Role of the polycomb protein Eed in the propagation of repressive histone marks

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Summary

Polycomb Group (PcG) proteins play an essential role in the epigenetic maintenance of repressive chromatin states. The gene silencing activity of the Polycomb Repressive Complex 2 (PRC2) depends on its ability to tri-methylate lysine 27 of histone H3 (H3K27) via the catalytic SET domain of the EZH2 subunit, and at least two other subunits of the complex: Suz12 and Eed. We show that the C-terminal domain of Eed specifically binds to histone tails carrying tri-methyl lysine residues associated with repressive chromatin marks and that this leads to the allosteric activation of the methyltransferase activity of PRC2. Mutations in Eed that prevent it from recognising repressive trimethyl-lysine marks abolish activation of PRC2 *in vitro* and, in *Drosophila*, reduces global methylation and disrupts development. These findings suggest a model for the propagation of the H3K27me3 mark that accounts for the maintenance of repressive chromatin domains and for the transmission of a histone modification from mother to daughter cells.

Introduction

A cell's fate is specified by its gene expression profile, often set early in development and maintained throughout the life-time of the cell by epigenetic mechanisms. The PcG group of proteins function by silencing inappropriate expression by maintaining a repressive epigenetic state[1]. It is thought that the PRC2-mediated trimethylation of lysine 27 on histone H3 plays a key role in marking repressive chromatin domains, while PRC1 is important for effecting transcriptional repression. Thus, once established, H3K27 trimethylation is the epigenetic mark for maintaining transcriptional repression. Mechanisms are therefore required to maintain faithfully this mark in repressed chromatin domains in non-dividing cells and to restore it after the two-fold dilution caused by DNA replication in dividing cells. However, it is not yet clear how PRC2 complexes recognize previously marked sites and how they propagate accurately these repressive marks to unmodified nucleosomes deposited during DNA replication.

The histone lysine methyltransferase (HKMT) activity of the PRC2 complex resides in the SET-domain containing protein Ezh2[2–5] but activity requires the other subunits of the core

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hydrophobic residues. In the case of H4K20 peptide, the only one of the four that bound to ΔEed and lacks an alanine at −2, its binding is facilitated by an alternative hydrophobic interaction between the leucine residue in the +2 position of the peptide with a second

significantly affect the enzymatic activity of PRC2 but trimethylated peptides activated it about seven-fold (Fig. 4A). Stimulation of enzymatic activity by H3K27me3 peptide reached a plateau around 100μM while half maximum stimulation is achieved around 30-40μM (Fig. S7), which is in good agreement with the dissociation constant determined for Δ

larvae with poorly developed brain and imaginal discs, which die when they pupate. This lethality is completely rescued by one copy of the wild-type *esc*>MYC-ESC transgene. In contrast, none of the aromatic cage mutant transgenes were able to rescue the lethality even when present in two copies (Fig. 5C), although the *esc*>MYC-ESC Phe-77Ala transgene alleviated the brain and imaginal disc phenotypes (data not shown). Of note, zygotic expression of the Phe-345Ala transgene impaired the contribution of a wild type *esc* indicating that this mutant acts as a dominant negative. The failure of the mutant Myc-ESC to rescue is not due to instability or to inability to be incorporated into a PRC2 complex: the ESC mutants were expressed at levels comparable to that of the wild type. Furthermore, immunoprecipitation experiments revealed that the mutant ESCs co-immunoprecipitated with endogenous E(Z) as efficiently as the wild type protein (Fig. 5D). To determine whether the mutant ESCs affected PRC2 function with respect to its gene targeting or activity, we performed chromatin immunoprecipitation followed by qPCR with the primer sets indicated in Figure 5E. Immunoprecipitation using anti-E(Z) shows that wild type Myc-ESC is nearly as effective as endogenous ESC (compare with the esc^{+} $escI^{-}$ chromatin) while PRC2 complex with Myc-ESC bearing mutations in the aromatic pocket is recruited less efficiently to the *Ubx* Polycomb Response Element (PRE) (Fig. 5F). Chromatin immunoprecipitation with anti-H3K27me3 antibodies also shows that wild type Myc-ESC is nearly as effective as endogenous ESC (*yw*) in trimethylating H3K27 in the *Ubx* upstream enhancer re512 t

