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Supplemental Data

HBO1 HAT Complexes Target Chromatin throughout Gene Coding Regions via Multiple PHD Finger Interactions with Histone H3 Tail

Nehmé Saksouk, Nikita Avvakumov, Karen S. Champagne, Tiffany Hung, Yannick Doyon, Christelle Cayrou, Eric Paquet, Mukta Ullah, Anne-Julie Landry, Valérie Côté, Xiang-Jiao Yang, Or Gozani, Tatiana G. Kutateladze, and Jacques Côté

Supplemental Experimental Procedures

Cell culture, plasmid and recombinant proteins

Tet-off retrovirus-transduced cell lines expressing FLAG-HBO1-TAP, HA-ING4-TAP and FLAG-ING5-TAP have been described previously (Doyon et al., 2006). The JADE1L, JADE3 and hEaf6 full-length cDNAs were obtained from ORIGENE. For construction of HA- or FLAG-tagged mammalian expression plasmids, HBO1, JADE1L/3, ING4/5 and hEaf6 were cloned by PCR into pcDNA3. For expression in bacteria, the PHD domains of JADE1, JADE3, ING4 and ING5 were cloned by PCR into the pGEX4T3 (GE Healthcare) and the recombinant proteins were purified following standard procedures. Details on the cloning procedure and primer sequences are available upon request. Mammalian expression vectors for HA- and FLAG-tagged JADE1S(1-509) isoform and its PHD mutants have been described (Panchenko et al., 2004). For transient transfections, $2x10^6$ cells (Hela S3 and 293T) were transfected with 8 μg of each expression plasmid by the calcium phosphate method. JADE1L and its PHD2 deletion mutant were cloned in the retroviral vector pRev-CMV-3XFLAG and

used to transduce HeLa S3 cells. JADE1L expression levels for the different isolated clones were measured and are low (example in Fig. S3C). Details on the cloning procedure (e.g., primer sequences) are available upon request. Nuclear localization of all transfected proteins was confirmed by immunofluorescence.

Antibodies, peptides, histones and chromatin

The following antibodies were used for western blots: anti-FLAG M2-R (1:2000) and anti-GST (1:2000) (Sigma); anti-HA 3F10 (1:2000) and 12CA5 (1:2000) (Roche); anti-TAP (1:3000) (Open Biosystems); anti-HBO1 (1:1000) (Santa Cruz Biotechnologies); anti-ING4 (1:1000), anti-ING5 (1:1000), anti-hEaf6 (1:500), anti-H3 (1:10,000), anti-H3K4me3 (1:5000) and anti-H3K36me3 (1:2000) (Abcam); anti-H3K56ac (1:5000) (Millipore).

Core histones and native H1-depleted chromatin were purified from HeLa or yeast cells as described (Altaf et al., 2007; Cote et al., 1995). Biotinylated histone peptides were synthesized, kindly donated by Dr. Bruno Amati (IEO, Milan) and Dr. Edwin Smith (Emory Univ.), or purchased from Millipore.

Anchorage-independent cell growth

1×10^5 HeLa S3 cells retrovirally transduced to express JADE1 constructs were suspended in DMEM/10%FBS/0.3% agar and plated on 100mm dishes in duplicate. Following 3 weeks of growth in a standard tissue culture incubator, four random fields on each plate were photographed using a light microscope with 4× magnification. Colonies in each field were counted using the AlphaImager software (Alpha Innotech, San Leandro, CA, USA). All isolated clones expressing the different constructs behave similarly in this assay.

Cell growth analysis

HeLa S3 cells retrovirally transduced to express JADE1L constructs were seeded at 4×10^4 cells per well of 6-well plates and allowed to grow in a standard tissue culture incubator. For each cell line, two independent wells were harvested on days 2, 5 and 7 post-seeding. Cells were counted using an Auto T4 Automatic cell counter (Nexcelom Bioscience, Lawrence, MA, USA). All isolated clones expressing the different constructs behaved similarly in this assay.

Affinity purification of the transiently transfected complexes

293T cells were harvested 48hrs post-transfection, washed twice with cold PBS and lysed in 1 ml/plate of lysis buffer [20mM HEPES pH 7.9, 150mM KCl, 5% glycerol, 1 mM DTT, 100μM ZnAcetate, 2 mM $MgCl_2$, 2mM EDTA, 0.2% (v/v) NP-40, 0.5 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM PMSF, 0.1 mM Na_2MoO_4, 0.1 mM Na_3VO_4, 10 mM β–glycerophosphate, 10 mM NaF and 10 mM Na butyrate] on ice for 30 min. The cell lysate was then clarified by centrifugation for 10 min at 12,000 g. The supernatant was incubated for 3 hrs or overnight with 40 μl of FLAG-M2 agarose (Sigma) at 4°C. The resin was recovered by centrifugation and then washed five times with 1 ml of the same buffer but containing 0.1 % (v/v) NP-40 and 300 mM KCl. Bound proteins were eluted with 100 μl of 400 mg/ml 3xFLAG peptide in elution buffer (20mM HEPES pH7.5, 100 mM KCl, 0.1% Triton, 5% glycerol). 20% of the eluate was resolved by SDS-PAGE and analyzed by Western Blot.

