Mechanism of Polycomb Group Gene Silencing

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The *Drosophila* trithorax-group (trxG) and Polycomb-group (PcG) proteins function in an antagonistic manner to maintain the transcriptionally active and silence states of target genes, respectively. Although they regulate numerous genes, mutant alleles of most trxG and PcG genes were first identified on the basis of homeotic phenotypes resulting from misexpression of Hox genes of the Antennapedia and bithorax gene complexes. *Drosophila* Hox genes, which encode transcription factors that regulate numerous downstream genes, must be continuously expressed in appropriate patterns throughout embryonic and larval development in order to assign segmental identities to cells along the anterior–posterior body axis. The expression patterns of the Hox genes are initially established in early embryos by activators and repressors encoded by gap and pair rule genes, but soon after Hox gene expression is initiated, these activators and repressors decay. It is during this window of time that trxG and PcG proteins somehow recognize the transcriptionally active or repressed states of Hox genes and become responsible for maintaining their expression states in cell lineages throughout embryonic and larval development. Thus, trxG and PcG proteins serve as molecular memory systems central to the process of cellular determination (Francis and Kingston 2001; Simon and Tamkun 2002).

Here we discuss our recent progress in understanding the mechanisms of PcG silencing, but, because PcG proteins function antagonistically to the trxG, we will first briefly describe the trxG and the mechanisms by which they help maintain transcriptional activity. The trxG comprises approximately 20 genes. Several encode components of the 2-MD Brahma (BRM) complex, which is a member of the SWI/SNF family of nucleosome remodeling complexes (Papoulas et al. 1998; Kal et al. 2000), and others encode proteins that are members of the SWI2/SNF2 family of ATPases, but are physically independent of the BRM complex (Daubresse et al. 1999; Ruhf et al. 2001). Two members of the trxG, Trithorax (Trx) and Abnormal small or homeotic discs-1 (ASH-1), contain SET domains [Su(var)3-9, Enhancer of zeste, Trx], conserved domains present in numerous chromatin proteins that possess histone lysine methyltransferase (HMTase) activity (Jenuwein et al. 1998; Rea et al. 2000). Both Trx and ASH-1 methylate histone H3 at lysine 4 (H3-K4) (Beisel et al. 2002; Byrd and Shearn 2003; Smith et al. 2004), a modification generally associated with gene activation (Bernstein et al. 2002). Trx coexists in the 1-MD TAC1 complex with dCBP, a histone acetyltransferase, and dSbf1 (Petruk et al. 2001).

Originally identified as regulators of *Drosophila* Hox genes, PcG homologs have since been identified across a wide phylogenetic spectrum, including *Caenorhabditis elegans*, *Arabidopsis thalania*, and mammals. A list of *Drosophila* PcG proteins and their mammalian homologs are provided in Table 1. Thus, PcG proteins appear to be an evolutionarily conserved gene-silencing system that has been adapted for the regulation of different genes and developmental purposes. The *Drosophila* PcG comprises approximately 15 genes, many of which encode components of multiprotein complexes. The Polycomb repressive complex 1 (PRC1) contains the PcG proteins Polycomb (Pc),

Table 1. A List of Known PcG Proteins in *Drosophila* and Mammalians

Drosophila proteins	Human proteins	Mouse proteins
Sequence-specific DNA-binding proteins		
Pho	YY1	Yy1
Phol	YY1	Yy1
Esc-E(z) complex		
Esc	EED	Eed
E(z)	EZH1	Ezh1/Enx2
	EZH2	Ezh2/Enx1
Su(z)12	SUZ12	Suz12
PRC1 complex		
Pc	HPC1/CBX2	M33/Cbx2
	HPC2/CBX4	Mpc2/Cbx4
	HPC3/CBX8	
Ph	HPH1/EDR1	Mph1/Rae28/Rae28
	HPH2/EDR2	Mph2/Edr2
	HPH3/EDR3	
dRing/Sce	RING1/RNF1/RING1A	Ring1/Ring1a
	RING1B/RNF2	Ring1b/Rnf2
Psc	BMI1	Bmi1
	ZFP144/RNF110	Mel18/Zfp144/Rnf110
	ZNF134	Znf134/Mblr
Undefined function		
Asx	ASXL1	
	ASXL2	
Crm		
Mxc		
Scm	SCML1	Scmh1
	SCML2	Scmh2
Pcl	hMTF2	MTF2
	PHF1	
Sxc		

