labeling resistant to the low concentration of α-amanitin corresponding to pol III transcription can also be seen in wild-type but not $TBP^{-/-}$ nuclei (Fig. 4B and fig. S6B). Hence, loss of TBP leads to a shutdown of pol I and pol III transcription.

We show that depletion of TBP results in a concomitant and proportional loss of pol I and pol III but not pol II transcription. The contrasting effects of TBP depletion show that these polymerases have a differential dependency on TBP and are indicative of a TBP-independent mechanism for pol II transcription (*6*). This conclusion would appear to contradict previous experiments, in which inactivation of TBP in zebrafish embryos before the onset of zygotic transcription resulted in the absence of H5 labeling, indicating a major loss of zygotic transcription (*3*). However, in our experiments TBP is depleted only after zygotic transcription has begun. This observation, together with the observed growth arrest of $TBP^{-/-}$ cells and the growth and cell cycle defects of $TBP^{+/-}$ chicken DT40 cells (*14*), show that TBP is required for initiation of zygotic transcription and for cell proliferation, which also involves de novo initiation of gene expression during the cell cycle and after the mitotic division. In contrast, the high levels of pol II transcription seen in our experiments suggest that TBP is not required for transcription reinitiation and maintenance of gene expression in growth-arrested cells. Hence for many genes, there may be a distinct requirement for TBP at the de novo activation and reinitiation stages of transcription.

Distinct pathways for initiation and reinitiation have indeed been proposed based on in vitro studies where a subset of the general transcription factors remains at the promoter to form a scaffold facilitating reinitiation (*15*). Whereas TFIID is among the general factors that remain at the promoter in vitro, our results suggest that persistent transcription of many cellular genes can take place independently of TBP in vivo. The TFTC (TPB-free, TAF-containing) complex, containing a subset of TBP-associated factors but no TBP, has previously been shown to substitute for TFIID in vitro (*16*) and has recently been implicated in TBP-independent interferon-stimulated transcription in cultured cells (*17*). Here we provide genetic evidence for TBP-, TLF-independent pol II transcription mechanisms in vivo. It is possible that the TFTC complex or another transcription factor may play a role in the high levels of pol II transcription that we observe in the $TBP^{-/-}$ cells.

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Supporting Online Material

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

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Polycomb group (PcG) proteins play important roles in maintaining the silent state of HOX genes. Recent studies have implicated histone methylation in long-term gene silencing. However, a connection between PcG-mediated gene silencing and histone methylation has not been established. Here we report the purification and characterization of an EED-EZH2 complex, the human counterpart of the *Drosophila* ESC-E(Z) complex. We demonstrate that the complex specifically methylates nucleosomal histone H3 at lysine 27 (H3-K27). Using chromatin immunoprecipitation assays, we show that H3-K27 methylation colocalizes with, and is dependent on, E(Z) binding at an *Ultrabithorax* (*Ubx*) Polycomb response element (PRE), and that this methylation correlates with *Ubx* repression. Methylation on H3-K27 facilitates binding of Polycomb (PC), a component of the PRC1 complex, to histone H3 amino-terminal tail. Thus, these studies establish a link between histone methylation and PcG-mediated gene silencing.

Maintenance of the spatially restricted expression pattern of HOX genes in both flies and vertebrates is controlled by PcG and trithorax group (trxG) proteins (*1*). Biochemical and genetic studies indicate that PcG proteins exist in at least two separate protein complexes, the Polycomb repressive complex 1 (PRC1) and the ESC-E(Z) complex. These two complexes function in a cooperative manner to maintain long-term gene silencing (*2*, *3*). Although components of both protein complexes are required to maintain the silenced state, the function of PRC1 appears to depend on the ESC-E(Z) complex (*4*). Recent studies on the covalent modifications of the histone NH_2-terminal tails have given rise to the "histone code" hypothesis (*5*). One of the covalent modifications, histone lysine methylation, has emerged as an important player in regulating gene expression and chromatin function (*6*). Histone lysine methylation occurs on lysines 4, 9, 27, 36, and 79 of H3 and on lysine 20 of H4. Biochemical and genetic studies indicate that methylation of different lysine residues, with the exception of H3-K79 (*7*–*9*), is catalyzed by different SET domain–containing proteins (*6*).