Peptide and GST pull-down assays

Concentrations of purified recombinant proteins were normalized by gel electrophoresis and staining. Concentrations of biotinylated peptides were verified by gel and anti-biotin

western blots. Briefly, 500 ng of biotinylated histone peptides were incubated with 1 µg of candidate binding protein in 100 µl of binding buffer [300 mM KCl, 50 mM Tris pH 7.5, 0.05% (v/v) NP-40, 100 µg/ml BSA, 1mM PMSF] at 4 degrees for 3 hours to overnight. After 1 h of incubation with streptavidin Dynabeads (Invitrogen), the beads were washed four times with binding buffer and subjected to Western Blot analysis with an anti-GST antibody.

Chromatin binding and histone acetyltransferase assays with protein complexes

Purified native chromatin (oligonucleosomes, 100-500 ng) was incubated with immunoprecipitated proteins bound to FLAG beads (approximately 10 µl of FLAG-JADE1 complexes on beads) essentially as described (Shi et al., 2006) in a final volume of 50 µl in pull-down buffer (25 mM HEPES pH 7.5, 100 mM KCl, 10 % glycerol, 100 µg / ml BSA, 0.1 % (v/v) NP-40, 0.5 mM DTT and protease inhibitors) for 2 hrs at 4°C. Supernatants were removed and the resin was washed 4 times with pull-down buffer. Input (50%) and bound material were analyzed by western blotting with anti-histone antibodies.

HAT assays with histone peptides (300 ng), core histones or chromatin (500 ng) prepared from HeLa or yeast cells were performed essentially as described (Utley et al., 2005). Briefly, protein complexes were incubated with or without substrate in 15 µl final volume of 50 mM KCl, 50 mM Tris (pH 8), 1 mM DTT, 5 % glycerol, 10 mM Na-Butyrate and 0.1 mM EDTA with 0.5 µl of [^{3}H] acetyl-CoA (0.25 µCi/µl, 4.9 Ci/mmol for 30 min at 30°C. Each reaction was spotted onto p81 filters, washed three times with 50 mM Na Carbonate (pH 9.2) and processed for scintillation counting. Alternatively,

the reaction was separated on an 18% SDS–PAGE, treated with Amplify (PerkinElmer) for 30 min, dried and exposed to autoradiographic film.

Fluorescence spectroscopy

Tryptophan fluorescence spectra were recorded at 25˚C on Fluoromax3 spectrofluorometer. The samples of 10 μM wild type PHD fingers containing progressively increasing concentrations (up to 2 mM) of histone peptides were excited at 295 nm. Emission spectra were recorded between 305 and 405 nm with a 0.5 nm step size and a 1 s integration time and averaged over 3 scans. The K_Ds were determined by fitting to the quadratic function using Equation 1, where F_i is the fluorescence change, F_s is the fluorescence change at saturation of X_T, Y_T is the peptide concentration and X_T is the total protein concentration. The K_D value was averaged over three separate experiments.

Equation 1:
$$F_i = \frac{\left\{F_s\left[\left([X_T]+[Y_T]+K_D\right)-\sqrt{\left([X_T]+[Y]_T+K_D\right)^2-\left(4[X_T][Y_T]\right)}\right]\right\}}{2[X_T]}$$

Chromatin immunoprecipitation (ChIP) assays

Briefly, $2x10^8$ asynchronously growing cells were untreated or treated with 200ng/ml of doxorubicin for 24hr (Sigma). Crosslinks were generated with 1% formaldehyde, and crosslinked chromatin was extracted in Lysis buffer (50mM Tris-Hcl pH8; 10mM EDTA; 0,5% SDS; 5mM Na-Byturate and 10μM Zinc Chloride). Chromatin was then sheared by sonication (Diagenode Bioruptor) on ice to an average length of 500bp. After pre-clearing with a mix of protein A/G sepharose beads (4°C for 1 hour), 200μg of chromatin was used for immunoprecipitation with appropriate antibodies [FLAG M2-R (Sigma), H3 trimethyl-K4

(Abcam), H3 trimethyl-K36 (Abcam), H4 acetyl-K8 (Abcam and Upstate), H3 acetyl-K14 (Upstate), and H3 (C terminus, Abcam)]. After overnight incubation at 4°C, beads were extensively washed and immunoprecipitated complexes were eluted in buffer E (50mM sodium bicarbonate; 1% SDS). Cross-links were reversed overnight at 65°C. The following day, samples were treated with proteinase K and the DNA was extracted using phenol-chloroform. Quantitative real-time PCR was performed using SYBR Green I (LightCycler 480, Roche). Enrichment for a specific DNA sequence was calculated using the comparative Ct method. Background non-specific signal was subtracted using untagged cell lines. In the case of post-translational modifications of histones, data were further normalized to total histone H3 signal to correct for nucleosome density. The numbers presented with standard errors are based on two independent experiments. Primers used in the PCR reactions (available upon request) were analyzed for linearity range and efficiency with a LightCycler (Roche) in order to accurately evaluate occupancy (percent of IP/input).