when the *Drosophila* Eed orthologue ESC bears mutations in the aromatic cage, the recruitment of PRC2 to the PRE is less effective, as shown by the drop in E(Z) binding to the *bxd* PRE, the massive reduction in the global level of H3K27me2/3 and by the phenotype of the Phe-77Ala and Phe-345Ala mutants. Our chromatin modification assays suggest that a major effect of Eed binding to repressive methyl-lysine marks is the stimulation of PRC2 methyltransferase activity, thus providing a mechanism for the propagation of this mark. Thus, when PRC2 is recruited to appropriate chromatin domains, the presence of pre-existing H3K27me3 marks on neighbouring nucleosomes activates the complex to carry out further methylation of unmodified H3K27 (Supplementary Fig. 1). Accordingly, a PcG target gene that had been repressed in one cell cycle will tend to be repressed again in the following cell cycle, and previously active genes will be left unmodified at H3K27. We propose that the ability to recognize a previously established mark that triggers its renewal is a feature that will be found in other epigenetic mechanisms mediated by histone modifications.

On-line methods

Protein Expression and Purification

Residues 78-441 of Eed (ΔEed) were cloned into pGEX-4T vector (Amersham Biosciences) and expressed in *E. coli*. Proteins were prepared as N-terminal glutathione-S-transferase (GST) fusion proteins and cleaved from GST with human α-thrombin (Haematologic Technologies, Inc.). Proteins were purified further using size exclusion chromatography (Superdex 200, GE Healthcare) in buffer containing 50 mM Tris-HCl pH 8.7, 150 mM NaCl and 3 mM TCEP. Site-directed mutants of ΔEed were generated with the ExSite protocol (Stratagene) and purified in a similar manner. Crystallographic and binding studies were carried out using a construct containing the mutation Met-370Thr, however the binding properties of this construct are identical to those of the 'wild type' construct. Peptides were synthesised and purified by reversed phase HPLC at the University of Bristol Peptide Synthesis Facility. Peptide masses were verified by mass spectrometry.

Crystallography

For crystallisation trials, protein solutions were prepared as either ΔEed alone at 2.5 mg/ml or as a complex solution at 1.5 mg/ml with peptide at a 7-fold higher molar ratio. All protein solutions contained TCEP at 15 mM concentration. Crystals were grown at 18°C using the vapour diffusion technique in hanging drops. Drops were prepared by mixing equal volumes of ΔEed protein alone with reservoir solution containing 4.0–4.1 M formate and 0.6–0.7 M NDSB-195, or by mixing equal volumes of ΔEed protein complex with 3.7–3.9 M formate solution. Crystals were transferred into mother liquor with 5 to 10% glycerol prior to flash cooling in liquid nitrogen. Diffraction data for the ΔEed-only native and selenomethionine crystals were collected at the Daresbury synchrotron on beamline 10.1 at the peak wavelength for selenium. Diffraction data for the H1K26me3, H3K9me3 and H4K20me3 protein complex crystals were collected using an in-house MicroMax 007HF rotating anode coupled to a RaxisIV^{++} detector. Data for H3K27me3 was collected at Diamond Light Source on beamline I04 at a wavelength 0.97 A. Data were integrated using Denzo and scaled with Scalepack[28]. Phases for the selenomethionine-substitued ΔEed structure were generated and extended using the single wavelength anomalous dispersion (SAD) method and SOLVE[29] and RESOLVE[30] programs. Phases from RESOLVE were used to autobuild a model with ARP/wARP[31] in warpNtrace mode. The protein complex crystal structures were solved by molecular replacement using AMoRe[32] and the selenomethionine-substitued ΔEed structure as the search model. Standard refinement was carried out with refmac5[33] and CNS[34] together with manual model building with O[35] and Coot[36]. Figures were created with Pymol (DeLano Scientific; http://pymol.sourceforge.net/).

Binding studies

Histone peptide binding experiments were performed by competition fluorescence spectroscopy and Isothermal Titration Calorimetry (ITC). All fluorescence emission spectra were measured using a dansyl labelled peptide (sequence: KKKARK(Me3)SAGAAK-dansyl) at 20¼ C in 50 mM Tris-HCl pH 8.7, 150 mM NaCl and 3 mM TCEP. Measurements were recorded using a SPEX FluoroMax fluorimeter (excitation wavelength 330nm, emission wavelength 537nm). Binding of dansyl peptide to Eed was monitored by titrating excess Eed into 5 μM peptide. Dissociation constants for the unlabelled histone peptides were determined using a competition assay by adding excess unlabelled peptide to a complex of 35 uM Eed with 35 uM dansyl peptide and monitoring the subsequent reduction in fluorescence. ITC measurements were carried out by injecting peptide at 400–1000 μM into the ITC cell containing Δ77Eed at 40–100 μM. Experiments were performed at 20¼ C in 50 mM Tris-HCl pH 8.7, 150 mM NaCl and 3 mM BME.