polyhomeotic (Ph), Posterior sex combs (Psc), dRing1 (also known as Sex combs extra, Sce; Fritsch et al. 2003), in addition to Zeste (which has been also classified as a trxG protein), dSbf1, HSC4, and five general transcription factors (dTAFIIs 250, 110, 85, 62, and 42) (Saurin et al. 2001). A second complex, referred to as Esc-E(z), contains the PcG proteins Extra sex combs (Esc), Enhancer of zeste [E(z)], and Suppressor 12 of zeste [Su(z)12], in addition to the histone-binding protein NURF-55. The histone deacetylase HDAC1 (Rpd3) has been identified in some forms of the complex (Tie et al. 2001; Czermin et al. 2002), but is absent from others (Müller et al. 2002). The human counterparts of both complexes have been purified and the core components are found to be conserved (Cao et al. 2002; Levine et al. 2002).

To fully understand the molecular mechanism of PcG-mediated gene silencing, several major questions must be addressed. (1) How is the repressed state of target genes initially recognized? (2) What are the mechanisms by which PcG proteins repress transcription? (3) How is the silenced state faithfully transmitted through many cycles of cell division? Here, we describe our recent studies aimed at addressing the latter two questions. In particular, we will discuss the role of sequence-specific DNA-binding PcG proteins Pleiohomeotic (Pho) and Pho-like (Phol) and H3-K27 methylation by ESC-E(Z)/EED-EZH2 complexes in maintenance of transcriptional silencing. We will also examine the roles of both catalytic and noncatalytic subunits of this HMTase complex and how they contribute to H3-K27 methylation. In addition, we will discuss the mechanisms by which PcG proteins may repress transcription, including the contribution of a novel enzymatic activity associated with the PRC1 complex.

MATERIALS AND METHODS

All materials used and methods described in the studies presented here have been previously described as indicated throughout the text.

RESULTS

Purification and Characterization of the EED-EZH2 HMTase Complex

Histone tails are rich in covalent modifications that include acetylation, methylation, ubiquitination, and phosphorylation (van Holde 1988). While acetylation on lysine residues generally correlates with gene activation, methylation on lysine residues results in either gene activation or repression depending on the particular lysine residues that are methylated (Zhang and Reinberg 2001; Lachner et al. 2003). In an attempt to understand the function of histone methylation, we have been using a systematic biochemical approach to purify and characterize histone methyltransferases from HeLa cells (Fang et al. 2003). Of the six HMTases that we have characterized so far, the EED-EZH2/ESC-E(Z) complex is of particular interest because of its roles in diverse biological processes including PcG silencing, X-inactivation, germ-line development, stem cell pluripotency, and cancer (Cao and Zhang 2004a).

By following a nucleosomal histone H3 methyltransferase activity, we had previously purified a protein complex of about 500 kD from HeLa cells (Cao et al. 2002). The complex is composed of five subunits including EZH2, SUZ12, AEBP2, EED, and RbAp48 (Fig. 1a). A similar protein complex was also purified independently by several other groups (Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002). RbAp48 is a WD40-repeat protein initially identified as a Rb-binding protein (Qian et al. 1993). Subsequent studies revealed the presence of this protein in many protein complexes involved in histone modification and nucleosome remodeling, consistent with the notion that this protein is a histone-binding protein (Verreault et al. 1998). AEBP2 is a zinc finger transcriptional repressor that may contribute to targeting of the complex to specific genes (He et al. 1999). EZH2, EED, and SUZ12 are PcG proteins (Table 1). Since, with the exception of AEBP2, the composition

