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Histone Methyltransferase Activity of a *Drosophila* Polycomb Group Repressor Complex

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Summary

Polycomb group (PcG) proteins maintain transcriptional repression during development, likely by creating repressive chromatin states. The Extra Sex Combs (ESC) and Enhancer of Zeste [E(Z)] proteins are partners in an essential PcG complex, but its full composition and biochemical activities are not known. A SET domain in E(Z) suggests this complex might methylate histones. We purified an ESC-E(Z) complex from *Drosophila* embryos and found four major subunits: ESC, E(Z), NURF-55, and the PcG repressor, SU(Z)12. A recombinant complex reconstituted from these four subunits methylates lysine-27 of histone H3. Mutations in the E(Z) SET domain disrupt methyltransferase activity in vitro and HOX gene repression in vivo. These results identify E(Z) as a PcG protein with enzymatic activity and implicate histone methylation in PcG-mediated silencing.

Introduction

The Polycomb (PcG) and trithorax group (trxG) proteins of *Drosophila* provide a paradigm for understanding how heritable transcriptional states are maintained during development (Francis and Kingston, 2001; Simon and Tamkun, 2002). These two sets of proteins provide long-term memory of gene activity states after initial on/off decisions are made by transiently acting transcription factors. In general, PcG proteins maintain gene silencing while trxG proteins maintain active states. Together, PcG and trxG proteins maintain expression patterns of HOX genes and other developmental control genes, which are critical for normal embryonic and post-embryonic pattern formation. PcG and trxG proteins and the complexes they form are highly conserved in mammals, where they are needed for embryonic and hematopoietic development and are also implicated in oncogenic mechanisms (Jacobs and van Lohuizen, 2002).

PcG and trxG proteins are thought to provide memory of transcriptional activity states through alterations of chromatin structure. Several trxG proteins are components of the *Drosophila* SWI/SNF nucleosome-remodeling complex (Kal et al., 2000; Papoulas et al., 1998) and a Trithorax-containing complex has histone acetyltransferase activity (Petruk et al., 2001). Thus, specific chromatin-modifying enzyme complexes are implicated in trxG maintenance of active states. In contrast, mechanisms by which PcG proteins affect chromatin structure are less well understood. In particular, enzymatic activities have not been identified for PcG proteins. Furthermore, little is known about how PcG- or trxG-mediated chromatin states are propagated through DNA replication and mitosis. Characterization of PcG and trxG complexes and mechanistic studies of their activities are essential to understanding these epigenetic mechanisms.

Two biochemically distinct types of PcG protein complexes have been characterized in *Drosophila*: Polycomb repressive complex 1 (PRC1) and the ESC-E(Z) complex. PRC1 contains the PcG proteins Polycomb (PC), Polyhomeotic (PH), Posterior Sex Combs (PSC), and dRING1 as well as additional polypeptides (Saurin et al., 2001; Shao et al., 1999). Biochemical studies show that PRC1 or PCC, a reconstituted complex containing these four PcG subunits, can block chromatin remodeling by human SWI/SNF on nucleosomal templates (Francis et al., 2001; Shao et al., 1999). Binding studies suggest that the remodeling block results from PRC1-nucleosome interactions that occlude SWI/SNF access (Francis et al., 2001). These in vitro experiments, together with the opposing role of fly SWI/SNF components as HOX gene activators in vivo, suggest that PRC1 may repress through a noncatalytic mechanism by restricting access to target gene chromatin.

Initial characterization of the ESC-E(Z) embryonic complex showed that it contains the PcG proteins Extra Sex Combs (ESC) and Enhancer of Zeste [E(Z)] as well as NURF-55, a protein implicated in histone binding (Ng et al., 2000; Tie et al., 2001). NURF-55 is the fly homolog of RbAp48, which copurifies with a human histone deacetylase (HDAC; Taunton et al., 1996). This connection, together with physical interactions between ESC, E(Z), NURF-55, and fly HDAC1 have led to the suggestion that the *Drosophila* ESC-E(Z) complex might function as a histone deacetylase (Tie et al., 2001). However, to

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date, HDAC activity has not been reported for the purified fly ESC-E(Z) complex.

The presence of a SET domain in the E(Z) protein (Jones and Gelbart, 1993) suggests that the ESC-E(Z) complex might have histone methyltransferase activity. This homology domain, originally recognized in the *Drosophila* Su(var)3-9, E(Z), and Trithorax proteins (Jones and Gelbart, 1993; Tschiersch et al., 1994), is a feature of many eukaryotic chromatin proteins (reviewed in Lachner and Jenuwein, 2002). Pioneering work on the SET domain of human SUV39H protein showed that this domain has histone methyltransferase (HMTase) activity with specificity for lysine 9 (K9) in the histone H3 tail (Rea et al., 2000). This H3-K9 modification has since been implicated in targeting of heterochromatin proteins (Bannister et al., 2001; Lachner et al., 2001). Subsequently, studies on several other SET domain proteins showed that these also possess HMTase activities with different lysine residue specificities (e.g., Nakayama et al., 2001; Nishioka et al., 2002; Strahl et al., 2002; Wang et al., 2001a), although the biological roles of only a few of these are known (e.g., Nakayama et al., 2001).

We purified an embryonic ESC-E(Z) complex and find that it contains four core subunits: ESC, E(Z), NURF-55, and the PcG protein, SU(Z)12. A complex reconstituted from these four proteins has H3-K27-directed HMTase activity. Residues in the E(Z) SET domain are required for HMTase activity in vitro and HOX gene repression in vivo. These results identify a catalytic activity of a PcG complex and imply that histone methylation is part of the mechanism of PcG transcriptional memory during development.

Results

A Purified Embryonic Complex Contains ESC, E(Z), NURF-55, and SU(Z)12

Gel filtration chromatography detects the bulk of E(Z) protein associated with ESC in an embryonic complex that migrates at 600 kDa (Ng et al., 2000; Tie et al., 2001). To define its subunit composition and to study activities of the complex, we affinity-purified ESC-containing complexes from transgenic embryos expressing a FLAG-tagged ESC protein (Figure 1). This FLAG-ESC protein is expressed as a polypeptide of the expected size, approximately 50 kDa, and it rescues *esc* mutants (see Experimental Procedures). Embryonic nuclear proteins were fractionated by QAE-Sepharose ion exchange chromatography and fractions with peak levels of FLAG-ESC and E(Z) were pooled and subjected to immunoaffinity chromatography with an anti-FLAG (M2) resin. Purifications were performed in parallel from a FLAG-ESC transformant and from the nontransformant parental line *(y Dfw^{67c2})* as negative control. Western blots on fractions following immunoaffinity chromatography confirmed that FLAG-ESC and E(Z) are coenriched by this procedure (Figure 1B, column "E"). In contrast, boundary element associated factor (BEAF), a chromatin protein with no known connection to PcG proteins (Zhao et al., 1995), is not enriched in the FLAG-peptide eluted material.

Major bands migrating at 105, 90, 55, and 50 kDa were specific to FLAG-ESC affinity-purified fractions (Figure 1C). Mass spectrometry analysis identified these four proteins as SU(Z)12, E(Z), NURF-55, and ESC. SU(Z)12 is the product of a recently discovered PcG gene (Birve et al., 2001) and was not previously linked to PcG complexes. Thus, our analysis indicates the ESC-E(Z) complex from embryos consists of three proteins previously identified by coimmunoprecipitation, cofractionation, and purification studies, ESC, E(Z), NURF-55 (Jones et al., 1998; Ng et al., 2000; Tie et al., 2001), plus SU(Z)12. We cannot rule out the presence of additional proteins that do not stain efficiently with silver. The complex from nuclear extracts and from affinity-purified fractions migrates similarly on glycerol gradients (data not shown), suggesting that its composition is preserved through the purification.

Characterization of SU(Z)12 Association

Because SU(Z)12 represents a novel component of the ESC-E(Z) complex, we wished to independently verify its association. Polyclonal antisera against full-length (FL antibody) or amino acids 448–802 of SU(Z)12 (SAC antibody) were generated and affinity-purified. The antibodies were used to track SU(Z)12 enrichment during purification and to verify its presence in the embryonic complex (Figure 1B).

If SU(Z)12 is tightly associated with the ESC-E(Z) complex, we would expect coimmunoprecipitation and cofractionation with these partner components in crude nuclear extracts. Indeed, coimmunoprecipitation of SU(Z)12, E(Z), and FLAG-ESC, but not BEAF, is observed using either of the anti-SU(Z)12 antibodies or anti-E(Z) antibody (Figure 2A). Gel filtration chromatography shows that the bulk of embryonic SU(Z)12 cofractionates with E(Z) in a peak of about 600 kDa (Figure 2B), which corresponds to the gel filtration behavior previously determined for the ESC-E(Z) complex (Ng et al., 2000; Tie et al., 2001). Taken together, these results validate SU(Z)12-E(Z)-ESC association and suggest SU(Z)12 is a core component of the ESC-E(Z) complex. In agreement with this central role, *Su(z)12* loss-of-function mutations produce embryonic phenotypes that are as severe as loss-of-function for *esc* or *E(Z)* (Birve et al., 2001; Jones and Gelbart, 1990; Struhl, 1981).