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To understand the function of histone methylation, we attempted to identify histone methyltransferase (HMTase) using a systematic biochemical approach. During purification of the H4-K20–specific HMTase SET8 (*10*), we noticed that both the 0.3 M and 0.5 M P11 fractions derived from HeLa cell nuclear pellet contained high levels of HMTase activity toward nucleosomal histone H3 (Fig. 1A). To identify the enzyme(s) present in the 0.5 M P11 fraction, we fractionated the proteins in a DEAE5PW column, which separated the HMTase activities into two peaks (Fig. 1B). The present study focuses on the second peak. After fractionation on Phenyl Sepharose and Hydroxyapatite columns (Fig. 1C) (*11*), the active fractions were further purified through a gel filtration Superose 6 column. Analysis of the fractions derived from this column indicates that the HMTase activity elutes between fraction 47 and 50 with an estimated mass of about 500 kD (Fig. 1D). Silver staining of an SDS-polyacrylamide gel containing these fractions revealed that six major polypeptides copurify with the enzymatic activity. Because the largest protein band neither cofractionates with the HMTase

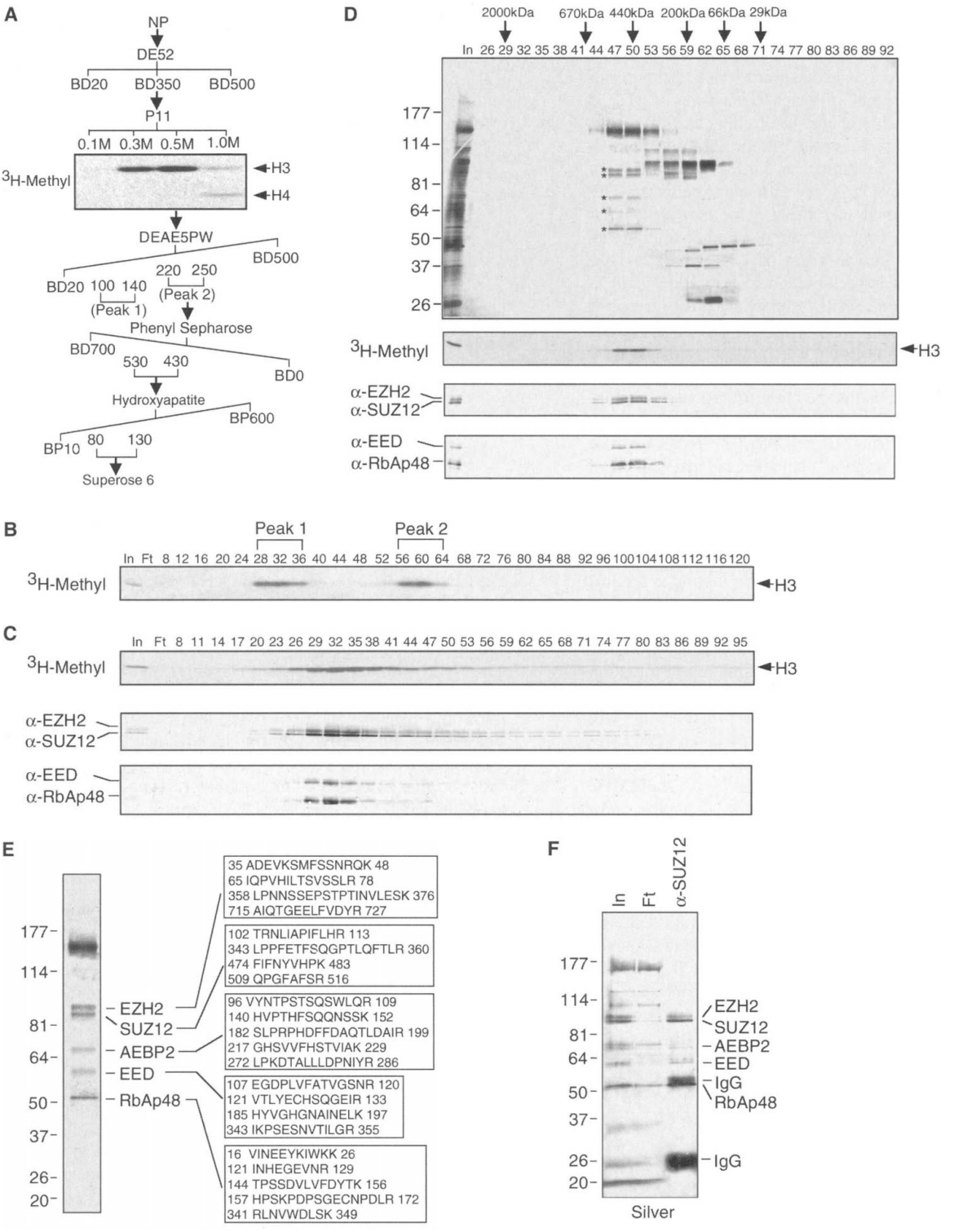

Fig. 1. Purification and identification of a nucleosomal H3-specific methyltransferase complex. (**A**) Schematic representation of the steps used to purify the H3-specific methyltransferase complex. Numbers represent the salt concentrations (mM) at which the HMTase activity elutes from the columns. (**B**) HMTase activities of the protein fractions derived from the DEAE5PW column. The two HMTase peaks are indicated. (**C**) HMTase activity and Western blot analysis of the protein fractions derived from the Hydroxyapatite column. Antibodies used for the Western blot are indicated. (**D**) Silver staining of a polyacrylamide-SDS gel (top panel), HMTase activity assay (second panel), and Western blot analysis (bottom two panels) of the fractions derived from the gel-filtration Superose 6 column. Each