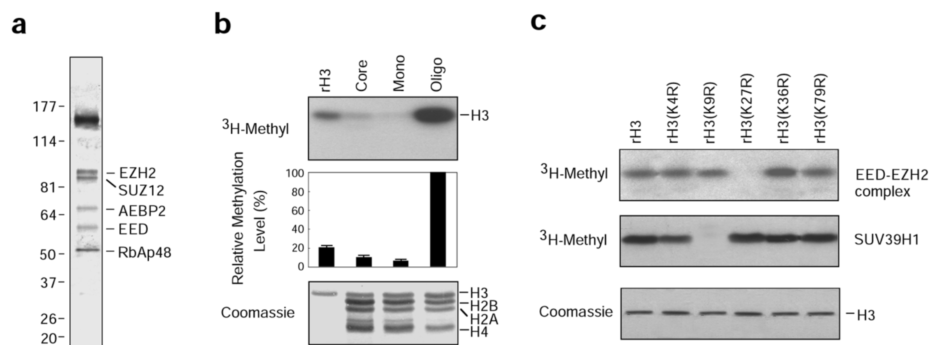

Figure 1. Purification and characterization of the EED-EZH2 histone methyltransferase complex. (*a*) Coomassie-stained polyacrylamide-SDS gel containing the purified EED-EZH2 complex. The identity of the proteins in the complex is indicated. The largest prominent protein is a contaminant. The protein size markers are indicated. (*b*) The EED-EZH2 HMTase complex prefers oligonucleosomal histone substrate. Equal amounts of the enzyme complex were used to methylate equal amounts of histone H3 alone, in octamer, mono-, and oligonucleosome forms (*bottom* panel). The *top* panel is an autoradiography of the *bottom* panel. Quantification of the autoradiography is presented in the *middle* panel. (*c*) EED-EZH2 complex methylates H3 at lysine 27. 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 on top of the panel. (Adapted, with permission, from Cao et al. 2002 [©AAAS].)

of this complex is conserved in the *Drosophila* ESC-E(Z) complex (Ng et al. 2000; Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002), we refer to it as the EED-EZH2 complex. The facts that most subunits of the complex belong to the PcG proteins and that EZH2 contains a SET domain suggest a potential link between the intrinsic HMTase activity of the complex and PcG silencing.

To understand the relationship between PcG silencing and the HMTase activity, we characterized the enzymatic activity further by determining its substrate specificity and the lysine residue on H3 that the complex methylates. Toward this end, equivalent amounts of isolated histone H3, histone H3 assembled with other core histones, and mono- or oligonucleosomes were subjected to methylation by equal amounts of the enzyme complex. Results shown in Figure 1b indicate that the enzyme complex has a strong preference for H3 in oligonucleosome form. To identify the lysine residue that the complex methylates, we generated H3 mutants in which each of the five potential methylation sites (K4, K9, K27, K36, and K79) was individually mutated. The effect of the mutations on the ability of H3 to serve as substrates for the enzyme complex was evaluated. As a control, the ability of these H3 mutants to serve as substrates for the H3-K9 methyltransferase SUV39H1 was also analyzed. Results shown in Figure 1c (top panel) indicate that mutation on K27 completely abolished the ability of H3 to serve as a substrate, whereas mutations on other sites had little effect. As expected, only mutation of K9 affected the SUV39H1-mediated H3 methylation (Fig. 1c, middle panel). These results strongly suggest that H3-K27 is the target site of methylation for the complex. To further verify the result, oligonucleosomes were subjected to methylation. After purification, the methylated H3 was subjected to microsequencing followed by liquid scintillation counting. This again revealed that K27 is the target site (Cao et al. 2002). Therefore, we conclude that the EED-EZH2 complex prefers oligonucleosomal substrates and methylates H3-K27.

H3-K27 Methylation Is Required for PRE Binding by PC and *Ubx* Gene Silencing

To study the function of H3-K27 methylation in vivo, we generated a polyclonal antibody that recognizes methylated, but not nonmethylated, H3-K27 (Cao et al. 2002). Using this antibody, we evaluated whether the *Drosophila* ESC-E(Z) complex is responsible for H3-K27 methylation in vivo. Previous studies have identified an *E(z)* temperature-sensitive allele, $E(z)^{61}$, which contains a Cys-to-Tyr substitution (C603Y) in the cysteine-rich region immediately preceding the SET domain (Carrington and Jones 1996). At 18ºC (permissive temperature), the protein functions normally and $E(z)^{61}$ homozygotes exhibit no detectable mutant phenotype and maintain wild-type expression patterns of Hox genes, such as *Ubx* (Jones and Gelbart 1990; Carrington and Jones 1996). However, at 29ºC (restrictive temperature), *E(Z)61*protein fails to bind to chromatin leading to disruption of chromosome binding by Polycomb (PC) and other PRC1 components (Rastelli et al. 1993; Platero et al. 1996). As a result, $E(z)^{61}$ produces multiple homeotic phenotypes because of derepression of Hox genes (Jones and Gelbart 1990). Therefore, if E(Z) is responsible for H3-K27 methylation in vivo, we expect partial or complete loss of H3-K27 methylation when $E(Z)^{61}$ mutants are shifted from 18ºC to 29ºC. Results shown in Figure 2a confirm this prediction and demonstrate that H3-K27 methylation is dramatically decreased in the $E(z)^{61}$ embryos at 29ºC (middle panel). However, these conditions do not affect H3-K9 methylation (top panel). Therefore, we conclude that functional E(Z) protein is required for H3-K27 methylation in vivo.