Tests for HDAC1 Association

Recent studies suggest links between histone deacetylase 1 [HDAC1, also called RPD3 (De Rubertis et al., 1996)] and PcG repressors in *Drosophila* (Chang et al., 2001; Poux et al., 2001; Tie et al., 2001), including physical interactions among ESC, E(Z), NURF-55, and HDAC1 (Tie et al., 2001). We therefore tested whether HDAC1 cofractionates with the ESC-E(Z) complex in our procedure. HDAC1 is readily detected in the input material but, unlike ESC, E(Z), and SU(Z)12, is not enriched in FLAG-ESC affinity fractions when compared to wild-type (Figure 1B). In addition, mass spectrometric analysis of a gel slice encompassing the 68 kDa region [the gel migration position for embryonic HDAC1 (Chen et al., 1999)] from a FLAG-ESC purified sample failed to identify peptides corresponding to HDAC1. Since it is possible that our purification conditions do not preserve HDAC1 association, we also performed coimmunoprecipitation tests on unpurified nuclear extracts. HDAC1

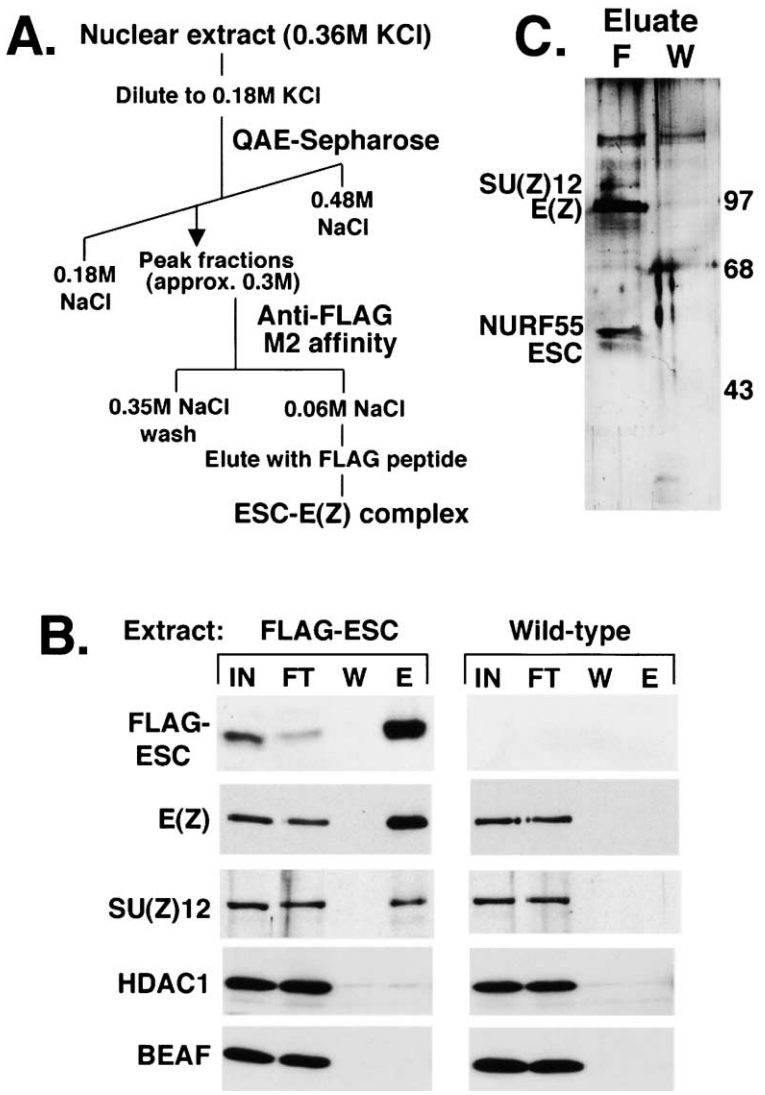


Figure 1. Purification of ESC-E(Z) Complex from Fly Embryos

(A) Purification scheme from transgenic embryos expressing FLAG-ESC.

(B) Western blots to test enrichment of indicated proteins during the final anti-FLAG M2 affinity step. Left image shows samples from FLAG-ESC line and right image shows samples from nontransgenic control. IN, input from peak Q-Sepharose fractions; FT, flowthrough; W, final wash; E, material eluted with FLAG peptide.

(C) Proteins in purified samples from FLAG-ESC (F) or wild-type control (W) detected by silver staining. The four indicated proteins, present in F and absent in W, were identified by mass spectrometry.

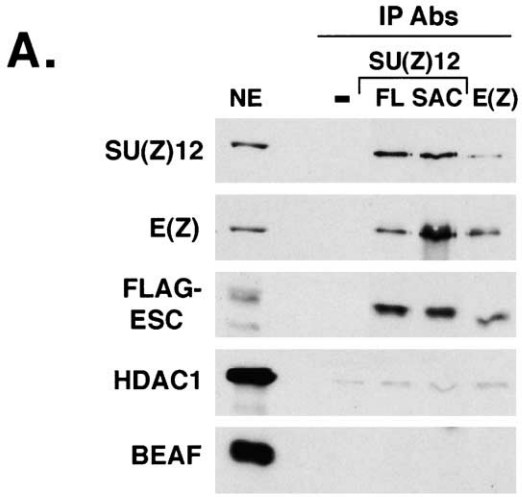
signals in the anti-SU(Z)12 and anti-E(Z) immunoprecipated samples (Figure 2A) are not significantly elevated over the mock-precipitated control. Thus, we do not observe a robust interaction between components of the ESC-E(Z) complex and HDAC1.

Reconstituted ESC-E(Z) Complex Has Histone Methyltransferase Activity

To determine whether ESC, E(Z), NURF-55, and SU(Z)12 form a stable complex and whether this complex has HMTase activity, we reconstituted the complex using recombinant proteins expressed in Sf9 cells from baculoviruses. When FLAG-ESC, E(Z), NURF-55, and SU(Z)12 were coexpressed, a stable complex containing each subunit could be isolated by FLAG affinity-purification (Figure 3A). This complex was stable in buffers containing up to 2 M KCl. Figure 3B shows that this recombinant ESC-E(Z) complex [rESC-E(Z)] can methylate histone H3 using either polynucleosomes or free histones as a substrate; the complex is at least four times more active on nucleosomes than free histones. Under the conditions used, the preparations transferred the methyl group from approximately one molecule of SAM to histone H3 for each complex in the reaction. To verify that the HMTase activity is derived from the ESC-E(Z) complex, we further purified the recombinant complex by glycerol gradient sedimentation. The peak of HMTase activity coeluted with the ESC-E(Z) complex (Figure 3C) and the specific activities are similar in gradient-purified fractions versus the affinity-purified gradient input material. We conclude that the rESC-E(Z) complex has HMTase activity specific for histone H3. A preparation of ESC-E(Z) complex isolated from embryos also had H3-directed MTase activity (data not shown), consistent with the function of the recombinant complex.

In addition to H3, we note that E(Z) and SU(Z)12 were also methylated in our reactions (Figure 3D). This methylation activity is stimulated by histones or chromatin and the levels of E(Z) and SU(Z)12 methylation correlate well with the level of histone methylation in the reaction. Thus, like other enzymes that covalently modify histones (e.g., Imhof et al., 1997), rESC-E(Z) can also methylate other proteins.

We also purified FLAG-E(Z) as an isolated protein and



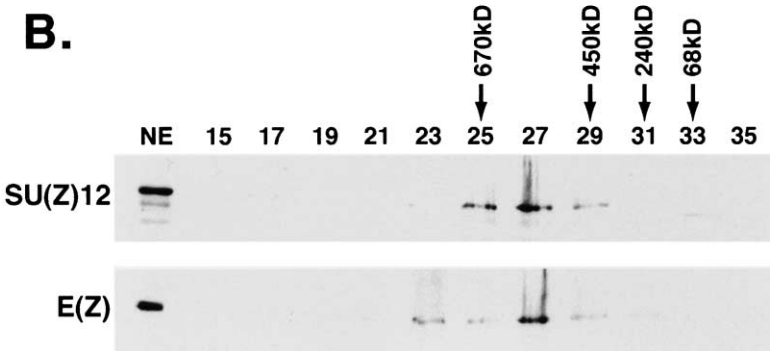


Figure 2. Tests for Proteins Associated with ESC-E(Z) Complex

(A) Coimmunoprecipitations on embryo nuclear extracts. Western blots to detect indicated proteins in immunoprecipitates obtained with the antibodies indicated at top. "−" indicates mock-precipitation lacking antibody.

