. (*B*) Demethylase assays were carried out as above with addition of either 10 μM 5PP-IP_5 or 1/3PP-IP_5 for 10 min at 37 °C. The graph represents the mean ± SE of three experiments.

1. Wissmann M, et al. (2007) Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. *Nat Cell Biol* 9(3):347–353.

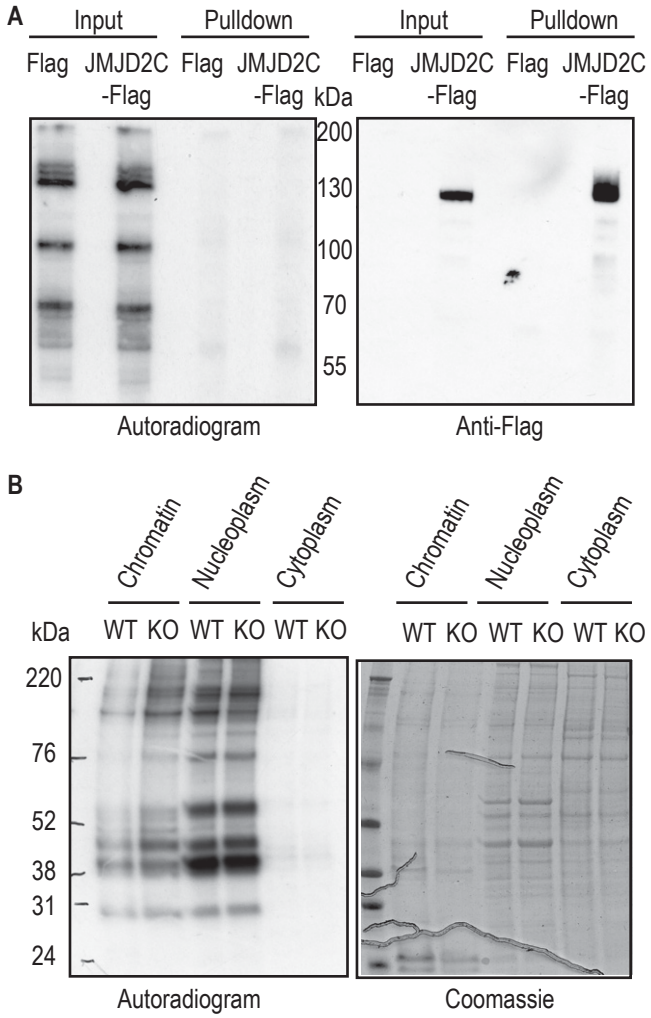

Fig. S10. IP_7 pyrophosphorylates a number of chromatin associated proteins. (*A*) IP_7 does not pyrophosphorylate JMJD2C. Full-length JMJD2C-Flag was immunoprecipitated from cells and was incubated with $5\beta[^{32}P]IP_7$ along with 5% inputs. The reactions were resolved by SDS/PAGE and membranes were exposed to autoradiography for 48 h and subsequently immunoblotted with anti-Flag antibody. The lack of a specific band for JMJD2C-Flag on the autoradiogram either from the input or immunoprecipitated sample indicates a failure to detect pyrophosphorylation of JMJD2C by IP_7. (*B*) $5\beta[^{32}P]IP_7$ pyrophosphorylates a number of histone-associated proteins. Wild-type (WT) and *ip6k1*$^{-/-}$ (KO) MEFs were fractionated into cytoplasmic, nucleoplasmic, and histone extracts following the high-salt method incubated with $5\beta[^{32}P]IP_7$ and resolved by SDS/PAGE. Parallel gels were either stained with Coomassie blue or exposed to autoradiography for 14 h. A number of both nucleoplasmic and histone-associated proteins were pyrophosphorylated by $5\beta[^{32}P]IP_7$. The increased signal of pyrophosphorylated proteins in the histone extract of the *ip6k1*$^{-/-}$ cells from exogenously $5\beta[^{32}P]IP_7$ is consistent with decreased levels of endogenous pyrophosphorylated histone-associated proteins in *ip6k1*$^{-/-}$ MEFs compared with wild-type cells. The experiment was repeated two times.

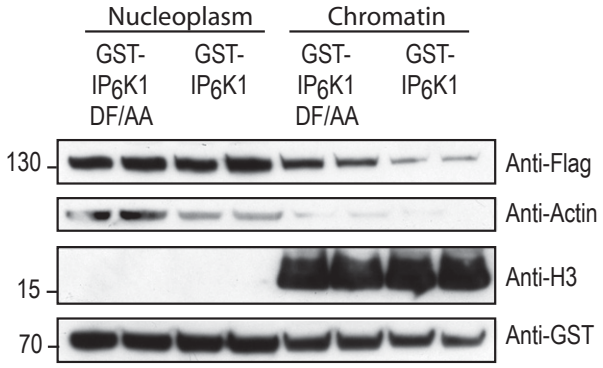

Fig. S11. JMJD2C chromatin association is dependent on IP6K1 catalytic activity. HEK293T cells were transfected with either GST-IP6K1 or GST-IP6K1 DF/AA and JMJD2C-Flag was separated into chromatin or nucleoplasmic (soluble) fractions. Western blot analyses were performed with either anti-Flag, anti-GST, anti-histone H3, or anti-actin antibodies.