

Role of Histone H3 Lysine 27 Methylation in Polycomb-Group Silencing

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Supplementary Material

Supplementary materials and methods:

Purification of the EED-EZH2 complex. Solubilization of HeLa nuclear pellet protein and chromatography on DEAE52 and phosphocellulose P11 columns were performed using a previously described procedure (2). The P11-BC500 fraction was dialyzed against buffer D [50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 2 mM DTT, 0.2 mM PMSF, and 25% glycerol] containing 20 mM ammonium sulfate (BD20), then loaded onto an HPLC-DEAE-5PW column (TosoHaas, 45 ml) equilibrated with BD20. Proteins bound to the column were eluted with a 12 column-volume (cv) linear gradient from BD20 to BD500. The HMTase activity was separated into two peaks on this column. The second peak fractions were pooled and adjusted to BD700 using saturated ammonium sulfate and loaded to Phenyl Sepharose column (Pharmacia), then eluted with a 10 cv linear gradient from BD700 to BD0. The active fractions were pooled and dialyzed in buffer P [5 mM Hepes-KOH (pH 7.5), 40 mM KCl, 0.01% Triton X-100, 0.01 mM CaCl₂, 1 mM DTT, 0.5 mM PMSF, and 10% glycerol] containing 10 mM potassium phosphate (BP10), loaded onto a 1 ml hydroxyapatite column. The bound proteins were eluted with 20 cv linear gradient from BP10 to BP600. The peak fractions were pooled, the proteins concentrated by ammonium sulfate precipitation, and then fractionated on a Superose-6 column (Pharmacia). The HMTase activity elutes between 670 and 440 kDa.

Protein identification, HMTase assay and substrate preparation. The fractions containing the H3-HMTase activity from the Superose 6 column were bound to 40 µl of phosphocellulose beads and resolved on a gradient SDS-PAGE. After Coomassie staining, each band was excised and digested with trypsin, partially fractionated, and the resulting peptide mixtures analyzed by matrix-assisted laser-desorption / ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS) (3), and also using an electrospray ionization (ESI) triple quadrupole MS/MS instrument modified with an ultra-fine ionization source (4). Selected masses from the MALDI-

TOF spectra were used to search the GenBank database. MS/MS spectra were inspected for yⁿ ion series to compare with the computer-generated fragment ion series of the predicted tryptic peptides. HMTase assay and quantification were performed as described (5). HeLa or chicken core histones, mono- and oligonucleosomes were purified as described (6). Histone mutants were generated by PCR based mutagenesis and confirmed by sequencing. Recombinant histones were purified as described (7).

Antibodies and ELISA. Antibodies against EED, EZH2, and SUZ12 were generated in rabbits using recombinant 6xhis-EED (35-535), 6xhis-EZH2 (aa406-746), and SUZ12 (aa560-739) as antigen, respectively. Antibodies against H3-mK27 were generated in rabbits using KLH-conjugated H3 peptide TKAARKmSAPAT (aa 22-32) containing dimethyl-K27. All the above antibodies were affinity purified before using. The methyl-K27 antibody was first purified using a methyl-K27 H3 peptide and further purified by incubating with an K27-unmethylated H3 peptide. Antibodies against RbAp48, H3-mK4, H3-mK9, H4-mK20, and E(Z) have been previously described (2, 6, 8).

For ELISA analysis, 100 ng of unmodified or K27-dimethylated histone H3 peptides (aa 19-35), or equivalent amounts of H3-K9 dimethyl (aa 1-21) or trimethyl (aa 1-16) peptides were incubated overnight in 100 mM NaHCO₃ (pH 9.6) in a 96-well ELISA plate at 4⁰C. After washing with PBS containing 0.05% Tween-20 (PBT), nonspecific bindings were blocked with 200 μl PBT containing 1% BSA for 1-2 hrs at 37⁰C. Following 3 washes with PBT, serially diluted antibodies were added and incubated for 2 hrs at 37⁰C. After washing with PBT 3 times, an HRP-conjugated rabbit secondary antibodies (1:5000) were added, incubated at 37⁰C for 1 hr. Finally 100 μl substrates TMB (Pierce) were added to each well and incubate for 15 min at room temperature before stopped by adding 100 μl 1 M H₂SO₄. The absorbance was measured at 450 nm.