Methyl lysine analog production

Pseudo-lysine (ϕK) containing histones were generated by a modification of known literature methods[12]. In brief; proteins to be modified (5–10mg) were weighed into 1.5 ml siliconized Eppendorf microcentrifuge tubes and 950 μl alkylation buffer (4 M guanadinium chloride, 1 M HEPES, 10mM D/L-methionine pH 7.8, the solution is passed through a 0.22 micron filter and purged with argon prior to use) was added. Proteins that do not readily dissolve were sonicated for 10–15 min in a Branson 1510 ultrasonic cleaning bath at ambient temperature to effect dissolution. The resultant clear colorless solutions were treated with 20 μl of a 1 M DTT solution in alkylation buffer prepared just prior to use, and agitated at 37°C for 1hr. At the end of this period the fully reduced proteins were treated as indicated below.

1. Pseudo-lysine (ϕK-NH2): to the reduced histone was added 100 μl of a 1 M 2-chloroethylamine monohydrochloride solution in alkylation buffer prepared just prior to use. The mixture was agitated in the dark at 45°C for 2.5 hr. At the end of this period the mixture was treated with a second portion of DTT (10 μl of the above 1 M solution) and heated with agitation at 45°C for an additional 2.5 hr. At reaction was then quenched with BME (50 μl) and cooled to room temperature prior to purification as outlined below.
2. Pseudo-monomethyl lysine (ϕK-Me1): to the reduced histone was added 100 μl of0.

prepared just prior to use. The mixture was agitated in the dark at 50°C for 2.5 hr. At the end of this period the mixture was treated with a second portion of DTT (10 μl of the above 1 M solution) and heated with agitation at 50°C for an additional 2.5 hr. At reaction was then quenched with BME (50 μl) and cooled to room temperature prior to purification as outlined below.

Purification Scheme—A PD-10 column was pre-equilibrated with 0.1% BME in 18 water. This was loaded with the reaction mixture, the reaction tube was rinsed with an additional 1 ml of alkylation buffer and this was also added to the top of the column. The proteins were then eluted according to the manufacturers protocol for centrifugal isolation. The eluent was frozen and lyophilized prior providing the modified histones as crispy foams. A portion of each (~0.1 mg) was analyzed by RP-HPLC and MALDI-TOF mass spectrometry to insure product identity and homogeneity.

Chromatin and interaction experiment

Histone H3 variants with the respective point mutations (Lys to Cys at the position to be modified and Cys to Ala at position 110) were expressed in *E. coli*, purified from inclusion bodies, and solubilized in 7 M guanidine hydrochloride, 20 mM Tris pH 8, 10 mM DTT. After dialysis to replace guanidine hydrochloride with 7 M urea, histones were further purified by sequential anion and cation chromatography. Histone-containing fractions were pooled, dialyzed against 5 mM beta-mercaptoethanol, and lyophilized. Histones were reconstituted into octamer as previously described[37] and chromatin was formed by salt dialysis. To prevent unspecific binding to free histone in the pull-down experiment, chromatin was further purified on an agarose2 column. For interaction, 2 ug of chromatin was incubated with 2 ug of protein or complex of interest in Buffer A (50mM Tris pH8.0, 50mM NaCl, EDTA 1mM, NP40 0.1%) for 2 hours at 4¼C in the presence of NiNta beads (Eed) or M2-Beads (PRC2). Beads were extensively washed, eluted with 1X SDS-Page loading buffer and analyzed by western blot.

HKMT assay

HKMT assays were performed as previously described[38]. For autoradiography exposure, the conditions were as followed: 1.5 ug of chromatin, 100 ng of reconstituted PRC2 complex, peptide (5–40 μM), ^{3}H-SAM (0.3 μM) was used. For scintillation counting, the assay was performed as followed: 1.5 ug of chromatin, 50 ng of reconstituted PRC2 complex, peptide (100 μM), SAM (24.8 μM, ^{3}H-SAM/SAM 1/30 ratio) and 15 min, unless otherwise stated in the figure legend.