ChIP-chip

ChIP and chromatin input amplicons were prepared using the Sigma Genomeplex Whole Genome Amplification2 kit (protocol available at genomics.ucdavis.edu/farnham). ChIP-chip experiments were performed on Nimblegen ENCODE microarrays (HG17). This microarray contains over 384 000 50-mers covering the 30 megabases of the ENCODE regions with one probe every 38 bp. Arrays were scanned using an Axon 3000B scanner. The software SignalMap from Nimblegen was used using a cutoff ratio of 1.5 and a false discovery rate (FDR) of .2 to extract significant peaks over the ENCODE region. The FDR of 0.2 is considered indicative of a binding site according to the manufacturer. Briefly, the ratios are first log2 transformed and then scaled by using Tukey's biweight

mean to bring the global mean log2 ratio to a value of 0. The FDR is calculated by randomizing 20 times the entire array while counting peaks having a ratio over cutoff at random. The significance of the overlaps between significant peaks set S and different regions R like: H3K4me3 hits in HeLa cells, other significant peaks, gene and intergenic, 1000 random sets having the size and length of peaks in S, were generate at random and the number of overlapping peaks between S and R was compared to the random distribution.

Sequence alignments

Multiple sequence alignments were processed by Clustalw (INFOBIOGEN) and shading was realized with BOXSHADE 3.21.

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Figure S1. Endogenous JADE1S associates with HBO1 in vivo, and H3K4 methylation stimulates the acetyltransferase activity of HBO1 HAT complexes in an ING-dependent manner.

(A) Mass spectrometry analysis of tandem-affinity purified native HBO1 HAT complexes identifies both JADE1L and JADE1S protein bands. Silver-stained gel of tandem-affinity-purified HBO1 complex. Indicated bands were identified by MS/MS (Harvard Microchemistry Facility and Taplin Biological Mass Spectrometry Facility, Boston MA). The 63 kDa band corresponding to the reported size of native JADE1S on gel (Zhou et al., 2005) was found to contain peptides corresponding to JADE1 sequence. (B) ING4/5 PHD fingers preferentially bind H3 peptides methylated on lysine 4 but not on lysine 9. Peptide pull-down assays with the indicated biotinylated peptides and recombinant PHD fingers fused to GST were analyzed by western blot with anti-GST. (C-D) ING5 physical association is required for stimulation of HBO1 HAT activity on H3K4me tails. FLAG-HBO1 HAT was affinity-purified after co-transfection with the indicated cDNAs and tested in HAT assays on peptides.

Figure S2. JADE1 PHDs bind to histone H3 peptides with different specificities, and PHD2 is required for acetylation of native human chromatin by HBO1.

(A-B) Symmetric or asymmetric methylation of histone H3 arginine 2 has little or no effect on H3 tail binding by JADE1 or ING4/5 PHD finger domains. Western blot

analysis of peptide pull-down assays with the indicated peptides and recombinant PHD domains.

(C) Histone H3 tail is essential for HBO1 complexes to associate with native chromatin but not with free histones. The indicated JADE1 complexes (immunopurified from co-transfected cells like in figure 1) were tested for binding to free core histones purified from wild type or *set1* (no H3K4me) yeast cells. Bound histones were visualized by western blot with the indicated H3 antibodies. The presence of the N-terminal truncation of H3 is shown on the right (lines separate samples because some lanes were removed).

(D-E) Acetylation of human native chromatin but not free core histones by HBO1-JADE1S complexes requires PHD2. Immunopurified wild type and mutant FLAG-JADE1S dimeric complexes from cells co-transfected with HBO1 were normalized for HBO1 content and tested in HAT assays on human free core histones (D) and native oligonucleosomes (E). Liquid scintillation counts from duplicate assays are presented.

Figure S3. Effect of JADE1 expression in vivo and its functional relationship with H3K36me3-containing chromatin.

(A-B) Transient transfections of 293T cells with JADE1L/S increase the bulk levels of histone H4 acetylation, and this effect depends on PHD2. 48 hrs post-transfection histones were acid-extracted and analyzed by western blotting using total H3 as loading control.

(C) Retroviral transduced clones (HeLa) used in cell growth and ChIP experiments express similar levels of wild type JADE1L and PHD2 deletion mutant.

(D) HBO1 complexes can bind H3K36me3-enriched chromatin from coding regions, independently of ING5 presence. Chromatin binding assays were done as in figure 2B and analyzed by western blot.