(B) Superose 6 gel filtration analysis on embryo nuclear extracts. NE, nuclear extract. Fraction numbers are indicated at top and elution positions of molecular mass standards are indicated by arrows.

tested it for HMTase activity. The FLAG-E(Z) fraction methylates histone H3 (Figure 3E), but titrations of rESC-E(Z) versus FLAG-E(Z) alone show that the specific activity of our FLAG-E(Z) preparation is at least a thousand fold lower than that of the complex on a chromatin substrate (Figure 3E, top). FLAG-E(Z) is about four times more active on free histones than chromatin (Figure 3E), so that the difference in specific activity between FLAG-E(Z) and rESC-E(Z) is about 60-fold on a histone substrate. We cannot rule out the possibility that the very low activity of FLAG-E(Z) fractions could be due to improper folding of the overexpressed protein or trace contaminants. Since E(Z) production using the same system with its partners yields highly active preparations (Figure 3B), we favor the idea that assembly with other subunits of the ESC-E(Z) complex somehow enhances HMTase activity of E(Z). The fact that intact complex prefers arrays as substrate, while isolated E(Z) prefers free histones, also supports this idea.

Four lysines have been demonstrated to be sites of H3 methylation in vivo: K4, K9, K27, and K36 (reviewed in Zhang and Reinberg, 2001). To determine which residue(s) are methylated by rESC-E(Z), we performed in vitro methylation on polynucleosomes and then used Edman degradation to identify radiolabeled residues. This analysis identified K27 as the major site of methylation by rESC-E(Z) (Figure 4A).

To independently address the lysine specificity, we performed methylation reactions on recombinant versions of H3 bearing mutations either in K9 or K27. rESC-E(Z) methylates wild-type and K9A mutant histone H3 with similar efficiency but mutation of K27 to A decreases methylation by at least 50-fold (Figures 4B and 4C). These results, together with the N-terminal sequencing, identify the ESC-E(Z) complex as an HMTase that uses H3-K27 as its preferred substrate.

Residues within a highly conserved (H/R)XXNHS motif are required for HMTase of other SET domain proteins (Rea et al., 2000; Strahl et al., 2002; Wang et al., 2001a). To determine whether the HMTase activity of rESC-E(Z) depends on the E(Z) SET domain, we constructed point mutations predicted to disrupt activity. Thus, E(Z) proteins bearing missense mutations in R699 or H703 (underlined residues) were assembled into recombinant complexes and assayed for activity. Figure 5 shows that these mutant proteins form stable complexes but that the HMTase activity is disrupted by all four mutations tested. Methylation of SU(Z)12 and E(Z) was also disrupted by these mutations. We conclude that the HMTase activity of the ESC-E(Z) complex is mediated by the SET domain of E(Z).

Mutations that Impair E(Z) HMTase Disrupt HOX Gene Silencing In Vivo

If HMTase activity of the ESC-E(Z) complex is required for transcriptional repression, then mutations that inacti-

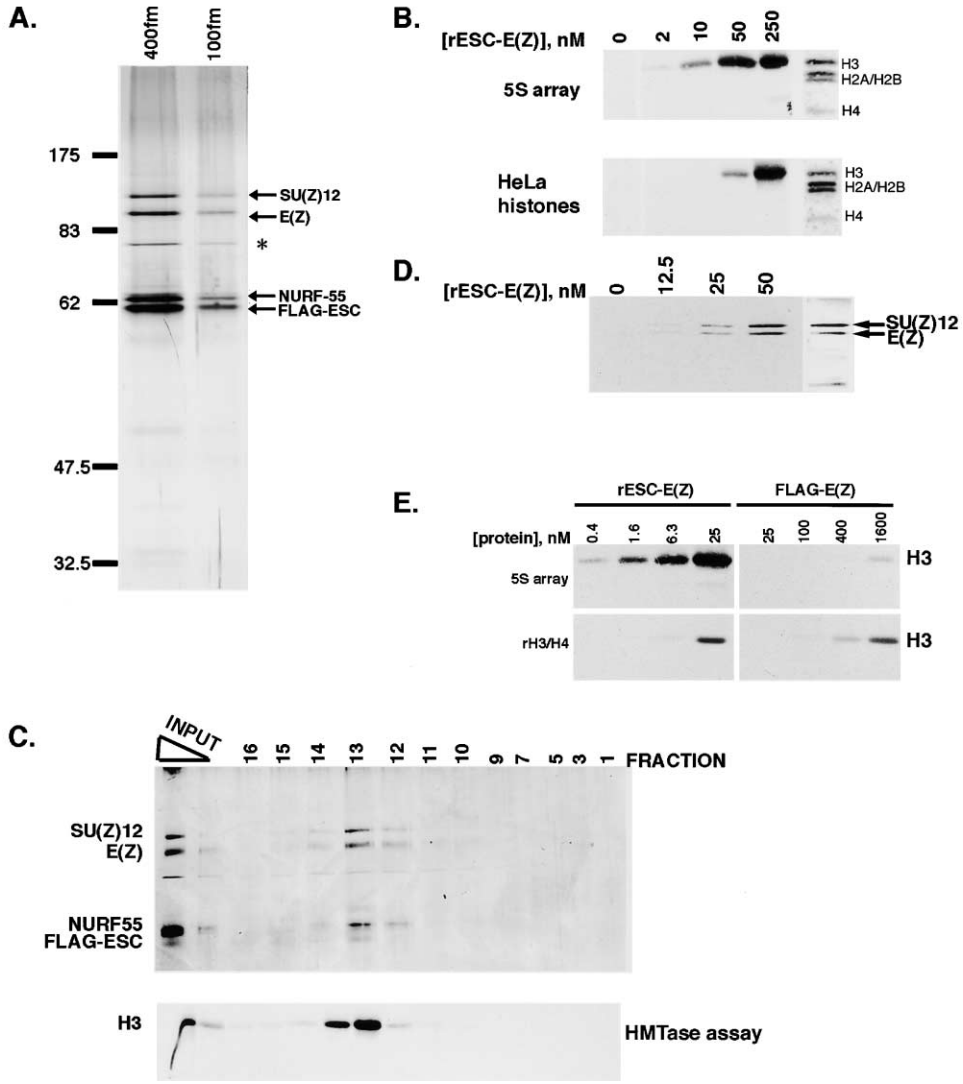


Figure 3. Reconstituted ESC-E(Z) Complex Has Methyltransferase Activity

(A) rESC-E(Z) affinity-purified via FLAG-ESC contains SU(Z)12, E(Z), NURF-55, and FLAG-ESC. 100 or 400 fm of complex were resolved by 10% SDS-PAGE and stained with silver. Asterisk indicates a contaminant that is likely to be HSP70.

(B) rESC-E(Z) has methyltransferase activity. In the presence of a nucleosomal array (upper image) or free histone (lower image) substrate, rESC-E(Z) methylates histone H3. Right-hand lane in both images shows amido black stain of MTase reactions. Substrate concentration in both images is ~250 nM nucleosomes or histone octamers (~500 nM H3).

(C) Histone methyltransferase activity cofractionates with rESC-E(Z). 50 ug of affinity purified rESC-E(Z) were separated on a 10%–35% glycerol gradient. Fractions were assayed for HMTase activity on a nucleosomal array substrate at 134 nM nucleosomes (lower image); corresponding fractions were resolved by 10% SDS-PAGE and stained with silver (upper image). Input lanes are 12 and 3 nM rESC-E(Z) in the HMTase assay.

(D) Methylation of SU(Z)12 and E(Z). MTase reactions in the presence of a nucleosomal array substrate (160 nM nucleosomes) were resolved on a 10% gel; right-hand image is amido black stain of the blot at the highest protein concentration.

(E) FLAG-E(Z) has low HMTase activity. FLAG-E(Z) or rESC-E(Z) were titrated into HMTase reactions using a 5S array substrate (160 nM nucleosomes) or *Drosophila* recombinant H3/H4 substrate (~770 nM H3).

vate the HMTase should disrupt silencing in vivo. However, *E(z)* mutant alleles that disrupt the catalytic core of the SET domain have not been described. We therefore used a transgene rescue assay to test if the H703K and R699H mutations, which inactivate E(Z) HMTase in vitro (Figure 5), could repress the HOX gene *Ubx* in vivo. We have previously shown that *Ubx*, which is normally kept silent in wing imaginal discs, is derepressed in wing disc cell clones that are homozygous for PcG mutations (Beuchle et al., 2001).