SF9 culture, infection and complex purification

As previously described[38].

Antibodies

H3K27me1 (Millipore), H3K27me2 (Abcam ab24684 and ab6002), H3K27me2 and H3K27me3 (Gift from Thomas Jenuwein), total H3 (Abcam, ab1791), Flag (Sigma), Myc 9E10 (chemicon). Previously described antibodies were used for Eed[38] and E(Z)[31].

Fly strains and mutants

The *Df(1)y*^{1} *w*^{67c23} strain (yw) was used as wild type control and for P-mediated germ-line transformation. For transgene insertion at specific genomic sites, we used fly strains in which the •C31 integrase gene is inserted on the X chromosome and attP landing sites are located in 68E or 86Fb, gifts from K. Basler[39]. Mutant strains for *esc*^{6} and *esc*^{6}, *escl*^{d01514} were used as described previously[15] and detailed crossing schemes to test transgene function

are given in Supplementary Figures S9 and S10. ChIP with larval tissues were done using flies homozygous for esc>Myc-ESC in homozygous *esc*^{6}, *esc*^{d01514} background or flies homozygous for esc>Myc-ESC Phe77Ala in homozygous *esc*^{6}, *esc*^{d01514} (selected using a *CyO*, GFP balancer).

Transposon construction

ESC mutant transgenes were produced using the *esc*>Myc-ESC construct[15] as starting material. The esc>Myc-ESC Phe77Ala and esc>Myc-ESC Tyr338Ala constructs for conventional P-mediated germ-line transformation were assembled in the pCaS-*escp* construct[15]. To generate transgenic lines for esc>Myc-ESC or esc>Myc-ESC Phe345Ala at the same chromosomal locations, we utilized the •C31 recombinase-mediated cassette exchange technique (RMCE)[40]. For recombinase-mediated cassette exchange, pCaSpeRattB plasmid was first generated by excising with *BamH*I the UAS and *hsp70* minimal promoter

frozen in liquid N2 and stored at −80°C. All steps for immunoprecipitation were performed at 4°C. An aliquot of sonicated chromatin was first precleared by mixing with Protein G Sepharose beads (GE helthcare) or Protein A Sepharose beads (Sigma) for 1 hr. After centrifugation, precleared chromatin was incubated with anti-Myc9E10 (Chemicon), anti-EZ or anti-H3K27me3 (Abcam, ab6002) overnight. Protein G or Protein A Sepharose beads were then added to allow binding to the antibody for 2 hr and then washed 5 times with RIPA buffer, once with LiCl buffer (10 mM Tris-Cl (pH 8.0), 250 mM LiCl, 0.5% NP-40, 0.5% Sodium deoxycholate and 1 mM EDTA) and twice with TE. The beads were resuspended in 100 μl of TE, and treated with 0.1 mg/ml RNase A at 37°C for 30 min. After supplementing with SDS (0.5% final), the beads were treated with 0.5 mg/ml Proteinase K at 37°C overnight and subsequently at 65°C for 6 hr. The immunoprecipitated DNA was recovered by phenol-chloroform extraction and ethanol precipitation, and then dissolved in H_2O. Control mock immunoprecipitations were done in the same way except that no antibodies were added to the reaction mixture. Real-time PCR quantification of immunoprecipitated DNA was performed as previously described[31]. The input DNA extracted from the same sonicated chromatin aliquots as above was used to plot a standard curve. Primers were as follow: for *bxd* PRE (FM4 and FM6), FM4.1, 5′-AGCAATTTGTCACCGCAAGG-3′, FM4.2, 5′-GGATTTTGAGTGCGTTCTTCC-3′, FM6.1, 5′-CCAACGGAAAAGCGAGTGG-3′, and FM6.2, 5′-GCACTAAACCCCATAAAAGTC-3′; for PBX enhancer, PBX-enh-5′, GAAAACACACAAGTGCAG-3′ and PBX-enh-3′, 5′-GGAGATCCTAAAACATGC-3′; for UBX promoter, U-up1.1, 5′-ATTCGCGAGATACCAATGCC-3′ and U-up1.2, 5′-ATTCGCGAGATACCAATGCC-3′; for *white* locus, W2.1, 5′-ATGCCACGACATCTGACC-3′ and w2.3, 5′-AATGCCAGACGCTTCCTTTC-3′. The quantity obtained by real-time PCR was corrected to obtain the percentage of input.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome Regulation by Polycomb and Trithorax Proteins. Cell. 2007; 128:735–745. [PubMed: 17320510]
2. Czermin B, et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell. 2002; 111:185–96. [PubMed: 12408863]
3. Cao R, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science. 2002; 298:1039–43. [PubMed: 12351676]
4. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. 2002; 16:2893–905. [PubMed: 12435631]
5. Muller J, et al. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell. 2002; 111:197–208. [PubMed: 12408864]