(E) Presence of the H3K36me3 mark in native yeast chromatin does not greatly affect the HAT activity of JADE1S/L complexes. HAT assays were performed with JADE1L and JADE1S complexes as in figure 2D using wild type and *set2* mutant (no K36 methylation) chromatin purified from yeast cells. Note that JADE1L complex has an overall higher activity compared to JADE1S on wild type chromatin (due to high K4me3 content) and stronger acetylation of H3 (as in Fig. 2D). The lower level of H3 acetylation by JADE1L on *set2* chromatin might be relevant but may also be due to the lower amount of full-length H3 in this chromatin prep.

Figure S4. JADE1L HAT complexes are associated with the coding regions of genes in human cells.

Graphical summary of the data obtained by ChIP-chip assay with JADE1L wild-type and PHD2 mutant proteins in the presence or absence of doxorubicin (200ng/ml for 24hrs). Significant detected peaks of binding are presented in relation to relative distance (bp) from transcription start/end sites (TSS/TES) of the genes present on the ENCODE arrays. The dashed vertical lines represent the median of gene lengths present on the array.

Figure S5. JADE1L binds to multiple loci within several genes.

(A) ChIP-chip data obtained on a cluster of 4 genes on human chromosome 1. H3K4me3 and H3K36me3 signals obtained by ChIP-chip on the same ENCODE sequences in HeLa

cells are also depicted (Koch et al., 2007; Lian et al., 2008). Values are presented as log2 ratio of IP/input. The part of the graph below a log2 ratio of 1 is shaded to highlight regions of significant binding (more than two-fold enrichment). Note that the doxorubicin treatment does not greatly affect the specific signals obtained, in contrast to the Menin1 gene presented in figure 4D.

(B-D) UCSC browser window presentation of the data obtained on the *EXT1* (B) and *FOXP4* (C) genes as well as on the entire *HOXA* cluster (D). Only the significant peaks are presented as well as the K4me3 and K36me3 signals.

Figure S6. Summary model of the HBO1 complexes assembled by the two JADE1 isoforms and their functional features for binding to specific chromatin regions and histone acetylation specificity.

Table S1. Affinities of JADE1 first PHD finger domain (PHD1) for histone H3 peptides and the effect of lysine 4 and 36 methylation (measured by tryptophan fluorescence).

H3 peptide	**Kd of JADE1 PHD1 (μM ± SD)**
H3 (aa1-12) K4me0	25.7 ± 1.2
H3 K4me1	65.9 ± 8.7
H3 K4me2	291 ± 56
H3 K4me3	547 ± 82
H3 (aa30-44) K36me0	322 ± 44
H3 K36me3	48.2 ± 5.7

Table S2. Affinities of JADE3 PHD finger domains for histone H3 peptides and the effect of lysine 4 trimethylation (measured by tryptophan fluorescence).

PHD finger	**Kd with H3 (aa1-12) (μM ± SD)**	**Kd with H3 K4me3 (μM ± SD)**
JADE3 PHD1	21.2 ± 1.3	517 ± 89
JADE3 PHD2	7.1 ± 3.1	ND

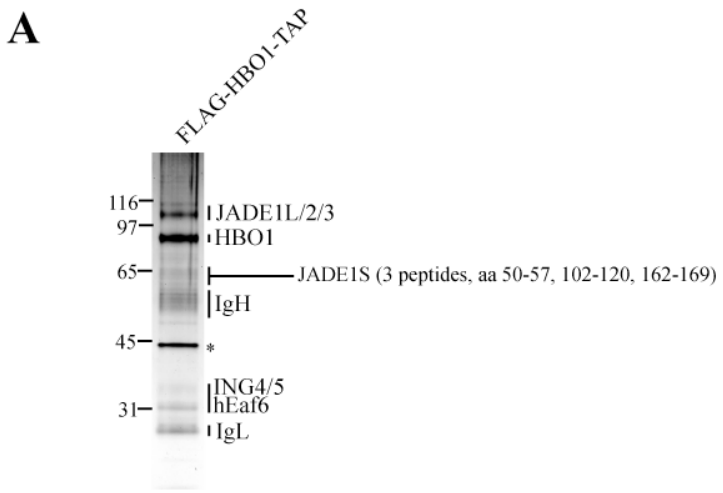
ND : not determined

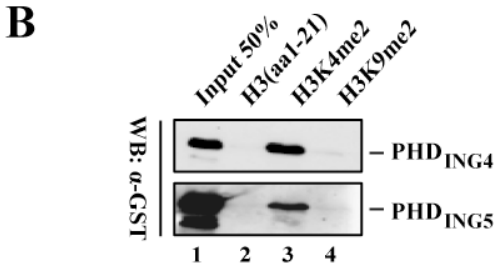
Table S3. Number of significant peaks of JADE1L binding detected by ChIP-chip experiments using Nimblegen high-density human ENCODE tiled arrays in the absence or presence of genotoxic agent doxorubicin.