As a first step, we analyzed clones of *E(z)* mutant cells for loss of HOX gene silencing in imaginal discs. For this analysis, four recently identified *E(z)* loss-of-function alleles, $E(z)^{731}$, $E(z)^{328}$, $E(z)^{1025}$, and $E(z)^{2434}$, were used (see Experimental Procedures). Since all four alleles gave comparable results, we focus here on results obtained with $E(z)^{731}$, an allele that produces no detectable E(Z) protein (Figure 6C). We generated clones of $E(z)^{731}$ homozygous cells and assayed for loss of HOX gene silencing by immunostaining wing discs for UBX and

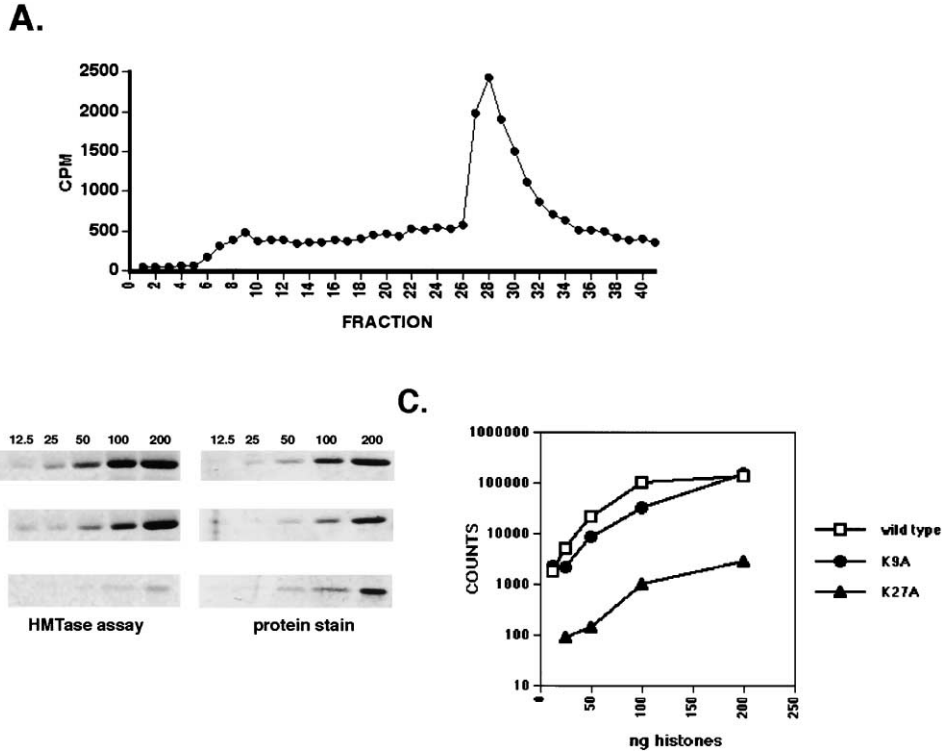


Figure 4. rESC-E(Z) Methylates Lysine 27 of H3

(A) N-terminal sequencing of rESC-E(Z) methylated chromatin indicates that methylation occurs on lysine 27. CPM from each cycle of Edman degradation are plotted against fraction (cycle) number. The major increase in CPM released occurs at residue 27; the CPM released at cycle 28 and beyond are likely due to accumulation of partially cleaved products over many cycles (see Experimental Procedures).

(B) Mutation of H3-K27 disrupts methylation by rESC-E(Z). rESC-E(Z) (125 nM) was tested for HMTase on wild-type, K9→A, or K27→A histone H3/H4 preparations in 10 μl reactions.

(C) Graph of phosphorimager analysis of histone titrations; experiment is representative of titrations carried out with four preparations of rESC-E(Z).

ABD-B. Mutant clones were identified by absence of a GFP-expressing marker gene. We found that *E(z)*^{731} mutant clones show widespread misexpression of UBX and ABD-B when assayed 96 hr after clone induction (Figure 6A). We then examined the kinetics of derepression; UBX is stably silenced until 48 hr after clone induction, when misexpression first becomes detectable in a few cells in the wing pouch (Figure 6A). ABD-B remains silenced 48 hr after clone induction and first becomes detectable at 72 hr. These results show that *E(z)* is required to maintain silencing of different HOX genes in imaginal discs.

We next tested whether a regular supply of wild-type E(Z), expressed from a heat-inducible *hsp70-E(z)* transgene, can rescue HOX gene silencing in *E(z)*^{731} mutant clones. We induced clones in larvae carrying the *hsp70-E(z)* transgene and, beginning at time of clone induction, we heat-shocked every 12 hr over a 96 hr period and then analyzed the clones for UBX. This regular supply of wild-type E(Z) completely restores HOX gene silencing; UBX is not detected in mutant clones containing the *hsp70-E(z)* transgene (Figure 6B, second image), whereas imaginal discs from similarly treated control larvae lacking the transgene show strong UBX misexpression (Figure 6B, top).

We then generated *hsp70-E(z)*^{H703K} and *hsp70-E(z)*^{R699H} transgenes to analyze their rescuing capacity in the same assay. Both the E(Z)^{H703K} and the E(Z)^{R699H} proteins incorporate into stable ESC-E(Z) complexes which show little or no HMTase activity (see Figure 5). The *hsp70-E(z)*^{H703K} and *hsp70-E(z)*^{R699H} transgenes were introduced into the *E(z)*^{731} mutant background and tested as described above for the wild-type transgene. Both mutant E(Z) proteins fail to repress in vivo; UBX misexpression in these *E(z)*^{731} clones is comparable to that of control clones from animals lacking any *hsp70-E(z)* transgene (Figure 6B). To confirm that the E(Z)^{H703K} and E(Z)^{R699} mutant proteins are expressed stably in vivo, we performed Western blots on *E(z)*^{731}/*E(z)*^{731} mutant larvae carrying either the *hsp70-E(z)*, the *hsp70-E(z)*^{H703K} , or the *hsp70-E(z)*^{R699H} transgene. E(Z) is not detected in the non-heat-shocked larvae, suggesting that stable E(Z) protein is not produced from the *E(z)*^{731} allele (Figure 6C, see also Experimental Procedures). Extracts from heat-shocked larvae show that full-length E(Z) proteins are indeed produced from each of the three transgenes at comparable levels (Figure 6C). Thus, the failure of the E(Z)^{H703K} and E(Z)^{R699H} proteins to provide HOX gene repression is likely due to their lack of HMTase activity.

Discussion

Understanding the role of PcG proteins in maintaining gene expression patterns requires understanding the

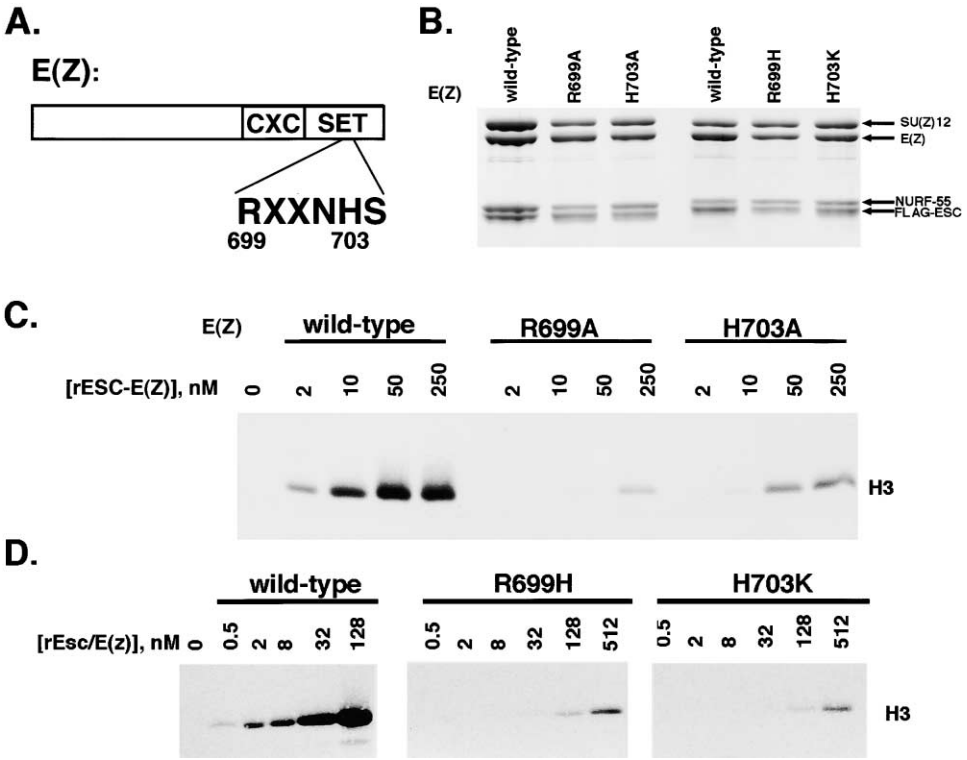


Figure 5. Conserved Residues in the E(Z) SET Domain Are Required for Methyltransferase Activity

(A) Schematic of E(Z) showing location of SET domain residues R699 and K703 that were mutated. CXC represents cysteine-rich pre-SET domain.