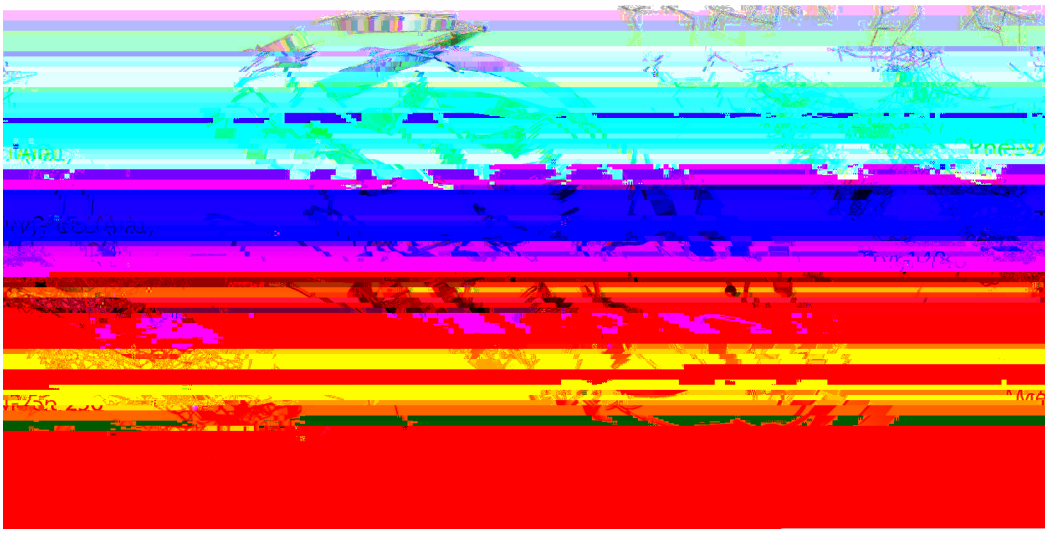

Figure 1. Trimethyl-lysine binding to an aromatic cage on Eed

Ribbons representation of the Eed/H3K27me3 complex where Eed is coloured grey and the histone peptide is coloured yellow with its methyl-lysine side chain shown in stick representation. The Cα positions of the aromatic cage are shown as blue circles, and the Cα position of tyrosine 358 by a red circle. The bottom panel shows the methyl-lysine binding site with 2fo-fc electron density for the four cage residues and the H3K27me3 peptide. Designed mutations to the cage are shown in red in parentheses. The side-chain of methionine 256 is also shown; this is equivalent to Met-236 in esc which has been identified from classical genetic screens in Drosophila as essential for the function of Eed.