of the five copurifying proteins is indicated by an asterisk. The elution profile of the protein markers is indicated at the top. The positions of the protein size markers on SDS–polyacrylamide gel electrophoresis are indicated on the left. (**E**) Identification of the polypeptides present in the HMTase complex. The identities of the polypeptides are indicated. Some of the identified peptides from each band are listed. Numbers correspond to the amino acid number of the indicated protein. (**F**). Silver-stained polyacrylamide-SDS gel demonstrating coimmunoprecipitation of the five components. In and Ft represent input and flow-through, respectively. The positions of the protein size markers are indicated on the left.

activity in the Hydroxyapatite column (fig. S1), nor coimmunoprecipitates with the other components (Fig. 1F), we conclude that it is not a part of the HMTase protein complex.

To identify the proteins that copurify with the HMTase activity, we excised the protein bands and analyzed them by a combination of peptide mass fingerprinting and mass spectrometric sequencing (*10*). In addition to RbAp48, a polypeptide present in many protein complexes involved in histone metabolism, several human PcG proteins, including EZH2 (*12*), SUZ12 (*13*), and EED (*14*), were identified in the HMTase complex (Fig. 1E). A zinc finger transcriptional repressor named AEBP2 was also identified (*15*). Whether this protein is involved in targeting the complex remains to be determined. EZH2 contains a SET domain, a signature motif for all known histone lysine methyltransferases except the H3-K79 methyltransferase DOT1 (*7–9*) and is therefore likely to be the catalytic subunit. However, recombinant EZH2 made in *Escherichia coli* or baculovirus-infected SF9 cells has no detectable HMTase activity, indicating that either a posttranslational modification or other components in the complex are required for the HMTase activity. This is consistent with previous results in which a partial EZH2 protein containing the SET domain was used (*16*).