Previous studies have demonstrated that transcriptional silencing of the *Ubx* gene requires both the ESC-E(Z) and the PRC1 complexes, in addition to a *cis*-acting Polycomb response element (PRE), to which the two complexes bind. To understand the functional relationship 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 (Francis et al. 2001), to the major *Ubx* PRE (PRE_D) by chromatin immunoprecipitation (ChIP) (Fig. 2b). Analysis of S2 tissue culture cells revealed a precise colocalization of E(Z), H3-K27 methylation, and PC binding to the PRE_D region (Cao et al. 2002). Importantly, disruption of the ESC-E(Z) complex by RNAi resulted in greatly reduced E(Z) binding, H3-K27 methylation, and concomitant loss of PC binding to the PRE (Cao et al. 2002), suggesting that ESC-E(Z)-mediated H3-K27 methylation contributes to PRE binding by PC. We also performed similar experiments using dissected wing imaginal discs from homozygous $E(z)^{61}$ larvae, which had been either reared continuously at 18ºC or shifted from 18ºC to 29ºC ~48 hours prior to dissection. Results shown in Figure 2c (left panels) demonstrate that at permissive temperatures, as in S2 cells, $E(Z)^{61}$ binding, H3-K27 methylation, and PC binding colocalize at the PRE_D region. At restrictive temperatures, however, loss of $E(Z)^{61}$ binding is concomitant with loss of H3-K27 methylation and PC binding (Fig. 2c, right panels). In contrast, similar changes in H3-K9 methylation were not observed under the same conditions (Fig. 2c). Similar inactivation of an *E(z)* temperature-sensitive allele during larval development has been shown to result in significant derepression of *Ubx* in wing discs (LaJeunesse and Shearn 1996). Collectively, these data suggest that H3-K27 methylation plays an important role in the maintenance of *Ubx* gene silencing.

PC Chromodomain Recognizes Methyl-K27 of H3

The "histone code" hypothesis predicts that single or combinational histone modifications may serve as molecular marks that can be recognized by specific protein modules or domains that in turn direct the functional consequence of the modification (Strahl and Allis 2000; Turner 2000). Consistent with this hypothesis, the chromodomain of the heterochromatin protein HP1 has been demonstrated to specifically bind to H3 tails that are methylated at K9 by the HMTase SUV39H1 (Bannister et al. 2001; Lachner et al. 2001). Several lines of evidence