(B) rESC-E(Z) was prepared with E(Z) bearing one of four different mutations (R699→A, H703→A, R699→H, H703→K). 1.5 μg of each complex were separated by 10% SDS-PAGE and stained with colloidal Coomassie. The two sets of three complexes were prepared independently and are on different gels.

(C and D) SET domain mutations impair histone methyltransferase activity of rESC-E(Z). Wild-type or mutant rESC-E(Z) complexes were used in methyltransferase assays with nucleosomal array substrate (200 nM (C) or 160 nM (D) nucleosomes). Similar results were obtained with HeLa polynucleosomes or free histone substrates.

functions of PcG complexes. The following conclusions can be drawn from the work presented here: a functional methyltransferase complex can be reconstituted that contains the PcG proteins ESC, E(Z), and SU(Z)12; this complex can methylate residue K27 of histone H3; and E(Z) mutations that disrupt ability to methylate K27 also impair maintenance of HOX gene repression. These results suggest that methylation is a necessary function of the ESC-E(Z) complex and that methylated histone H3 is an epigenetic mark that helps propagate the PcG silenced state.

Histone Methyltransferase Activity of the ESC-E(Z) Complex

Our purification and reconstitution of an ESC-E(Z) complex revealed four core components: ESC, E(Z), SU(Z)12, and NURF-55. Genetic studies have shown that ESC, E(Z), and SU(Z)12 are key repressors of HOX genes in vivo (Birve et al., 2001; Jones and Gelbart, 1990; Struhl, 1981), while NURF-55 mutants have not been described, perhaps because it is found in many chromatin modifying complexes (Martinez-Balbas et al., 1998; Tyler et al., 1996). The lack of H3 methyltransferase activity in complexes containing E(Z) with SET domain mutations (Figure 5) implies that E(Z) has catalytic function in the complex. The essential roles of other subunits may be in targeting or modulating activity of the complex.

The ESC-E(Z) complex methylates H3 with primary specificity for K27 in both chromatin assembled from purified HeLa histones and in recombinant histones (Figure 4). The complex might also have H3-K9-directed activity since we consistently observe a small peak in this region following analysis by Edman degradation (Figure 4A). The low level of K9-directed methylation in these experiments could derive from a contaminating activity, although very little methylation of the K27A histone H3 mutant that has intact lysine 9 was observed (Figure 4B). It is also possible that only a subpopulation of HeLa histones, such as those bearing preexisting histone modifications, can be methylated at H3-K9 by the ESC-E(Z) complex. Thus, we conclude that the ESC-E(Z) complex methylates H3-K27 and we leave open the possibility that it can methylate H3-K9, or both K9 and K27, in certain contexts. It is worth noting that H3-K9 and H3-K27 are both embedded in the sequence ARKS where S can be phosphorylated in vivo. Furthermore, the mammalian enzyme, G9a, has dual specificity for H3-K9 and H3-K27 in vitro, although its H3-K9 activity appears more significant in vivo (Tachibana et al., 2002). In contrast, SUV39H appears specific for H3-K9 (Rea et al., 2000). Thus, it will be important to define molecular

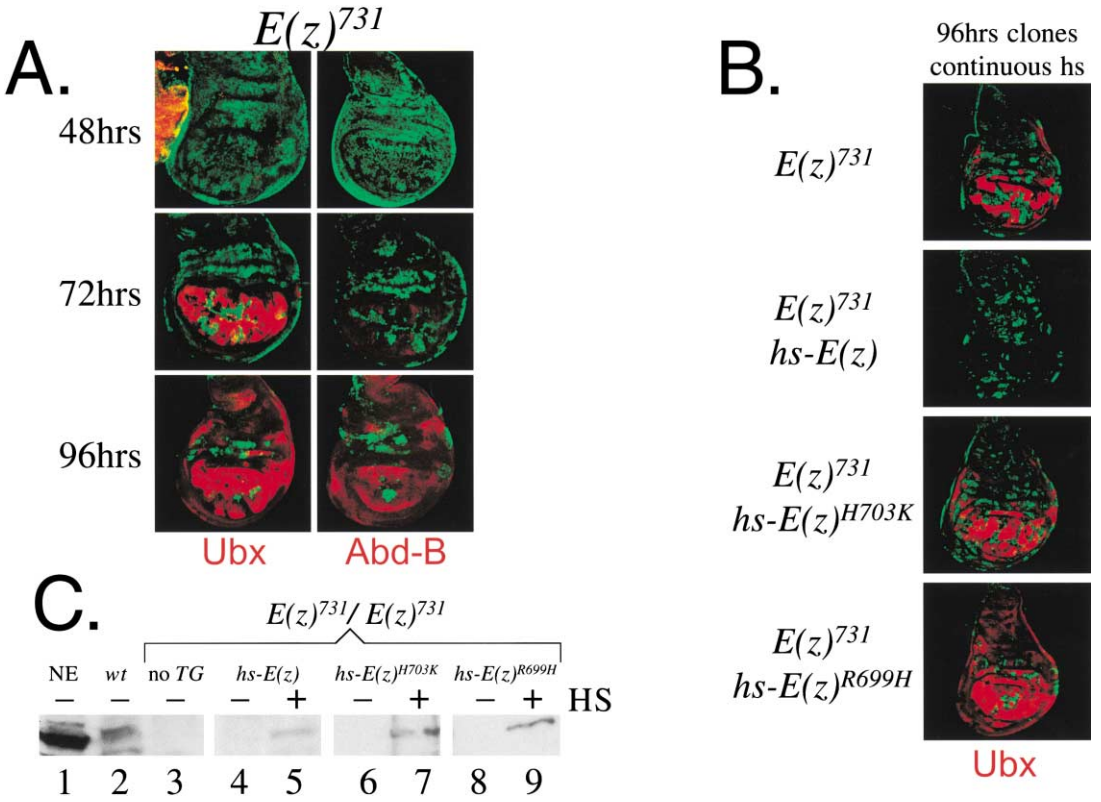


Figure 6. Requirement for E(Z) HMTase in HOX Gene Silencing.

(A) and (B) Wing imaginal discs with clones of cells homozygous for *E(z)*^{731} were stained with antibodies against UBX or ABD-B (red) and GFP (green); the *E(z)*^{731} mutant cells are marked by absence of GFP.

(A) UBX and ABD-B expression analyzed 48, 72, or 96 hr after clone induction.

(B) Wing discs with *E(z)*^{731} mutant clones from larvae that carry no transgene (top) or the indicated *hsp70-E(z)* transgenes. Discs were stained for UBX 96 hr after clone induction. Wild-type E(Z) completely rescues HOX gene silencing in mutant clones (compare top two images) whereas E(Z)^{H703K} and E(Z)^{R699H} fail to rescue (images 3 and 4).

(C) Western blots to detect E(Z) in nuclear extract from wild-type embryos (lane 1), total extract from wild-type (lane 2), or *E(z)*^{731} homozygous larvae without (lane 3) or with an *hsp70-E(z)* transgene (lanes 4–9). Lanes 5, 7, and 9 contain extracts from larvae that were heat-shocked for 1 hr. E(Z) protein is not detected in *E(z)*^{731} homozygotes (lanes 3–9) except in heat-shocked larvae bearing indicated transgenes. Comparable amounts of larval extract were loaded in each lane.

determinants that dictate ESC-E(Z) specificity for H3 sites in vivo, which might include other histone modifications or interactions with other proteins.

Role of Methylation in PcG Function

Histone H3 is an attractive candidate to be the primary functional target for the ESC-E(Z) complex in vivo. Previous work on heterochromatin proteins established a molecular model that links H3 methylation and gene repression. In this case, SUV39H HMTase methylates H3-K9 (Rea et al., 2000) and this modification creates a binding site for the chromodomain of HP1 (Bannister et al., 2001; Lachner et al., 2001). Similarly, H3 methylation by ESC-E(Z) might provide a binding site for another chromodomain protein, such as Polycomb (PC), a component of the PRC1 complex, whose chromodomain is required for chromatin localization in vivo (Messmer et al., 1992). Targeting of PRC1 via histone methylation performed by ESC-E(Z) is consistent with several in vivo results that imply synergy between these complexes (Simon and Tamkun, 2002). Rather than supplying binding sites for specific proteins, ESC-E(Z) mediated methylation could influence chromatin more generally by altering adjacent nucleosome interactions needed to package the chromatin fiber.

It is possible that proteins other than histone H3 are important targets for methylation by the ESC-E(Z) complex. We observe some methylation of E(Z) and Su(Z)12 in our reactions (Figure 3D), implying that the complex can methylate proteins other than H3. Methylation of this type might contribute to repression by as yet unknown means.