Although mammalian EZH2 and EED, and their respective homologs in *Drosophila* and *Caenorhabditis elegans*, are known to interact directly (*2*, *3*), the presence of SUZ12 in such a complex has not been previously reported. To verify that these proteins are components of the same protein complex, we generated antibodies against each of these proteins. Western blot analysis of the column fractions derived from the last two columns indicated that these proteins copurify with the HMTase activity (Fig. 1, C and D). To further confirm that the copurified proteins exist as a single protein complex, we immunoprecipitated the last column fractions (51 to 53) with an antibody to SUZ12. As shown in Fig. 1F, all five proteins coimmunoprecipitated. Because a protein complex containing *Drosophila* ESC and E(Z), respective homologs of EED and EZH2, has been previously named the ESC-E(Z) complex, we refer to the human counterpart as the EED-EZH2 complex. Although both EED-EZH2 and ESC-E(Z) complexes physically associate with HDACs (*17*, *18*), our purified complex neither contains any HDAC polypeptide nor possesses detectable HDAC activity. It is possible that a different protein complex containing EED, EZH2, and HDAC may exist. Alternatively, HDACs may be recruited to target sites through direct interaction with EED, yet may not exist as a stable subunit of EED-EZH2 complexes. Further work is needed to differentiate these possibilities.

To characterize the substrate specificity of the EED-EZH2 complex, we subjected equivalent amounts of histone H3 that exist alone, in complex with other core histones, and in mono- or oligonucleosome forms to methylation by equal amounts of the enzyme. As shown in Fig. 2A, the EED-EZH2 complex was capable of methylating all forms of histone H3, but showed a strong preference for H3 in oligonucleosome forms.

We next attempted to identify the residue methylated by the EED-EZH2 complex. Because oligonucleosomes are preferred substrates, they were subjected to methylation by the EED-EZH2 complex in the presence of S-adenosyl-L-[methyl-^{3}H]methionine (^{3}H-SAM). After purification, the labeled H3 was subjected to microsequencing followed by liquid scintillation counting. Neither K4 nor K9 released numbers of counts clearly greater than background. However, a small radioactive peak was detected in cycle 27 (fig. S2). Given that the recovery efficiency decreases with each microsequencing cycle, the detection of a small peak on cycle 27 indicates that K27 is likely to be the site targeted by the EED-EZH2 complex. To confirm this possibility, we mutated each of the five potential methylation sites on H3 and compared the effect of the mutation on the ability of H3 to serve as a substrate for the enzyme. As a control, the ability of these H3 mutants to be methylated by SUV39H1 was also analyzed. Mutation of K27 completely abolished the ability of H3 to serve as a substrate, whereas mutations of other sites had little effect (Fig. 2B, top panel). As expected, only mutation of K9 affected the SUV39H1-mediated H3 methylation (Fig. 2B, middle panel). These data, in combination with the in vivo results presented in Fig. 3, allow us to conclude that K27 is the predominant site, if not the only site, that is targeted for methylation by the EED-EZH2 complex.

To gain insight into the function of H3-K27 methylation in vivo, we generated a polyclonal antibody against a dimethyl-K27 H3 peptide. This antibody is highly specific for mK27 when evaluated by peptide competition and enzyme-linked immunosorbent assay (fig. S3A). Western blot analysis with the H3-mK27–specific antibody demonstrated that H3-K27 methylation occurs in a variety of multicellular organisms, including human, chicken, and *Drosophila*. However, it does not appear to occur in the budding yeast *Saccharomyces cerevisiae* (fig. S3B).