ChIP, RNAi, and histone Western. Formaldehyde-crosslinked chromatin was prepared from S2 cells (grown in SD medium supplemented with 10% fetal bovine serum, Gibco) and immunoprecipitations were performed using the ChIP Assay Kit according to the manufacturer's recommended protocol (Upstate Biotechnology). Pairs of PCR primers (each 20 nt in length)

were used for amplification of the following segments of the *Ubx* PRE region (coordinates refer to nucleotide positions in P1 clone DS03408, GenBank accession number L32205): 1, 36332 to 35662; 2, 35637 to 35140; 3, 35155 to 34624; 4, 34453 to 33990; 5, 34339 to 33884; 6, 34148 to 33664; 7, 33216 to 32935; 8, 32959 to 32420; 9, 32324 to 31915. Amplification of the *RpIII40* promoter region (coordinates 57 to 666, GenBank accession number X05709), which approximates the *RpIII40* pol1 region previously described (9), served as negative control. PCR scheme: 94°C for 2 min, once; 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, 30 times; 72°C for 5 min, once. The amplified DNA was separated on 1.5% agarose gels and visualized with ethidium bromide. For wing imaginal discs ChIP assays, wing discs were dissected from late third instar larvae from a homozygous *E(z)⁶¹* stock. Larvae were either maintained continuously at 18°C or shifted from 18°C to 29°C ~48 h prior to dissection. Discs were dissected in serum free SS3 medium (Sigma) and stored on ice until ready for formaldehyde fixation. Groups of 20 discs were fixed at a time, following the same protocol used for S2 cells, except that the fixation time was doubled to 20 min. Following lysis of cells, fixed lysates were pooled to ensure uniformity and chromatin from the equivalent of ~ 9 discs was immunoprecipitated with each antibody. One tenth of the DNA from each immunoprecipitation was used in each PCR reaction. 36 cycles were required in order to detect PCR products.

The template for synthesizing *esc* dsRNA was produced from total *Drosophila* embryo RNA by RT-PCR amplification of a 483 bp exonic region extending from 4 bp upstream of the *esc* ATG into exon 3 (GenBank accession number L41867). Both primers included T7 promoter sequence at their 5' ends. The PCR product was bi-directionally transcribed using the MEGA script T7 kit (Ambion), and complementary strands annealed by incubating the RNA at 65°C for 30 min and then slowly cooling to room temperature. Transfection (and mock transfection) of S2 cells was performed essentially as described (9) with the following modifications. 1.0×10^6 cells were initially transfected with ~5 µg *esc* dsRNA. After 3 days of growth, 1.0×10^6 of these cells were again transfected (or mock transfected), cultured another 3 days and then harvested.

For histone Western, embryos were collected from a homozygous *E(z)⁶¹* stock and an OregonR stock at 18°C (0 to 24 h) or at 29°C (0 to 12 h). Embryos were dechorionated in 50%

hypochlorite bleach and frozen. Histones were isolated (2), and quantified before analyzed by Western.

In vitro transcription/translation of PC and peptide pull-down assays. The PC cDNA was generated by PCR from a *Drosophila* embryo cDNA library and cloned into EcoRI/XhoI sites of the pCITE-3b vector, which allows *in vitro* transcription/translation by the T7 polymerase. The W47A and W50A mutations were generated by overlapping PCR and confirmed by DNA sequencing. Plasmid DNA (1µg) was transcribed and translated using the TNT-kit (Promega) following manufacture's instruction. For peptide pull-down assays, 20 ng of biotinylated histone H3 peptides (aa 19-35), that were either unmethylated or methylated on K27, were incubated with 10 µl streptavidin-conjugated Sepharose beads (Amersham Pharmacia Biotech) in PBS for 2 hrs at 4°C . After washing three times with binding buffer (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 20% glycerol) containing 200 mM KCl (BC200), the beads were mixed with 5 µl *in vitro* translated proteins and 300 µl BC200 for 60 min at 4°C. After extensive washing with binding buffer containing 500 mM KCl, the bound proteins were eluted in SDS loading buffer, resolved by SDS-PAGE and subjected to autoradiography.