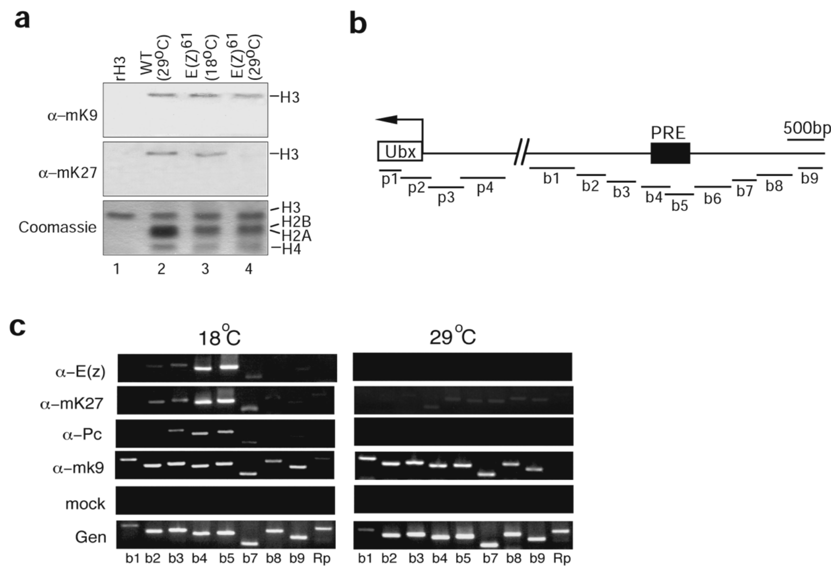

Figure 2. Loss of E(Z) function abolishes H3-K27 methylation, PC binding, and Ubx gene silencing. (*a*) H3-K27 methylation depends on functional E(Z) protein. Equivalent amounts (*bottom* panel) of histones purified from wild-type (lane *2*) and mutant $E(z)^{61}$ (lanes *3* and *4*) *Drosophila* embryos were probed with H3-2mK9- (*top* panel) or H3-2mK27- (*middle* panel) specific antibodies. (*b*) Schematic representation of the Ubx promoter and bxd PRE regions. The regions amplified by PCR in these and subsequent ChIP assays, p1–p4 and b1–b9, are depicted as horizontal lines below. (Adapted from Wang et al. 2004.) (*c*) ChIP assays demonstrate colocalization of E(Z) binding and H3-K27 methylation in $E(z)^{61}$ wing imaginal discs at 18ºC (*left* panel) and loss of binding in wing discs at 29ºC (*right* panel). Antibodies used in each assay are indicated on the *left*. Genomic DNA from pooled collection of wing imaginal discs was PCR amplified as controls for efficiencies of PCR primers. Numbers below the panels indicate the PCR primers used in each ChIP assay. Lanes *1–9* corresponding to the regions are as indicated in *b*; lane *10* is a PCR product of RpII140 promoter, which served as a negative control. (Adapted, with permission, from Cao et al. 2002 [©AAAS].)

suggest that the chromodomain of PC may recognize H3 tails methylated at K27, analogous to that of the HP1 binding to H3 tails methylated on K9. First, the chromodomain of PC is both necessary and sufficient for targeting PC, as well as other components of the PRC1 complex, to specific chromosomal locations in vivo (Messmer et al. 1992; Platero et al. 1995). Second, loss of E(Z) function abolishes H3-K27 methylation as well as PC binding to the *Ubx* PRE (Fig. 2c). Third, all the amino acids in HP1 chromodomain that are involved in methyllysine binding are conserved in the PC chromodomain. These lines of evidence prompted us to test the *Drosophila* PC protein, generated using the rabbit reticulocyte transcription/translation system, for its ability to bind to biotinylated H3 peptides with or without K27 methylation. Results shown in Figure 3a (top panel) indicated that methylation on K27 facilitates binding of PC to the H3 peptide. This binding is mediated through the chromodomain as mutations in two of the highly conserved amino acids within the chromodomain (W47A, W50A) abolished preferential binding of PC to the methylated peptide (Fig. 3a, middle panel). 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. 3a, bottom panel).

The above in vitro binding results were recently confirmed by structural studies in which the PC chromodomain in complex with an H3 peptide trimethylated on K27 was crystallized and the structure solved (Fischle et al. 2003; Min et al. 2003). The study revealed a conserved mode of methyl-lysine binding and provided structural basis for specific recognition of PC chromodomain to histone H3 methylated on K27, but not K9. As shown in Figure 3b, the *Drosophila* PC chromodomain consists of three β strands (β1–β3) and a carboxy-terminal helix (αA). The histone H3 peptide is bound in a cleft formed between the PC amino terminal to β1 and the loop connecting β3 and αA. Although the overall structures of PC and HP1 chromodomains are very similar (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002), differences between the two chromodomains are noticeable. For example, while the methyl-lysine-binding pocket of HP1 interacts with methyl-K9 via hydrophobic interaction, the corresponding aromatic residues on PC interact with methyl-K27 through cation–π interactions. In addition, unique interactions between Leu 20, Thr 22 of histone H3, and Arg 67 of PC were noticed. However, these interactions cannot account for the binding specificity of PC chromodomain to methyl-K27, but not methyl-K9, because only the main-chain atoms of histone H3 are involved in the interaction (Min et al. 2003).