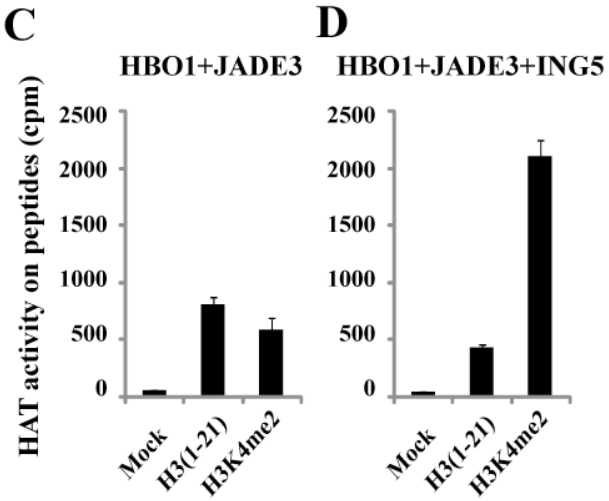
Protein ChIP	**# of significant peaks on Nimblegen human ENCODE arrays**
JADE1L	823
JADE1L PHD2 mutant	125
JADE1L + doxorubicin	905
JADE1L PHD2 mutant +doxorubicin	298

Table S4. Colocalization of JADE1L binding with reported peaks of histone H3 trimethylation on lysine 4 and 36 using Nimblegen ENCODE arrays. Numbers represent the fold enrichment compared to prediction based on random binding (value of 1).

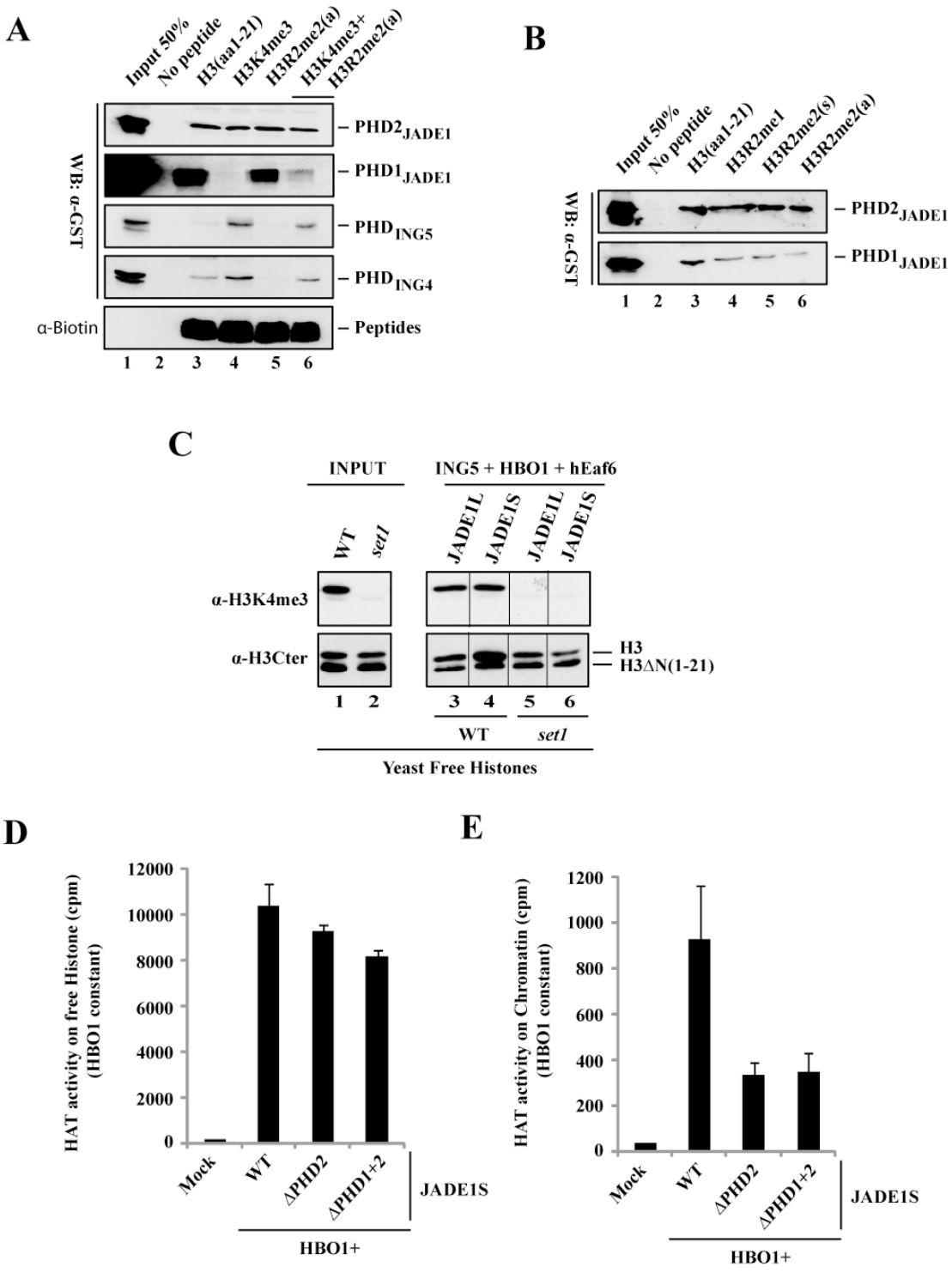
Protein ChIP	**Enrichment of colocalization with H3 K4me3 peaks**	**Enrichment of colocalization with H3 K36me3 peaks**
JADE1L	1.30	2.87
JADE1L PHD2 mutant	2.13	2.15
JADE1L + doxorubicin	1.61	2.60
JADE1L PHD2 mutant +doxorubicin	1.78	3.23