Supplementary references

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Supplementary Figure Legends

Supplemental Figure 1. The largest protein that co-elutes with the HMTase activity on Superose 6 does not co-fractionate with the HMTase activity on the Hydroxyapatite column. Shown is a silver stained polyacrylamide-SDS gel (top panel) and HMTase activity (bottom panel) of the fractions derived from the Hydroxyapatite column. While the majority of the HMTase activity is eluted between fractions 29-38, the contaminating protein, indicated by *, is present throughout the majority of the column fractions. The migration positions of the size markers in the SDS-PAGE are indicated on the left.

Supplemental Figure 2. The EED-EZH2 complex likely methylates H3-K27, but not H3-K4 or H3-K9 in oligonucleosomes. Oligonucleosomes were methylated by the EED-EZH2 complex in the presence of ^3H -SAM. The labeled H3 was gel-purified and subjected to N-terminal automated sequencing and ^3H -radioactivity eluted from each cycle was counted. The amino acids identified at each cycle of microsequencing are listed. Although the peak on K27 is small, it is significant due to the reduced recovery efficiency in each cycle (1). Assuming a 90% recovery efficiency for each cycle, the signal in cycle 27 would be only 15% (0.9^{18}) of what it was in cycle 9. Therefore, a peak of ~ 550 cpm at cycle 27 would be equivalent to a peak of ~3,667 cpm in cycle 9 (about 14-fold of what was observed in cycle 9).

Supplementary Figure 3. H3-K27 methylation occurs in a wide range of multicellular organisms. **(A).** Characterization of the H3-mK27 antibody. Western blot analysis of HeLa core histones using affinity purified H3-mK27 and H4-mK20 antibodies (Top panel). H4 serves as a loading control. The H3-mK27 antibody specifically recognizes H3 and is competed by the K27 methylated H3 (aa 19-35) peptides but not by K27 nonmodified (aa 19-35), or K9 dimethylated (aa 1-21) or K9 trimethylated (aa 1-16) H3 peptides. The amounts of competing peptides were adjusted to equal molar concentration equivalent to that of the K27 methylated H3 peptides (0.05 $\mu\text{g/ml}$). The same peptides used above were used for ELISA analysis of the H3-mK27 antibody. **(B).** H3-K27 methylation occurs in a wide range of multicellular organisms. Equivalent amounts of histones from different organisms indicated on top of the panel were analyzed by Western blot using the H3-mK27 or -mK4-specific antibodies.

Figure 1 (Cao et al.)

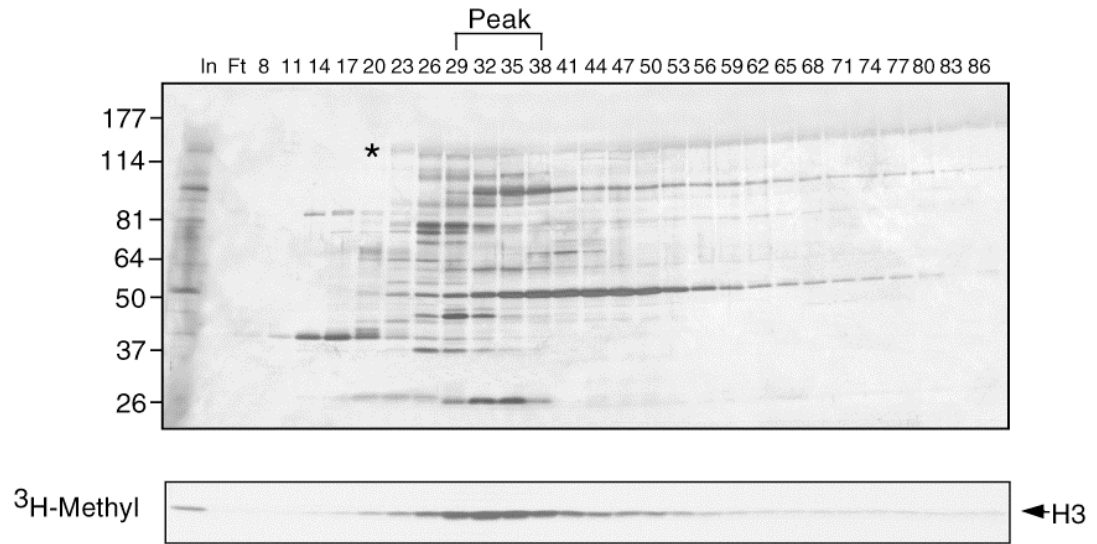


Figure 2 (Cao et al.)



Figure 3 (Cao et al.)