A careful examination of the cocrystal structure identified a potential chromodomain dimer that can account for the binding specificity of PC chromodomain to methyl-K27. As depicted in Figure 3c, the chromodomain dimer interacts via intermolecular hydrogen bonds between the main-chain atoms of Leu 64 and Arg 66, which appear to be specific to the PC family of proteins. An additional hydrogen bond can also form between Arg 66 and Val 61. The chromodomain dimer juxtaposes the two H3-binding clefts in an antiparallel fashion and results in histone–histone interactions involving Leu 20, Thr 22, and Ala 24

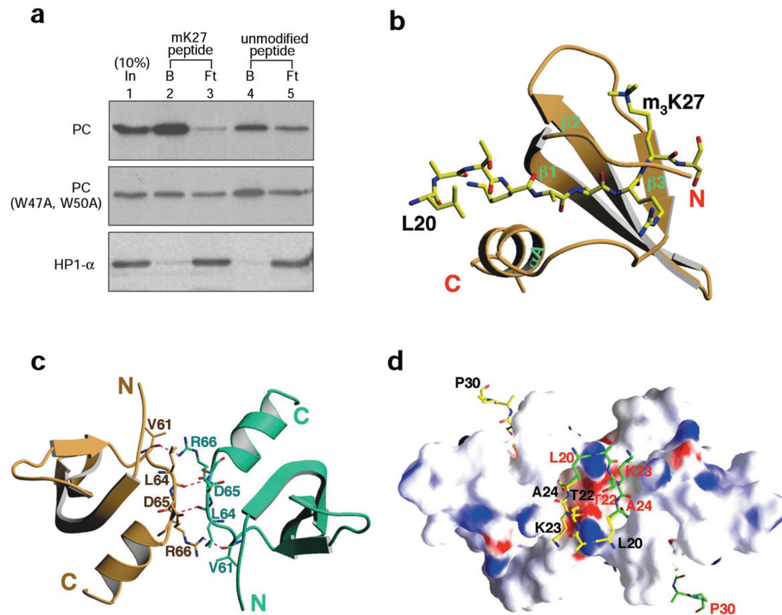

Figure 3. H3-K27 methylation facilitates binding of PC to H3 through its chromodomain. (*a*) Autoradiographs of peptide pulldown experiments. ^{35}S-labeled PC, PC mutant (W47A, W50A), and HP1-α were incubated with biotinylated H3 peptides (aa 19–35), which were either methylated or unmethylated at K27, in the presence of streptavidin-conjugated Sepharose beads. After extensive washing, the beads were boiled with SDS loading buffer and resolved in SDS-polyacrylamide gels. In: 10% of the total input used for the pulldown assays; B: bound; Ft: flowthrough. (*b*) Overall structure of the *Drosophila* PC chromodomain (aa 23–77) in complex with a histone H3 peptide (aa 19–33) trimethylated on K27. The choromodomain is shown in a ribbon diagram (*brown*), and the H3 peptide is shown as a ball-and-stick model (*red,* oxygen; *blue,* nitrogen; and *yellow,* carbon). (*c*) The PC chromodomain dimer. The PC chromodomain are shown in a ribbon representation (*brown* and *cyan*). Key residues involved in dimerization are shown in a bond model. Hydrogen bonds involving these residues are indicated with *broken lines*. (*d*) The PC chromodomain dimer juxtaposes the two binding sites of methyl-K27 of H3. The PC chromodomain dimer is shown as surface representation (*red,* negatively charged area; *blue,* positively charged area; *white,* neutral). Two bound H3-3mK27 peptides are shown in a ball-and-stick model. (Courtesy of Dr. Rui-Ming Xu.)

(Fig. 3d). This recognition mode can effectively exclude the binding of a histone H3 peptide encompassing methylated Lys 9, as the residues corresponding to Leu 20, Thr 22, and Ala 24 of H3 would be Arg 2, Lys 4, and Thr 5, respectively. Therefore, the key determinants that confer specific recognition of methyl-K27 by PC chromodomain are both the histone H3 sequence (Leu 20, Thr 22, and Ala 24) and the dimerization of the PC chromodomain.

H3-K27 Methylation Contributes to PC/PRC1 Recruitment

As described above, loss of E(Z) results in rapid loss of H3-K27 methylation and PC binding to the PRE_D region. Previous studies also suggest that the E(Z) complex can transiently interact with components of the PRC1 complex (Poux et al. 2001). Therefore, results from the above study cannot distinguish between the contribution of H3-K27 methylation and the physical interaction between the ESC-E(Z) complex and the PRC1 components in PC recruitment. However, ChIP analysis indicate that E(Z), PC, and trimethyl H3-K27 are also present near the *Ubx* promoter in wing imaginal discs (Fig. 4a). Following inactivation of $E(Z)^{61}$ and loss of the HMTase complex, H3-K27 methylation is maintained near the *Ubx* promoter for ~24 hours. PC also remains near the *Ubx* promoter in the absence of E(Z), but is finally lost when H3-K27 methylation is no longer detectable (Fig. 4b). Thus, PC binding correlates with H3-K27 methylation, but not with the physical presence of E(Z)-containing complex, consistent with H3-K27 methylation serving as a tag that is primarily responsible for recruiting PC-containing complexes.