Saksouk et al. Supplementary Figure 1



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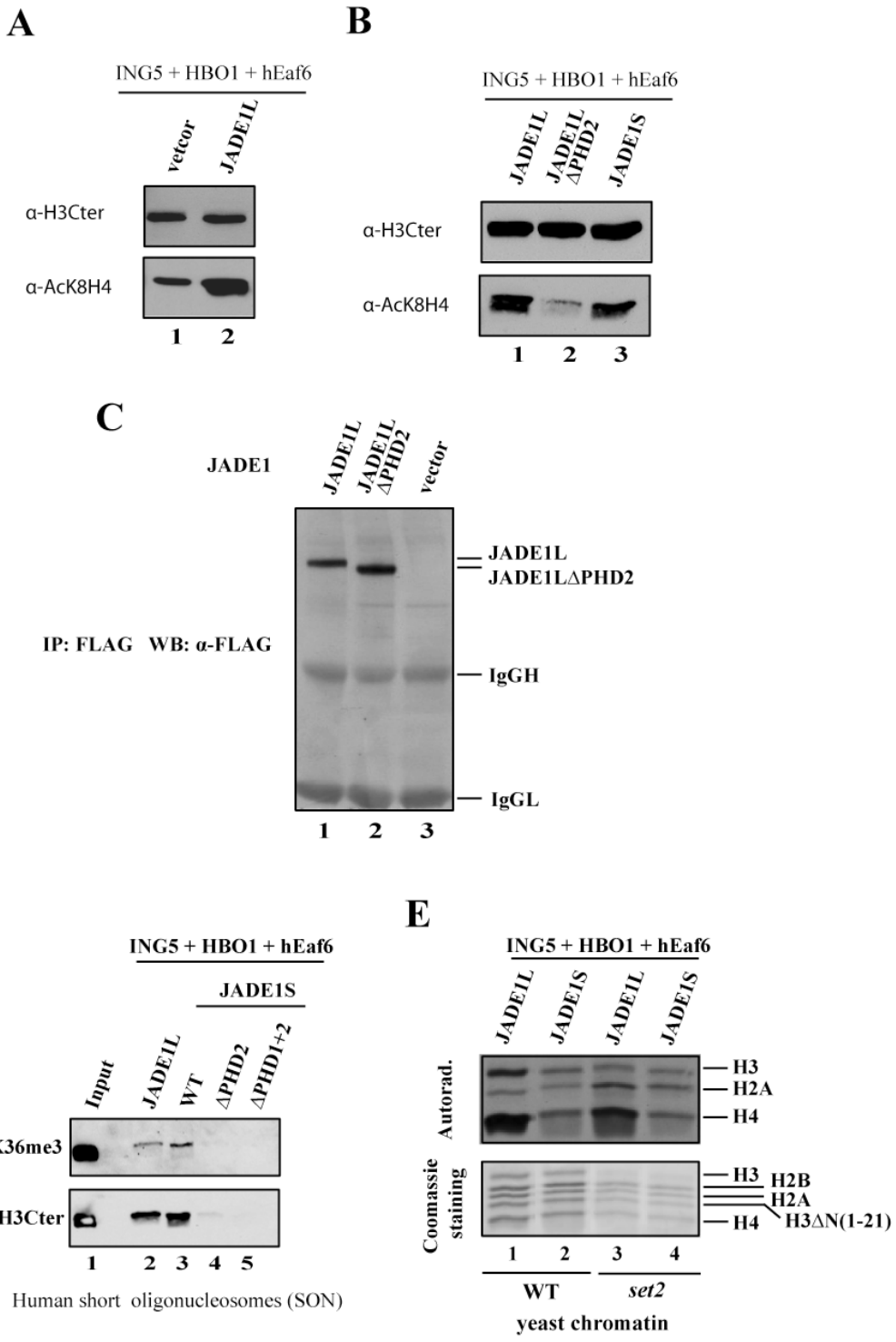
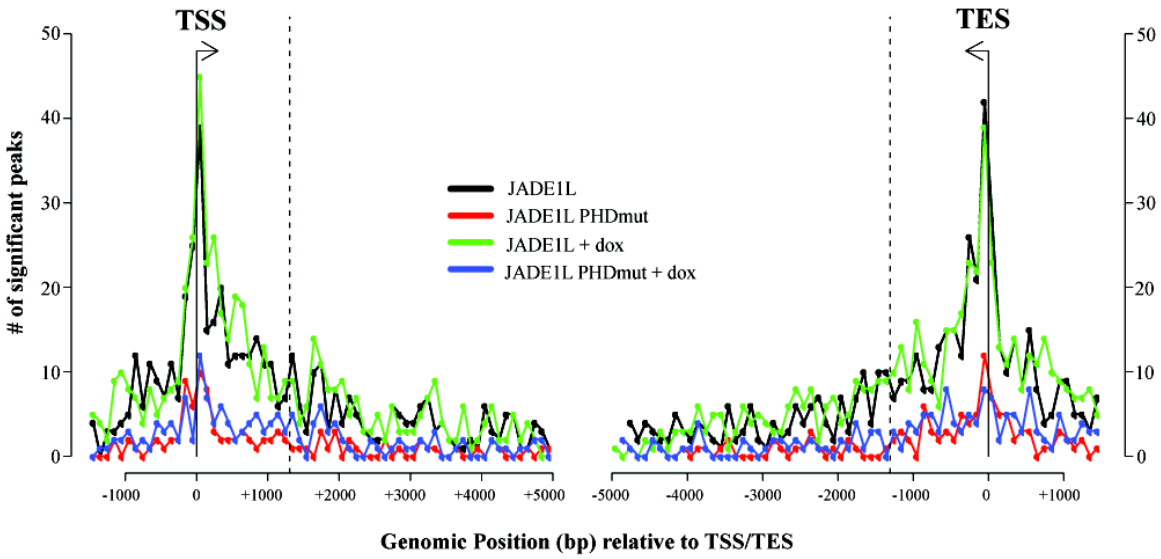