Given that both H3-K27 methylation as well as the EED-EZH2 counterpart exist in *Drosophila*, we examined whether the ESC-E(Z) complex is responsible for H3-K27 methylation in this organism. Previously, we have characterized several *E(z)* temperature-sensitive mutant alleles, one of which, $E(z)^{61}$, contains a Cys-to-Tyr substitution (C603Y) in the cysteine-rich region immediately preceding the SET domain (*19*). When reared continuously at 18°C (permissive temperature), $E(z)^{61}$ homozygotes exhibit no detectable mutant phenotype and maintain wild-type expression patterns of HOX genes, such as *Ubx* (*19*, *20*). However, at 29°C (restrictive temperature), $E(z)^{61}$ produces multiple homeotic phenotypes due to derepression of HOX genes (*20*), which correlates with loss of polytene chromosome binding by the E(Z)^{61} protein (*19*) and disruption of chromosome binding by Polycomb (PC) and other PRC1 components (*4*, *21*). Given that chromosome binding by E(Z)^{61} protein is abolished at 29°C (*19*), H3-K27 methylation should be correspondingly reduced in the mutants at 29°C, if E(Z) is responsible for H3-K27 methylation. Western blot analysis (Fig.

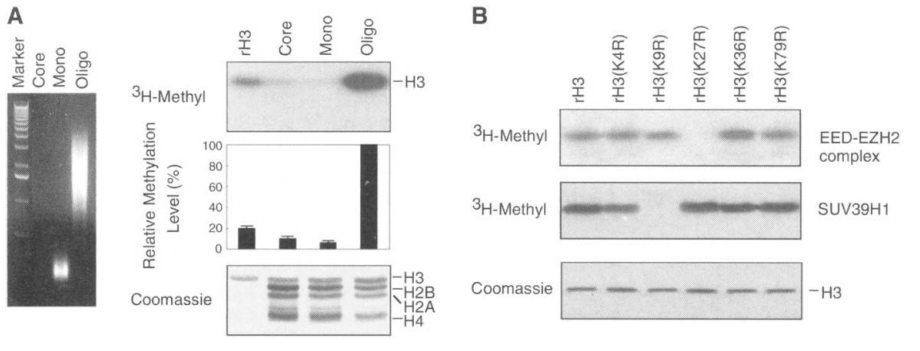

Fig. 2. The EED-EZH2 HMTase complex prefers oligonucleosomal histone substrate and methylates histone H3 at lysine 27. (**A**) Characterization of the substrate specificity of the EED-EZH2 complex. (Left) Ethidium bromide–stained agarose gel containing different substrates. (Right) Equal amounts of the enzyme complex were used to methylate equal amounts of histone H3 alone or in octamer, mono-, and oligonucleosome forms (bottom panel). The top panel is an autoradiograph of the experiment shown in the bottom panel. Quantification of the autoradiography is presented in the middle panel. (**B**) Determination of methylation site by the EED-EZH2 complex. Equal amounts of wild-type and mutant histone H3 (bottom panel) were methylated by EED-EZH2 complex (top panel) and SUV39H1 (middle panel), respectively. The lysines that were mutated are indicated at the top.

3A) of the histones from wild-type and $E(z)^{61}$ fly embryos at 18° and 29°C demonstrate that the H3-K27 methylation is abolished in the $E(z)^{61}$ embryos at 29°C (middle panel). However, these conditions do not have a detectable effect on H3-K9 methylation (top panel). Therefore, we conclude that functional E(Z) protein is required for H3-K27 methylation in vivo.

To understand the functional relation between E(Z)-mediated H3-K27 methylation and HOX gene silencing, we analyzed E(Z) binding, H3-K27 methylation, and recruitment of PC, a core component of the PRC1 complex, to the major PRE of the *Ubx* gene (*22*) in S2 tissue culture cells by chromatin immunoprecipitation (ChIP). Consistent with the involvement of E(Z) in H3-K27 methylation, ChIP analysis of a 4.4-kb region that includes this PRE (Fig. 3B) showed precise colocalization of E(Z) binding and H3-K27 methylation (Fig. 3C, left panel). In contrast, similar colocalization was not observed for mK9, indicating that H3-K9 methylation, or at least K9-dimethylation, is independent of E(Z) binding. This result is consistent with data presented in Figs. 2B and 3A. To further verify the importance of E(Z) binding for H3-K27 methylation, we attempted to disrupt ESC-E(Z) complex activity using RNA interference (RNAi). We reasoned that depletion of the ESC protein, a direct binding partner of E(Z) and a component of the ESC-E(Z) complex, would result in disruption of PRE binding by E(Z). Depletion of ESC with RNAi resulted in greatly reduced PRE binding by E(Z), loss of H3-K27 methylation, and concomitant loss of PC binding (Fig. 3C, right panel). Depletion of PC in S2 cells has been shown to result in derepression of *Ubx* (*23*). Therefore, these data collectively suggest that the ESC-E(Z) complex is critical not only for H3-K27 methylation, but also for PC binding to the PRE region, and that H3-K27 methylation is associated with *Ubx* repression.