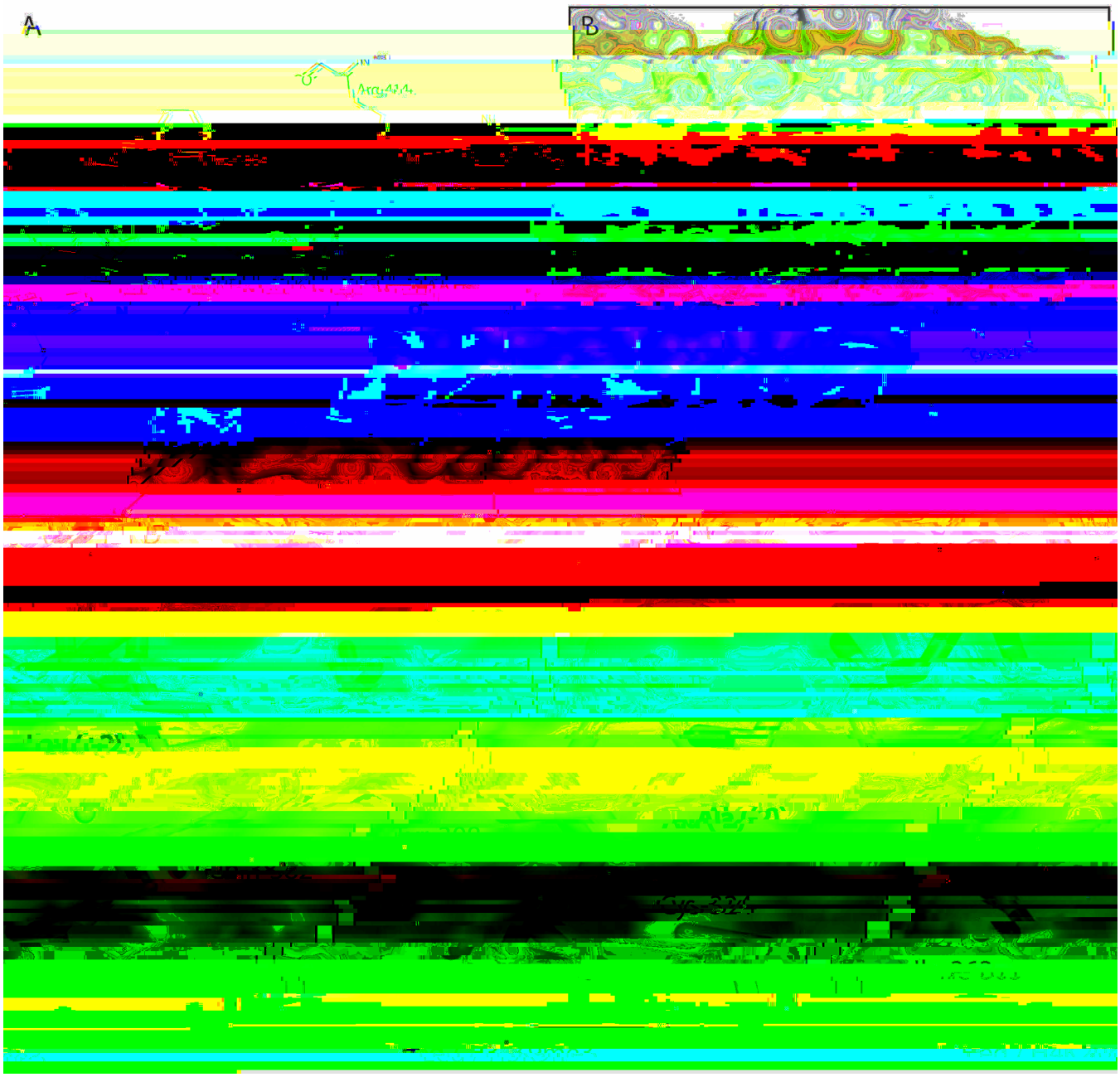

Figure 2. Interactions between Eed and trimethylated histone peptides

(A) Schematic representation of the interaction between Eed and H3K27me3. For clarity, the aromatic methyl-lysine binding cage has been omitted and the methylated lysine side-chain shown as a yellow circle. Hydrogen bonds from the main-chain carbonyl of the methyl-lysine, and the residue immediately N-terminal to it, with Eed are shown as dashed lines. The green hatching indicates the hydrophobic pocket on EED which accommodates the alanine side chain two residues before the methyl-lysine. **(B)** Eed is shown in surface representation with a composite of two of its cognate peptides shown in sticks representation and coloured yellow (H3K9me3) and pink (H4K20me3). (**C)** shows the pocket on Eed that accommodates Ala (−2) from the H3K9me3 peptide while (**D)** shows the other pocket that

contains and Leu (+2) from the H4K20me3 peptide. The Eed surface is coloured according to atom type.