Saksouk et al. Supplemental Figure 3



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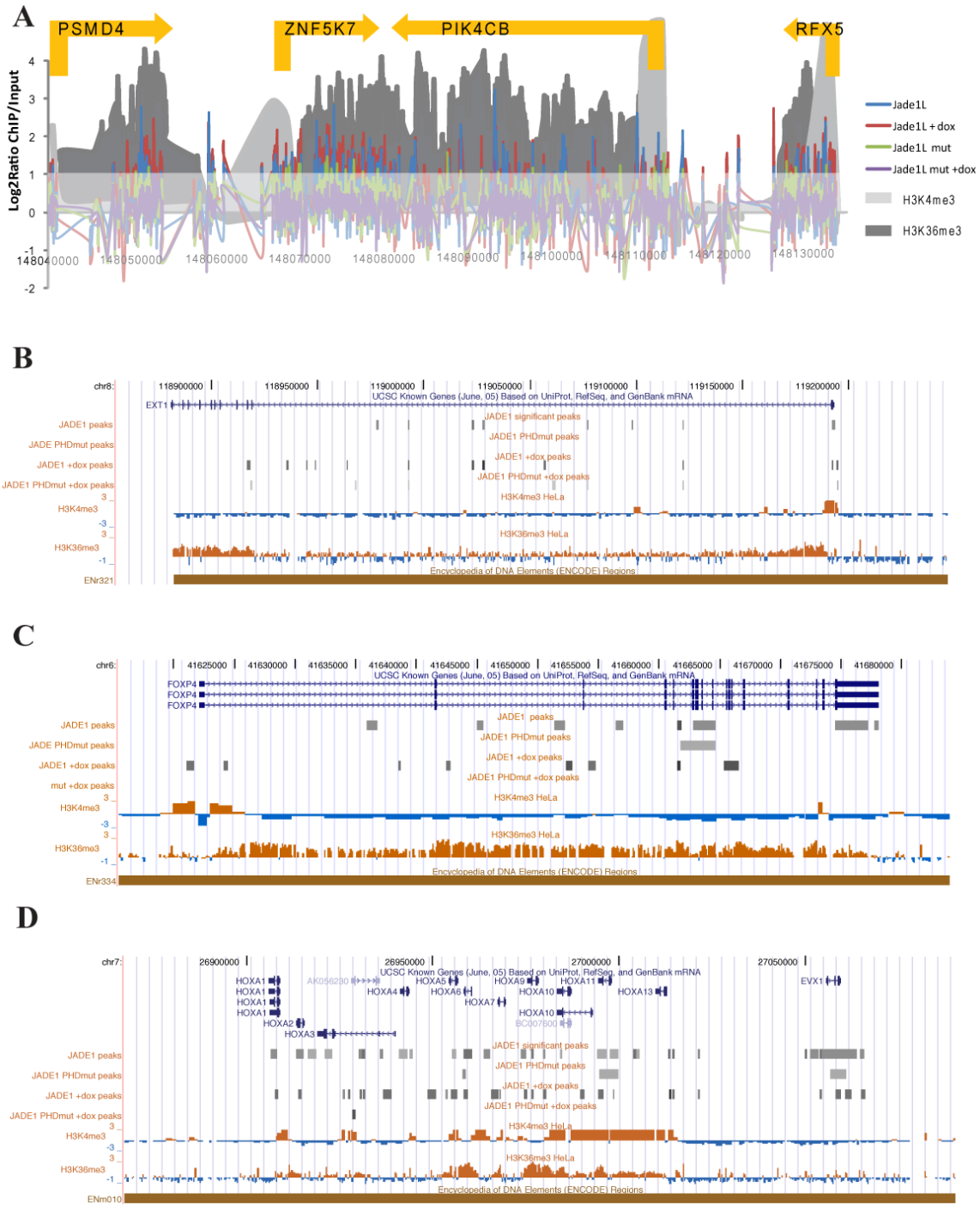