To examine the relation between E(Z) binding, H3-K27 methylation, and *Ubx* gene repression in vivo, we dissected wing imaginal discs from homozygous $E(z)^{61}$ larvae that had been either reared continuously at 18°C or shifted from 18° to 29°C ~48 hours before dissection, and analyzed E(Z) binding and H3-K27 methylation in the same *Ubx* PRE region by ChIP. Consistent with previous studies demonstrating disruption of polytene chromosome binding by both $E(Z)^{61}$ and PC proteins at 29°C (*19*), ChIP analysis showed loss of $E(Z)^{61}$ and PC binding to this PRE at restrictive temperature (Fig. 3D, right panel). In addition, H3-K27 methylation colocalizes with E(Z) binding at permissive temperature, but is lost along with E(Z) binding at 29°C. In contrast, similar changes in H3-K9 methylation were not observed under the same conditions (Fig. 3D). Under normal

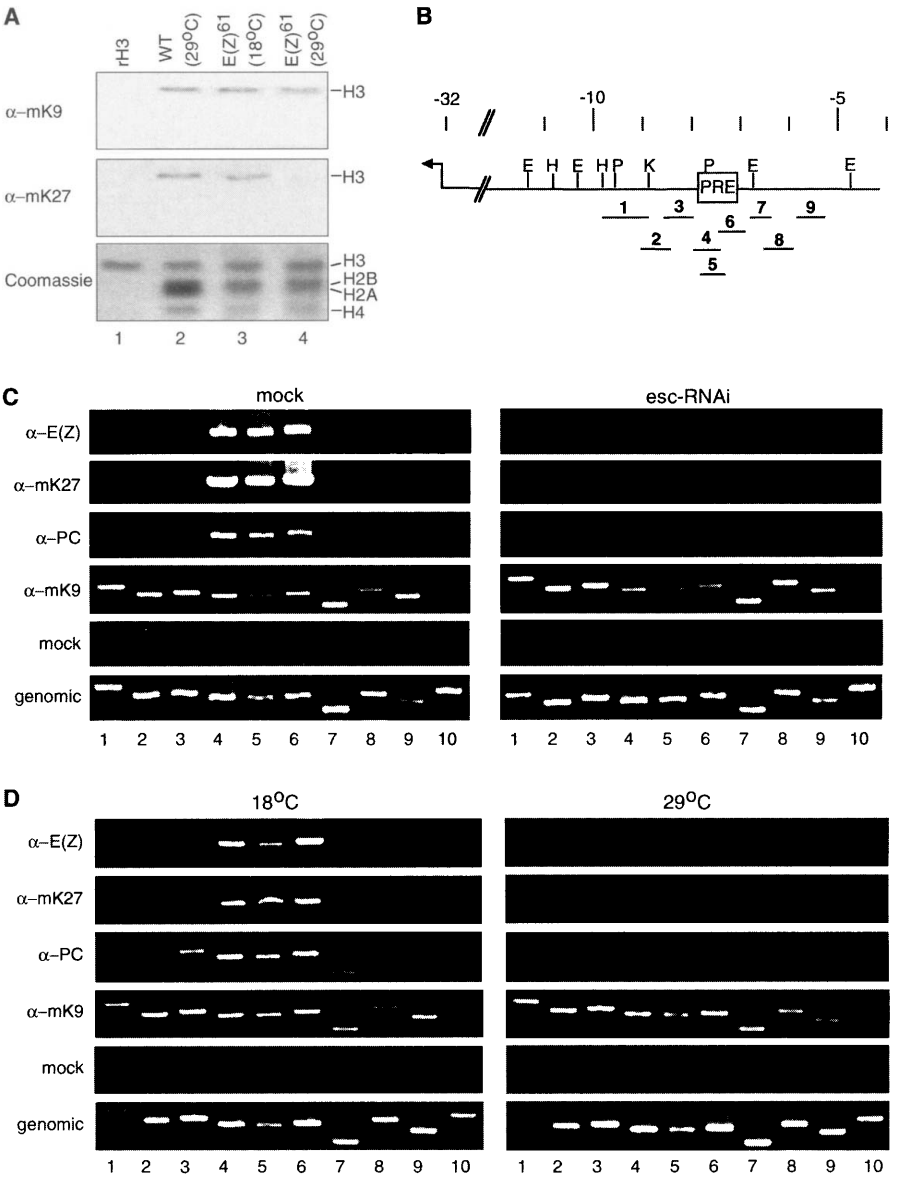

Fig. 3. Loss of E(Z) function abolishes H3-K27 methylation, PC binding, and *Ubx* gene silencing. (**A**) H3-K27 methylation depends on functional E(Z) protein. An equivalent amount (bottom panel) of histones purified from wild-type (lane 2) and mutant $E(z)^{61}$ (lanes 3 and 4) *Drosophila* embryos were probed with H3-mK9 (top panel)– or H3-mK27 (middle panel)–specific antibodies. (**B**) Map of the *Ubx* upstream regulatory region, including locations of the major PRE (*22*) and regions amplified by polymerase chain reaction (PCR) in ChIP assays. The scale above the map is according to a published numbering system (*34*) with the *Ubx* promoter