Besides histone methylation, genetic and biochemical tests link histone deacetylation to PcG-mediated repression (Chang et al., 2001; Poux et al., 2001; Tie et al., 2001; van der Vlag and Otte, 1999) and hyperacetylation to trxG-dependent active states (Cavalli and Paro, 1999; Petruk et al., 2001). It will be important to test for regulatory interactions, or crosstalk, among the various tail modifications (reviewed in Zhang and Reinberg, 2001). For example, H3-K27 acetylation would be anticipated to antagonize H3-K27 methylation, similar to the mutual exclusion of these two modifications on H3-K9. Likewise, interplay between phospho-H3-S28 and methyla-

tion of H3-K27 could resemble the inhibitory crosstalk between H3-S10 and H3-K9 (Rea et al., 2000). This possibility could enable kinases and phosphatases to make regulatory inputs to the PcG/trxG system. It will be important to determine whether additional PcG/trxG proteins possess or interact with histone-modifying activities, and also to define the histone modification states on PcG-repressed and trxG-activated genes in vivo.

Histone Methylation and Inheritance of the PcG Silenced State

The HOX genes of *Drosophila* provide the best example of heritable silencing by PcG proteins. Recent work showed that derepression of HOX genes after removal of PC or PSC in proliferating cells can be reversed if the depleted protein is resupplied within a few cell generations (Beuchle et al., 2001). These results led to the proposal that silenced HOX genes bear a heritable molecular mark that targets them for PcG repression and that this mark can be maintained for a few cell generations, even after HOX genes are derepressed. Could the proposed mark reflect, at least in part, H3-K27 methylation by the ESC-E(Z) complex? Histone lysine methylation is a very stable modification, so is well-suited for a long-term molecular mark (reviewed in Bannister et al., 2002). *E(z)* mutants show an unusually long delay before HOX misexpression is detected in imaginal disc clones (72 hr for robust misexpression of UBX; Figure 6) and a long delay is observed in temperature-shift experiments with an *E(z)* allele that behaves as a null at restrictive temperature (LaJeunesse and Shearn, 1996). In addition, of all the PcG mutants tested, only *Su(z)12* mutants show a comparably long delay in release from silencing in imaginal disc clones (Birve et al., 2001). In contrast, removal of the PRC1 components PSC or PH triggers rapid loss of repression (Beuchle et al., 2001).

Conservation of ESC-E(Z)-SU(Z)12 Partnership

Remarkably, ESC, E(Z), and SU(Z)12 are conserved in many organisms, where they appear to function together as repressors in a wide array of developmental processes. Mammalian complexes that resemble the ESC-E(Z) complex (Sewalt et al., 1998) are implicated in multiple processes including early embryonic patterning, HOX gene regulation, and hematopoiesis (Lessard et al., 1999; O'Carroll et al., 2001; Schumacher et al., 1996). Intriguingly, mouse homologs of ESC and E(Z) associate with the inactive X chromosome in trophoblast stem cells, suggesting a direct role in X-inactivation (Mak et al., 2002; Wang et al., 2001b). In *C. elegans*, the ESC- and E(Z)-related proteins MES-6 and MES-2 form a stable complex (Xu et al., 2001) and are required for germline development and gene silencing (Holdeman et al., 1998; Kelly and Fire, 1998). The conserved partnership extends to plants, where proteins related to ESC, E(Z), and SU(Z)12 are cohort regulators in several *Arabidopsis* developmental pathways (e.g., Goodrich et al., 1997; Spillane et al., 2000; Yoshida et al., 2001). A striking example is VRN2, a SU(Z)12 relative, which is required for long-term gene silencing in response to vernalization (Gendall et al., 2001). Further work in these systems should address if histone methylation by ESC-E(Z) complexes represents an evolutionary ancient mechanism to mark chromatin for heritable repression during development.

Experimental Procedures

Antibodies and Western Blots

Rabbit anti-SU(Z)12 antibodies were produced against HIS_6 fusions to either full-length SU(Z)12 (amino acids 1–900, FL Ab) or SU(Z)12 amino acids 448–802 (SAC Ab), affinity-purified against SU(Z)12 (1-900), and used at 1:500. SU(Z)12 antibodies recognize bands of predicted sizes (105 kDa or 70 kDa, respectively) on Western blots of embryo extracts from wild-type or *Su(z)12*^{1} truncation mutants (Birve et al., 2001 and data not shown). Additional antibodies used were: anti-FLAG M5 (1:1000; Sigma), anti-E(Z) [1:1000; (Carrington and Jones, 1996)], anti-RPD3 [1:2000; (De Rubertis et al., 1996)] and anti-BEAF [1:100; (Zhao et al., 1995)]. HRP-conjugated secondary antibodies were from Jackson Immuno Laboratories (goat anti-mouse, 1:15,000), or BioRad (goat anti-rabbit, 1:10,000), and Western blots were developed using ECL (Amersham Pharmacia Biotech).

Generation and Testing of *FLAG-esc* Germline Transformants

A FLAG epitope was placed at the N terminus of *esc* coding sequences in the context of a 4.2 kb genomic fragment in the pCasper4 vector. This construct is identical to wild-type and HA-tagged *esc* transgenes that rescue *esc* mutations in vivo (Jones et al., 1998; Simon et al., 1995) and was used to generate transformants in a *y Df(1)w*^{67c23} background. *FLAG-esc* inserts were tested as described (Simon et al., 1995) and rescued viability to adulthood. A transformant line containing two X-linked inserts was made homozygous and used to prepare embryo nuclear extracts.

Purification of ESC-E(Z) Complex from Transgenic *Drosophila* Embryos

All steps were performed at 4°C unless otherwise indicated. Nuclear extracts were prepared from 0–24 hr *FLAG-esc* or *y Df(1)w*^{67c23} embryos as described (Ng et al., 2000) with a final salt concentration of 0.36 M KCl. Extracts were diluted to 0.18 M KCl with QCB (25 mM Tris [pH 7.6], 1 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, 10 mM NaF, 1 mM ammonium molybdate, 1 mM benzamidine, 0.1 mM PMSF, and 2 μg/ml aprotinin) and centrifuged to remove particulates. Up to 100 mg of nuclear proteins were fractionated on a Q-Sepharose (Amersham Pharmacia Biotech) column in QCB with a linear gradient from 0.18 to 0.48 M NaCl. Peak fractions for FLAG-ESC and E(Z) were pooled, supplemented with Tween-20 (0.1%) and fresh protease inhibitors (1 mM benzamidine, 0.1 mM PMSF, 2 μg/ml aprotinin, 0.5 μg/ml antipain, and 1 μg/ml each of leupeptin, chymostatin, and pepstatin A), and incubated overnight with 100 μl of anti-FLAG M2-agarose beads (Sigma). Beads were packed into a column, washed at room temp with 5 ml of QCB-0.35 M NaCl and 1 ml QCB-0.06 M NaCl, and eluted for 20 min at room temp with 100 μl aliquots of QCB-0.06 M NaCl containing 0.4 mg/ml of FLAG peptide (DYKDDDDK, Sigma). QCB used at these steps also contained 0.1% Tween-20 and protease inhibitors as above.

Mass Spectrometry Analyses of Proteins

Approximately 0.25 to 0.5 pmol of purified ESC-E(Z) complex was resolved by SDS-PAGE and components were stained with silver. Protein bands were excised and processed for mass spectrometric fingerprinting using protocols from the Mass Spectrometry Consortium at the University of Minnesota (http://biosci.cbs.umn.edu/mass_spec/). Proteins were destained, digested with trypsin (Promega), and peptides were purified on a μC18 Ziptip (Millipore). Peptide masses were obtained by MALDI-TOF using a Biflex III instrument (Bruker). Experimental masses were compared to predicted masses for tryptic peptides of proteins in the NCBInr *Drosophila* database using the Matrix Science Mascot Peptide Mass Fingerprint search engine (http://www.matrixscience.com). Proteins from two independent preparations were analyzed; one was produced by purification as in Figure 1A and the other by direct M2 affinity-purification of embryo nuclear extracts. The total number of peptides

identified for each protein was as follows: ESC: 10, E(Z): 10, NURF-55: 7, SU(Z)12: 14.

Immunoprecipitations and Gel Filtration

300 μg of embryo nuclear extract was incubated with 25 μl anti-SU(Z)12 or 100 μl anti-E(Z) antibodies for 2 hr at 4°C. Immunoprecipitates were recovered with protein A-agarose beads (Roche), washed five times with 350 mM NaCl, 10 mM Hepes [pH 7.6], 0.1% Tween-20, and proteins were eluted with SDS sample buffer. Superose 6 gel filtration analysis was performed on nuclear extracts from 0 to 24 hr *FLAG-esc* embryos as described (Ng et al., 2000).