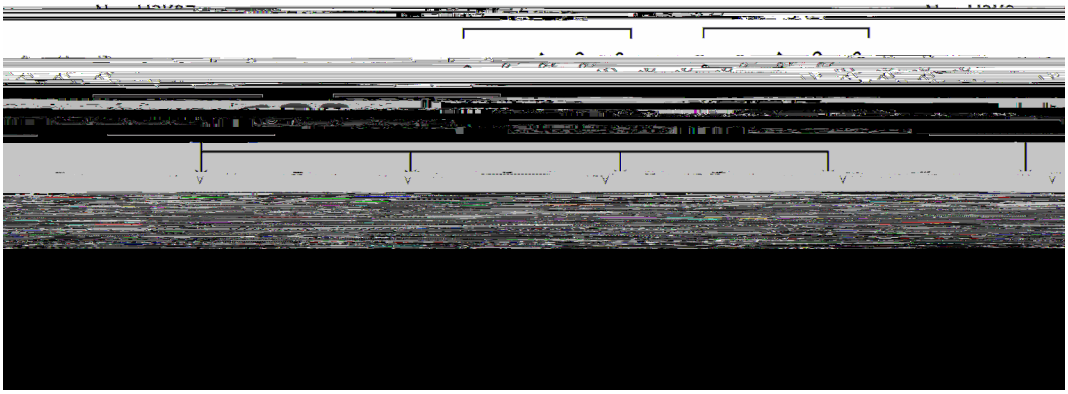

Figure 3. Eed and PRC2 interaction with Chromatin

Pull-down experiment to analyze the interaction between Eed full-length, PRC2-Ezh2 wild type or reconstituted with Eed Tyr-365Ala and H3K27 modified chromatin (left) or between PRC2-Ezh2 wild type and H3K9 modified chromatin (right). Note that “beads only” control for interaction H3K9 modified chromatin is not shown but was identical to the control shown in the figure.

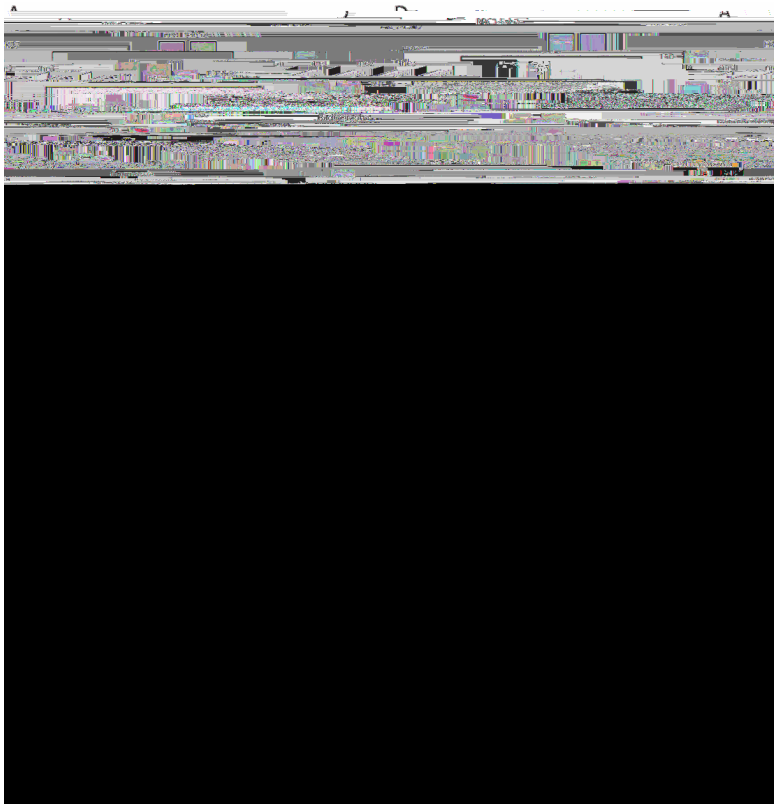

Figure 4. Peptide mimicking repressive marks stimulates PRC2 activity

(A) Left, coomassie blue staining of reconstituted PRC2-Ezh2 complex. Right, HMT assay with PRC2-Ezh2 alone or in the presence of 10 and 40 μM H3K27, unmodified, mono, di or tri methylated peptides. **(B)** Titration of the methyl donor (S-adenosyl-Methionine) in the presence of H3K27me0/1/2/3 peptides (**C**) Nucleosome titration in the presence of H3K27me0/3 peptides. (**D**) Left, Coomassie blue staining of reconstituted PRC2-Ezh2 Tyr365Ala complex and right, HMT assay with the corresponding complex in the same condition as (**A**) **(E)** Relative PRC2 histone methyltransferase activity in the presence of various peptides as indicated in the legend. (**F**) Table indicating the peptides used for the stimulation study as well as their Kd values (μM) for ΔEed binding (**G**) Relative PRC2 histone methyltransferase activity in the presence of various peptides as indicated in the legend.