at −32. The box indicates the position of PRE_D, and the horizontal lines below the map indicate the PCR-amplified regions. Restriction sites in this region are labeled as follows: E, Eco RI; H, Hind III; P, Pst; K, Kpn I. (**C**) Colocalization of E(Z), H3-K27 methylation, and PC to the PRE in mock-transfected S2 cells (left panel) and concomitant reduction of E(Z), H3-K27 methylation, and PC binding to the PRE in *esc*-RNAi–treated cells (right panel). (**D**) ChIP assays demonstrate colocalization of E(Z) binding and H3-K27 methylation in $E(z)^{61}$ wing imaginal discs at 18°C (left panel) and loss of binding in wing discs at 29°C (right panel). (C and D) Antibodies used in each assay are indicated on the left. Genomic DNA from each S2 cell culture or pooled collection of wing imaginal discs was amplified by PCR as a control for efficiencies of PCR primers. Numbers below the panels indicate the PCR primers used in each ChIP assay. Lanes 1 to 9 corresponding to the primer regions amplified are indicated in (B); lane 10 is a PCR product of RpII140 promoter, which served as a negative control.

conditions, *Ubx* is not expressed in wing discs due to PcG-mediated silencing (*24*). Similar inactivation of an *E(z)* temperature-sensitive allele during larval development has been shown to result in derepression of *Ubx* in wing discs (*25*). Thus, *Ubx* PRE-associated nucleosomes appear to be targeted by E(Z)-mediated H3-K27 methylation, which correlates with PC binding and repression of *Ubx*. Collectively, these data suggest that H3-K27