Protein Expression and Purification

Baculovirus production was performed with the Bac-to-Bac system (GibcoBRL), using full-length cDNAs for ESC, E(Z), SU(Z)12, and NURF-55 inserted into pFastBac1. Sf9 cells were infected for 46–48 hr with FLAG-E(Z) virus or viruses for all four complex components. Whole-cell extracts were prepared according to the method of Ito et al. (1999). 1 ml M2 anti-FLAG beads (Sigma) were used for 10 ml of extracts. Binding was carried out for 2–4 hr at 4°C in extraction Buffer F (20 mM Tris-Cl [pH 8.0], 500 mM NaCl, 20% glycerol, 4 mM $MgCl_2$, 0.4 mM EDTA, and 2 mM DTT) with 0.05% NP40, 10 μM $ZnCl_2$ and the following protease inhibitors: 0.4 mM PMSF, 50 μg/ml TLCK, 20 μg/ml aprotinin, 32 μg/ml benzamidine, 20 μg/ml leupeptin, 4 μg/ml pepstatin, and 20 μg/ml phenanthroline. Beads were washed twice with Buffer F + 0.05% NP40, and then with BC buffer (20 mM Hepes [pH 7.9], 0.4 mM EDTA, and 20% glycerol) with 0.05% NP40, 0.2× protease inhibitors, 0.5 mM DTT and up to 2 M KCl. Beads were loaded into a column, equilibrated in BC buffer with 300 mM KCl, and complex was eluted with 0.4 mg/ml FLAG peptide (DYKDDDDK) in BC300 for 1–2 hr. Complexes were estimated to be 85% pure by colloidal blue (Novagen) or silver staining, and molarity was calculated using a molecular weight of 283.5 kDa, which assumes one copy of each subunit per complex.

For glycerol gradient purification, 10%–35% glycerol gradients (in 50 mM Tris-Cl [pH 8.0], and 1 mM EDTA) were centrifuged 18 hr at 35,000 RPM in a Beckman Sw55 Ti rotor and resolved into ~300 μl fractions.

E(Z) SET domain and histone H3 mutations were generated using the Quick Change Mutagenesis Kit (Stratagene). Mutant rESC-E(Z) complexes were prepared in parallel with a wild-type control and at least twice for each complex.

MTase Assays

HeLa core histones, 5S nucleosomal arrays, and recombinant *Drosophila* H3/H4 were prepared as described (Levenstein and Kadonaga, 2002; Sif et al., 2001). MTase reaction conditions were: 12 mM Hepes, [pH 7.9], 0.24 mM EDTA, 12% glycerol, 4 mM DTT, 2.5 mM $MgCl_2$, 0.5–1 μM ^{3}H-SAM (NEN), and 60–110 mM KCl. Reactions were incubated 60 min at 30°C, stopped with SDS-sample buffer, resolved by 18% SDS-PAGE, and transferred to Immobilon-P (Millipore). Blots were stained with amido black (Sigma) and exposed to a tritium phosphorimager screen (Molecular Dynamics) or sprayed with EN^{3}HANCE (NEN) and exposed to film. Maximal ^{3}H-methyl incorporation was determined using saturating concentrations of nucleosomal templates by scintillation counting of histone H3 bands. One microgram of rESC-E(Z) transferred 2–5.5 pmol ^{3}H-SAM in a 60 min reaction, which corresponds to 0.7–1.8 pmol SAM transferred/pmol rESC-E(Z).

Edman degradation was performed on histone H3 excised from blots of 100 μl reactions containing 160 pmol of nucleosomes in the 5S array, ~30 pmol of rESC-E(Z), and 1.6 nmol ^{3}H-SAM; methylation site was determined by scintillation counting of Edman degradation fractions. We interpret the results in Figure 4A to mean that rESC-E(Z) methylates only K27, although there is also significant CPM release for residue 28 and beyond. Amino acid sequencing suggests this reflects accumulation of n + 1 (and longer) products due to incomplete coupling or cleavage reactions.

E(z) Alleles, *hs-E(z)* Transgenes, and Rescue Assays in Imaginal Disc Clones

E(z)^{731}, *E(z)^{328}*, *E(z)^{1025}*, and *E(z)^{2434}* were induced with EMS on an *FRT2A* chromosome (D. Beuchle, C. Fritsch, C.-m. Chen, G. Struhl and J. Müller, unpublished data) and contain the following molecular lesions: E(z)^{731} and E(z)^{328}: W638stop (TGG to TGA); E(z)^{1025}: Q553stop (CAG to TAG); E(z)^{2434}: Q404stop (CAG to TAG). Imaginal disc clones homozygous for any of the four alleles show strong misexpression of HOX genes but no obvious proliferation defects. We previously reported that clones homozygous for *E(z)^{63}*, a protein-negative *E(z)* allele (Carrington and Jones, 1996), show cell proliferation defects but only subtle misexpression of HOX genes (Beuchle et al., 2001). However, rescue tests on *E(z)^{63}* mutants with *E(z)* transgenes (J. Müller, unpublished data) suggest that the *E(z)^{63}* chromosome contains additional mutations that may have masked the PcG phenotype.

hs-E(z) transformants expressing wild-type E(Z) protein, kindly provided by Gunter Reuter, have been described (Laible et al., 1997). *hs-E(z)^{H703K}* and *hs-E(z)^{R699H}* contain the full-length E(Z) ORF, plus 91 bp of 5′-UTR and 88 bp of 3′-UTR, inserted into CaSpeR-hs. The H703K (CAT to AAG) and R699H (CGG to CAT) mutations were generated by PCR.

E(z) mutant clones in imaginal discs were generated and analyzed by antibody staining as described (Beuchle et al., 2001); in all cases, the *Minute* technique (i.e., *M(3)^{i55}*) was used (see Beuchle et al., 2001). For rescue tests, strains were constructed to ensure that animals analyzed carried the *hs-E(z)* transgene. Transgenes were expressed by 1 hr heat shocks (37°C) applied every 12 hr over a 96 hr period. For each transgene, two independent *hs-E(z)* insertions were tested and produced the same result.

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References

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature *410*, 120–124.

Bannister, A.J., Schneider, R., and Kouzarides, T. (2002). Histone methylation. Dynamic or static? Cell *109*, 801–806.

Beuchle, D., Struhl, G., and Muller, J. (2001). Polycomb group proteins and heritable silencing of *Drosophila* Hox genes. Development *128*, 993–1004.

Birve, A., Sengupta, A.K., Beuchle, D., Larsson, J., Kennison, J.A., Rasmuson-Lestander, A., and Muller, J. (2001). *Su(z)12*, a novel *Drosophila* polycomb group gene that is conserved in vertebrates and plants. Development *128*, 3371–3379.

Carrington, E.A., and Jones, R.S. (1996). The *Drosophila* enhancer of zeste gene encodes a chromosomal protein: examination of wild-type and mutant protein distribution. Development *122*, 4073–4083.

Cavalli, G., and Paro, R. (1999). Epigenetic inheritance of active

chromatin after removal of the main transactivator. Science *286*, 955–958.

Chang, Y.L., Peng, Y.H., Pan, I.C., Sun, D.S., King, B., and Huang, D.H. (2001). Essential role of *Drosophila* Hdac1 in homeotic gene silencing. Proc. Natl. Acad. Sci. USA *98*, 9730–9735.

Chen, G., Fernandez, J., Mische, S., and Courey, A.J. (1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in *Drosophila* development. Genes Dev. *13*, 2218–2230.

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K., and Spierer, P. (1996). The histone deacetylase RPD3 counteracts genomic silencing in *Drosophila* and yeast. Nature *384*, 589–591.

Francis, N.J., and Kingston, R.E. (2001). Mechanisms of transcriptional memory. Nat. Rev. Mol. Cell. Biol. *2*, 409–421.

Francis, N.J., Saurin, A.J., Shao, Z., and Kingston, R.E. (2001). Reconstitution of a functional core polycomb repressive complex. Mol. Cell *8*, 545–556.

Gendall, A.R., Levy, Y.Y., Wilson, A., and Dean, C. (2001). The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in *Arabidopsis*. Cell *107*, 525–535.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997). A polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. Nature *386*, 44–51.

Holdeman, R., Nehrt, S., and Strome, S. (1998). MES-2, a maternal protein essential for viability of the germline in *Caenorhabditis elegans*, is homologous to a *Drosophila* polycomb group protein. Development *125*, 2457–2467.

Imhof, A., Yang, X.J., Ogryzko, V.V., Nakatani, Y., Wolffe, A.P., and Ge, H. (1997). Acetylation of general transcription factors by histone acetyltransferases. Curr. Biol. *7*, 689–692.

Ito, T., Levenstein, M.E., Fyodorov, D.V., Kutach, A.K., Kobayashi, R., and Kadonaga, J.T. (1999). ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. Genes Dev. *13*, 1529–1539.

Jacobs, J.J., and van Lohuizen, M. (2002). Polycomb repression: from cellular memory to cellular proliferation and cancer. Biochim. Biophys. Acta *1602*, 151–161.