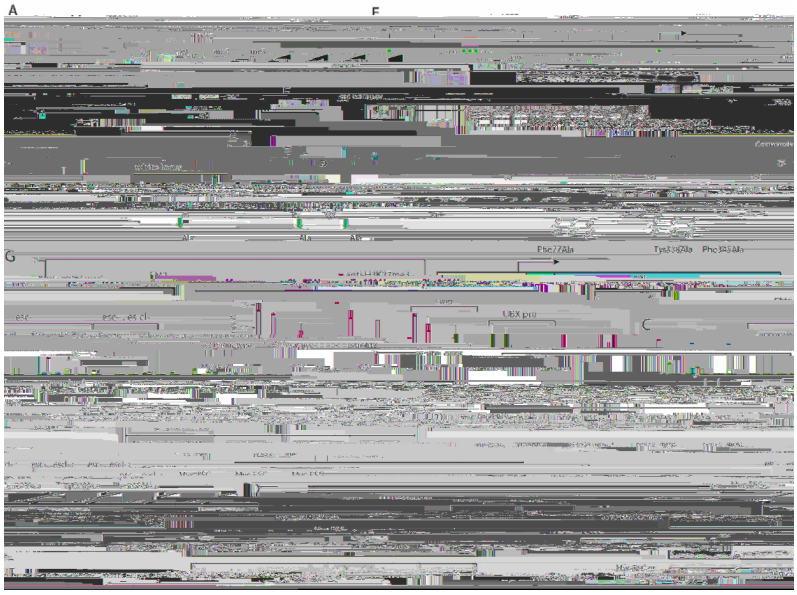

Figure 5. The aromatic cage in *Drosophila* ESC is important for its *in vivo* function
(A) Left, Coomassie blue staining of reconstituted dPRC2-Ez complex. Right, HMT assay with dPRC2-Ez and H3K27, unmodified, mono, di or tri methylated peptides. **(B)** Top**,** amino acid residues Phe-77, Tyr-338 and Phe-345 that were mutated to alanine in *Drosophila* ESC and corresponding residues in Eed. Bottom, schematic representation of transposon constructs. The Myc-tagged ESC or its mutants are expressed under the control of the *esc* gene promoter. **(C)** Rescue experiment. Details of the crossing schemes are shown in Supplementary Figures S9 and S10. Several independent lines were examined for each transgenic construct and showed the same phenotype except for one line of Myc-ESC Tyr338Ala. In the case of Myc-ESC Phe345Ala, transgenes were inserted at •C31 *att* sites at 68E and 86Fb, respectively. For direct comparison, wild type Myc-ESC lines were also established at the same chromosomal location and showed the same results as other wild type Myc-ESC lines established by conventional P-element transformation **(D)** ESC aromatic cage mutation does not impair binding to E(Z). Both Myc-ESC and Myc-ESC Phe-77Ala co-immunoprecipitated E(Z) from ovarian extracts of heterozygous esc^{-} $escl^{-}$ flies. The double Myc-ESC bands are caused by the well known phosphorylation of ESC. **(E)** Scheme indicating the genomic location of primers used for ChIP. **(F)** ChIP analysis of E(Z) binding to the *bxd* PRE (FM4) in homozygous esc^{6}, $escl^{d01514}$ carrying the same Myc-ESC transgenes. In the presence of the aromatic cage mutations, E(Z) binding is strongly reduced. *yw* indicates the wild type stock with endogenous wild type ESC and ESCL. **(G)** ChIP analysis of the H3K27me3 distribution at four sites in the *Ubx* gene. H3K27me3 is drastically reduced in the presence of the aromatic cage mutations. **(H)** Histone H3K27 methylation in esc^{6}, $escl^{d01514}$ double mutant larvae expressing Myc-ESC transgenes. Total protein lysates from wild type and homozygous esc^{6}, $escl^{d01514}$ 3rd instar larvae expressing Myc-ESC transgenes were used for western blot analysis with anti-H3, anti-H3K27me2 and anti-H3K27me3 antibodies. The aromatic cage mutations Myc-ESC Phe77Ala and Myc-ESC Phe345Ala cause an almost complete loss of histone H3K27 di- and trimethylation while wild type Myc-ESC fully restores the loss of H3K27 di- and tri-methylation in esc^{6}, $escl^{d01514}$ double mutant larvae.