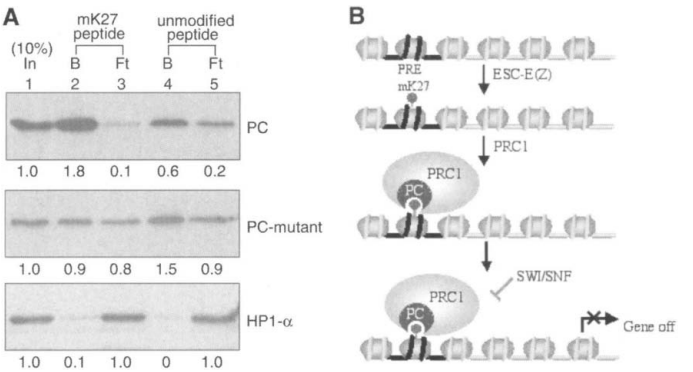

Fig. 4. H3-K27 methylation facilitates binding of PC to H3 through its chromodomain. **(A)** Autoradiographs of peptide pull-down assays. ^{35}S-labeled PC, PC mutant (W47A, W50A), and HP1-α were incubated with biotinylated H3 peptides (amino acids 19 to 35) that were either methylated or unmethylated at K27, in the presence of streptavidin-conjugated Sepharose beads. After extensive washing (see supplementary text), the beads were boiled with SDS loading buffer and resolved in SDS-polyacrylamide gels. "In" represents 10% of the total input used for the pull-down assays. "B" and "Ft" represent bound and flow-through, respectively. Numbers below each lane are quantified signals relative to inputs. **(B)** Model depicting the relation between ESC-E(Z)–mediated H3-K27 methylation and PcG silencing. PRE is represented by a thick line.

methylation plays an important role in the maintenance of *Ubx* gene silencing.

The chromodomain of the heterochromatin protein HP1 specifically binds to H3 tails that are methylated at K9 by the HMTase SUV39H1 (*26*, *27*). Given that PC contains a chromodomain and that loss of E(Z) function abolishes H3-K27 methylation as well as PC binding to the *Ubx* PRE (Fig. 3, C and D), it is possible that methylation of H3-K27 by ESC-E(Z) facilitates PRE binding by PC, analogous to the effect of H3-K9 methylation on nucleosome binding by HP1 (*26*, *27*). To test this possibility, we generated *Drosophila* PC using the rabbit reticulocyte transcription/translation-coupled system and incubated it with biotinylated H3 peptides with or without K27 methylation in the presence of streptavidin-conjugated Sepharose beads. Analysis by peptide pull-down assay indicated that methylation on K27 facilitates binding of PC to the H3 peptide (Fig. 4A, top panel; compare lanes 2 and 4). Binding of PC to the peptides is specific because the chromodomain-containing protein HP1 failed to bind to the same peptides under the same conditions (Fig. 4A, bottom panel).

Previous studies strongly suggest that the chromodomain of PC is necessary and sufficient for targeting PC to specific chromosomal locations in vivo because mutations in the PC chromodomain abolish the ability of PC to bind to chromatin in vivo (*28*). In addition, a chimeric PC/HP1 protein, in which the HP1 chromodomain is replaced by the PC chromodomain, binds to both heterochromatin and PcG target sites in euchromatin (*29*). To evaluate the contribution of the chromodomain in the preferential binding of PC to K27 methylated peptide, we generated a PC mutant in which two of the highly conserved amino acids Trp-47 and Trp-50 were changed to Ala. These two amino acids were chosen because the corresponding amino acids in the HP1 chromodomain have been shown to directly contact the methyl group of an H3-mK9 peptide (*30*, *31*). The mutant PC did not preferentially bind to the K27 methylated peptide (Fig. 4A, middle panel), suggesting that the chromodomain of PC is responsible for the preferential binding to the H3-mK27.

Collectively, our studies support a model in which ESC-E(Z)–mediated H3-K27 methylation serves as a signal for the recruitment of the PRC1 complex by facilitating PC binding (Fig. 4B). Recruitment of PRC1 in turn prevents the access of nucleosome remodeling factors, such as SWI/SNF (*2*, *32*), leading to the formation of a repressive chromatin state (Fig. 4B). Although this model is attractive, it does not exclude the possibility that protein-protein interaction also contributes to the recruitment of PRC1 to PREs. Indeed, a recent study indicates that PC transiently associates with the ESC-E(Z) complex during early embryogenesis (*33*). Our studies established a correlation between H3-K27 methylation and PcG silencing. Further work is needed to establish the exact role of H3-K27 methylation in PcG silencing.

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Materials and Methods
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