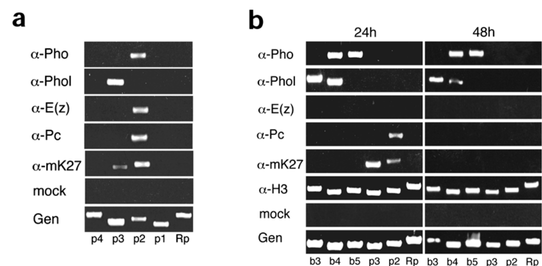

Figure 4. Pc binding at the *Ubx* promoter and PRE_D regions is dependent on H3-K27 methylation. (*a*) ChIP assays showing distribution of PcG proteins and H3-3mK27 in the *Ubx* promoter region in wing imaginal discs. Wing imaginal discs were dissected from $E(z)^{61}$ larvae reared continuously at 18ºC. (*b*) ChIP assays of wing imaginal discs from $E(z)^{61}$ larvae shifted from 18ºC to 29ºC (*left*) 24 hr or (*right*) 48 hr prior to dissection. α-H3, anti-histone H3 was used as a positive control in the ChIP assays shown in this figure and in Fig. 5. (Adapted, with permission, from Wang et al. 2004 [©Elsevier].)

Hierarchical Recruitment of PcG Complexes

PcG proteins maintain the transcriptionally silenced state of target genes through many cell cycles. Both initiation and maintenance of transcriptional silence require a *cis*-acting PRE. Reporter genes contained within P element constructs become derepressed after one to a few cell generations following deletion of a flanking PRE (Busturia et al. 1997; Sengupta et al. 2004). Several components of the PRC1 dissociate from chromosomes in tissue culture cells during mitosis (Buchenau et al. 1998). This suggests that proteins capable of binding directly to sites within PREs play important roles in repeatedly recruiting PcG proteins to PREs following mitosis. PREs contain binding sites for several sequence-specific DNA-binding proteins. These include GAGA factor, Pipsqueak (Psq), Pleiohomeotic (Pho), and Pho-like (Phol). GAGA factor and Psq both bind to GAGAG repeats and mutation of these sequences in PRE_D within the context of a P element transgene results in partial derepression of a reporter gene in embryos (Horard et al. 2000; Hodgson et al. 2001). However, GAGA factor and Psq may not be required for maintenance of PcG silencing in larvae, since mutation of PRE GAGAG sites does not affect repression of a reporter gene in imaginal discs (Fritsch et al. 1999).

Pho and Phol are homologs of human Yin Yang 1 (YY1), and are identical in their sequence-specific DNA-binding activities in vitro (Brown et al. 1998, 2003). Maternally expressed Pho is needed early in embryogenesis in order to establish PcG silencing, but individuals that are homozygous for null *pho* alleles (derived from heterozygous mothers) die as late pupae with relatively mild homeotic phenotypes and show only moderate derepression of Ubx in wing imaginal discs (Brown et al. 2003). The relatively mild zygotic phenotypes of *pho* mutants appear to be due to functional redundancy with *phol*. Although *phol* nulls are homozygous viable, *phol*;*pho* double mutants exhibit extensive *Ubx* derepression in wing imaginal discs and die as late larvae/early pupae (Brown et al. 2003). This suggests that Pho and Phol play important roles in maintaining PcG silencing during larval development and that their functions may be partially redundant. We have recently demonstrated that PHO and PHOL directly interact with E(Z) and/or ESC (Wang et al. 2004), which suggests that the ESC-E(Z) complex may be recruited to PREs through protein–protein interactions with PHO and/or PHOL. ChIP analysis of wing imaginal discs revealed the presence of PHO and PHOL in the PRE_D and *Ubx* promoter regions at sites that overlapped those of E(Z) and PC (Figs. 4a, 5a). Binding by neither E(Z) nor PC was affected in *phol* or *pho* mutant wing imaginal discs. However, binding by both E(Z) and PC is lost in *phol;pho* double mutants (Fig. 5b). Taken together with the role of H3-K27 methylation in PC recruitment described above, we propose the following hierarchical pathway of PcG recruitment. PHO and/or PHOL bind to sites within PREs and directly recruit ESC-E(Z) complex, which then methylates H3 at K27. The PC chromodomain then binds to the methylated H3-K27 tag, facilitating recruitment of PC-containing complexes such as PRC1 (Fig. 6).