Jones, R.S., and Gelbart, W.M. (1990). Genetic analysis of the enhancer of zeste locus and its role in gene regulation in *Drosophila melanogaster*. Genetics *126*, 185–199.

Jones, R.S., and Gelbart, W.M. (1993). The *Drosophila* polycomb-group gene enhancer of zeste contains a region with sequence similarity to trithorax. Mol. Cell. Biol. *13*, 6357–6366.

Jones, C.A., Ng, J., Peterson, A.J., Morgan, K., Simon, J., and Jones, R.S. (1998). The *Drosophila* esc and E(z) proteins are direct partners in polycomb group-mediated repression. Mol. Cell. Biol. *18*, 2825–2834.

Kal, A.J., Mahmoudi, T., Zak, N.B., and Verrijzer, C.P. (2000). The *Drosophila* brahma complex is an essential coactivator for the trithorax group protein zeste. Genes Dev. *14*, 1058–1071.

Kelly, W.G., and Fire, A. (1998). Chromatin silencing and the maintenance of a functional germline in *Caenorhabditis elegans*. Development *125*, 2451–2456.

Lachner, M., and Jenuwein, T. (2002). The many faces of histone lysine methylation. Curr. Opin. Cell Biol. *14*, 286–298.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116–120.

Laible, G., Wolf, A., Dorn, R., Reuter, G., Nislow, C., Lebersorger, A., Popkin, D., Pillus, L., and Jenuwein, T. (1997). Mammalian homologues of the polycomb-group gene enhancer of zeste mediate gene silencing in *Drosophila* heterochromatin and at *S. cerevisiae* telomeres. EMBO J. *16*, 3219–3232.

LaJeunesse, D., and Shearn, A. (1996). E(z): a polycomb group gene or a trithorax group gene? Development *122*, 2189–2197.

Lessard, J., Schumacher, A., Thorsteinsdottir, U., van Lohuizen, M., Magnuson, T., and Sauvageau, G. (1999). Functional antagonism of the polycomb-group genes eed and Bmi1 in hemopoietic cell proliferation. Genes Dev. *13*, 2691–2703.

Levenstein, M.E., and Kadonaga, J.T. (2002). Biochemical analysis of chromatin containing recombinant *Drosophila* core histones. J. Biol. Chem. *277*, 8749–8754.

Mak, W., Baxter, J., Silva, J., Newall, A.E., Otte, A.P., and Brockdorff, N. (2002). Mitotically stable association of polycomb group proteins eed and enx1 with the inactive x chromosome in trophoblast stem cells. Curr. Biol. *12*, 1016–1020.

Martinez-Balbas, M.A., Tsukiyama, T., Gdula, D., and Wu, C. (1998). *Drosophila* NURF-55, a WD repeat protein involved in histone metabolism. Proc. Natl. Acad. Sci. USA *95*, 132–137.

Messmer, S., Franke, A., and Paro, R. (1992). Analysis of the functional role of the polycomb chromo domain in *Drosophila melanogaster*. Genes Dev. *6*, 1241–1254.

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D., and Grewal, S.I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110–113.

Ng, J., Hart, C.M., Morgan, K., and Simon, J.A. (2000). A *Drosophila* ESC-E(Z) protein complex is distinct from other polycomb group complexes and contains covalently modified ESC. Mol. Cell. Biol. *20*, 3069–3078.

Nishioka, K., Rice, J.C., Sarma, K., Erdjument-Bromage, H., Werner, J., Wang, Y., Chuikov, S., Valenzuela, P., Tempst, P., Steward, R., et al. (2002). PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. Mol. Cell *9*, 1201–1213.

O'Carroll, D., Erhardt, S., Pagani, M., Barton, S.C., Surani, M.A., and Jenuwein, T. (2001). The polycomb-group gene Ezh2 is required for early mouse development. Mol. Cell. Biol. *21*, 4330–4336.

Papoulas, O., Beek, S.J., Moseley, S.L., McCallum, C.M., Sarte, M., Shearn, A., and Tamkun, J.W. (1998). The *Drosophila* trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. Development *125*, 3955–3966.

Petruk, S., Sedkov, Y., Smith, S., Tillib, S., Kraevski, V., Nakamura, T., Canaani, E., Croce, C.M., and Mazo, A. (2001). Trithorax and dCBP acting in a complex to maintain expression of a homeotic gene. Science *294*, 1331–1334.

Poux, S., Melfi, R., and Pirrotta, V. (2001). Establishment of polycomb silencing requires a transient interaction between PC and ESC. Genes Dev. *15*, 2509–2514.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593–599.

Saurin, A.J., Shao, Z., Erdjument-Bromage, H., Tempst, P., and Kingston, R.E. (2001). A *Drosophila* polycomb group complex includes zeste and dTAFII proteins. Nature *412*, 655–660.

Schumacher, A., Faust, C., and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature *384*, 648.

Sewalt, R.G., van der Vlag, J., Gunster, M.J., Hamer, K.M., den Blaauwen, J.L., Satijn, D.P., Hendrix, T., van Driel, R., and Otte, A.P. (1998). Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. Mol. Cell. Biol. *18*, 3586–3595.

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C.T., Bender, W., and Kingston, R.E. (1999). Stabilization of chromatin structure by PRC1, a polycomb complex. Cell *98*, 37–46.

Sif, S., Saurin, A.J., Imbalzano, A.N., and Kingston, R.E. (2001). Purification and characterization of mSin3A-containing Brg1 and hBrm chromatin remodeling complexes. Genes Dev. *15*, 603–618.

Simon, J.A., and Tamkun, J.W. (2002). Programming off and on states in chromatin: mechanisms of polycomb and trithorax group complexes. Curr. Opin. Genet. Dev. *12*, 210–218.

Simon, J., Bornemann, D., Lunde, K., and Schwartz, C. (1995). The extra sex combs product contains WD40 repeats and its time of

action implies a role distinct from other polycomb group products. Mech. Dev. *53*, 197–208.

Spillane, C., MacDougall, C., Stock, C., Kohler, C., Vielle-Calzada, J.P., Nunes, S.M., Grossniklaus, U., and Goodrich, J. (2000). Interaction of the *Arabidopsis* polycomb group proteins FIE and MEA mediates their common phenotypes. Curr. Biol. *10*, 1535–1538.

Strahl, B.D., Grant, P.A., Briggs, S.D., Sun, Z.W., Bone, J.R., Caldwell, J.A., Mollah, S., Cook, R.G., Shabanowitz, J., Hunt, D.F., and Allis, C.D. (2002). Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. Mol. Cell. Biol. *22*, 1298–1306.

Struhl, G. (1981). A gene product required for correct initiation of segmental determination in *Drosophila*. Nature *293*, 36–41.

Tachibana, M., Sugimoto, K., Nozaki, M., Ueda, J., Ohta, T., Ohki, M., Fukuda, M., Takeda, N., Niida, H., Kato, H., and Shinkai, Y. (2002). G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. Genes Dev. *16*, 1779–1791.

Taunton, J., Hassig, C.A., and Schreiber, S.L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408–411.

Tie, F., Furuyama, T., Prasad-Sinha, J., Jane, E., and Harte, P.J. (2001). The *Drosophila* polycomb group proteins ESC and E(Z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3. Development *128*, 275–286.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G., and Reuter, G. (1994). The protein encoded by the *Drosophila* position-effect variegation suppressor gene *Su(var)3–9* combines domains of antagonistic regulators of homeotic gene complexes. EMBO J. *13*, 3822–3831.

Tyler, J.K., Bulger, M., Kamakaka, R.T., Kobayashi, R., and Kadonaga, J.T. (1996). The p55 subunit of *Drosophila* chromatin assembly factor 1 is homologous to a histone deacetylase-associated protein. Mol. Cell. Biol. *16*, 6149–6159.

van der Vlag, J., and Otte, A.P. (1999). Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat. Genet. *23*, 474–478.

Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Borchers, C., Tempst, P., and Zhang, Y. (2001a). Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. Mol. Cell *8*, 1207–1217.

Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J.C., Nagy, A., and Magnuson, T. (2001b). Imprinted X inactivation maintained by a mouse polycomb group gene. Nat. Genet. *28*, 371–375.

Xu, L., Fong, Y., and Strome, S. (2001). The *Caenorhabditis elegans* maternal-effect sterile proteins, MES-2, MES-3, and MES-6, are associated in a complex in embryos. Proc. Natl. Acad. Sci. USA *98*, 5061–5066.

Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R., and Takahashi, S. (2001). EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in *Arabidopsis*. Plant Cell *13*, 2471–2481.

Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev. *15*, 2343–2360.

Zhao, K., Hart, C.M., and Laemmli, U.K. (1995). Visualization of chromosomal domains with boundary element-associated factor BEAF-32. Cell *81*, 879–889.