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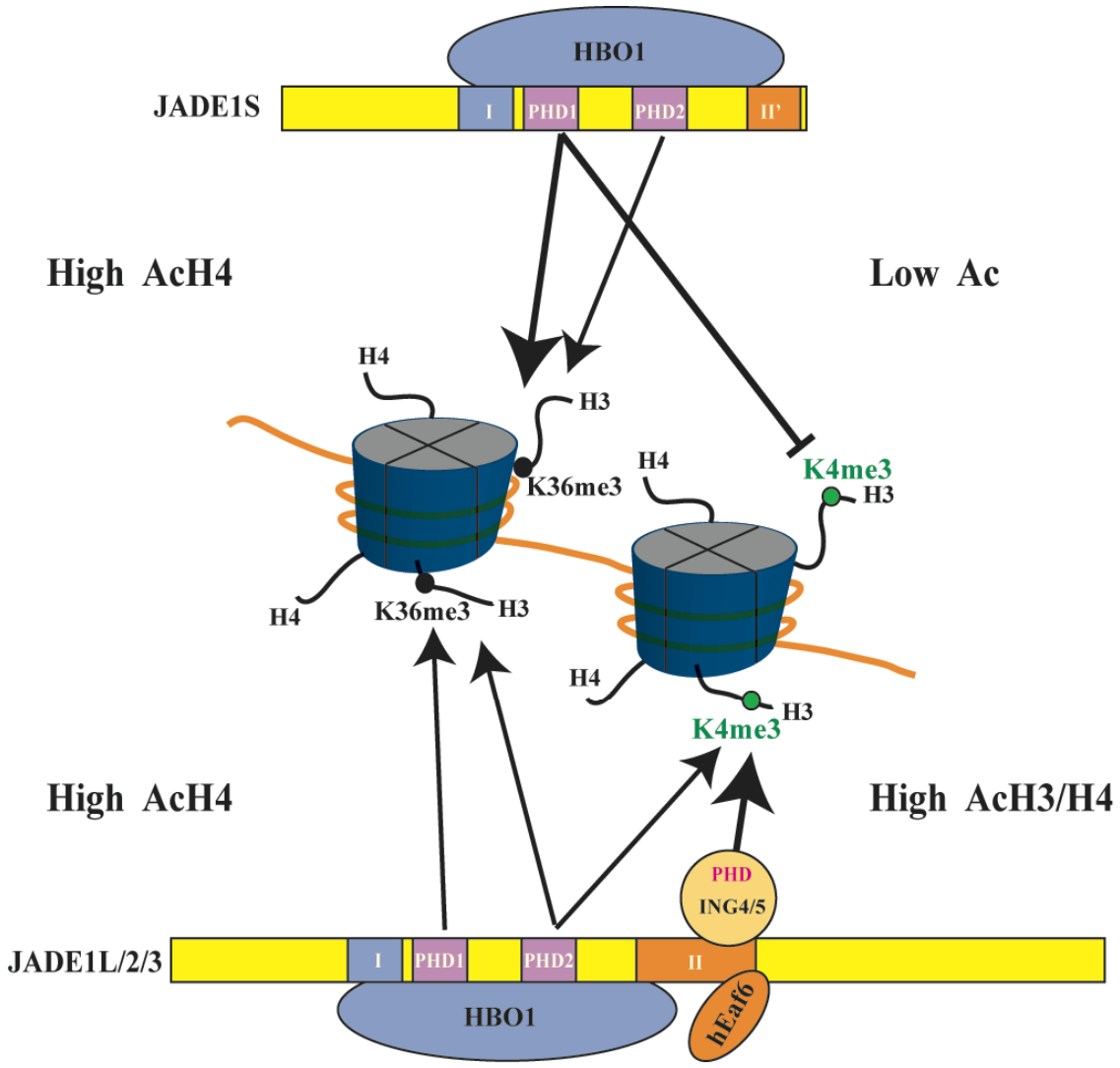

Saksouk et al. Supplemental Figure 6