In addition to their presence at the PRE_D region, Pc, E(z), Pho, and Phol are associated with discrete regions near the transcription start site (Fig. 4a). In the absence of

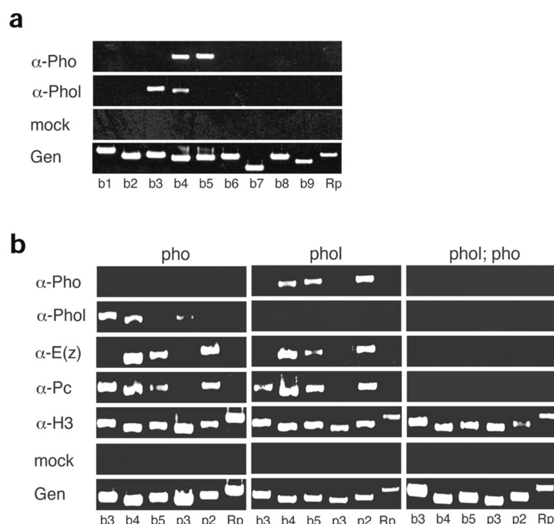

Figure 5. Pho and Phol are redundant for recruitment of E(z)- and Pc-containing complexes. (*a*) ChIP assays showing distribution of Pho and Phol in the PRE_D region in wing imaginal discs. (*b*) ChIP assays of wing imaginal discs dissected from pho^{1} (*left*), $phol^{81A}$ (*middle*), or pho^{1};$phol^{81A}$ (*right*) larvae. (Adapted, with permission, from Wang et al. 2004 [©Elsevier].)

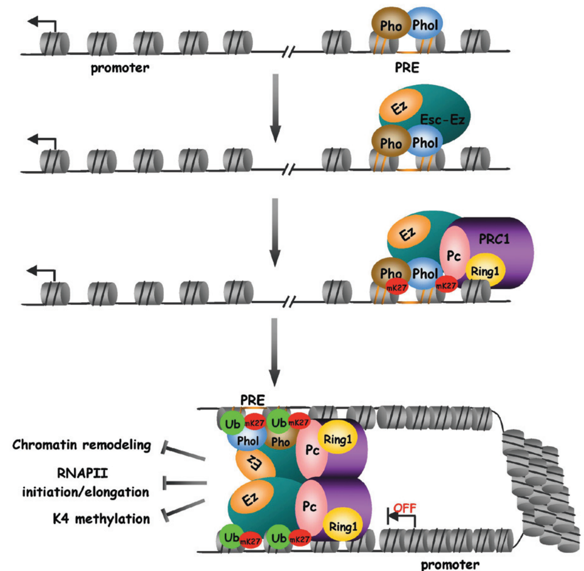

Figure 6. Model depicting the mechanism of PcG silencing. Binding of transcription factors, such as Pho and Phol, to PRE initiates the recruitment of the ESC-E(Z) complex, which methylates H3-K27. H3-3mK27, recognized by the chromodomain of PC, serves as a marker for the recruitment of the PRC1 complex. A yet-to-be-identified mechanism mediates loop formation bringing the PRE and associated PcG proteins into proximity of the transcriptional start site, which inhibits transcription by (1) interfering with chromatin remodeling, (2) directly inhibiting transcription initiation or elongation by RNAPII, and/or (3) ubiquitinating histone H2A, thereby inhibiting H3-K4 methylation by Trx and/or Ash1 complexes.

E(z), Pho and Phol remain at the PRE, but are no longer detected near the *Ubx* promoter (Fig. 4b). This is consistent with a model in which PcG proteins assemble at the PRE followed by the formation of a loop that brings them into contact with the promoter (Fig. 6). Assuming this model is correct, it is not clear what may mediate loop formation. PRC1 has been shown to be able to recruit chromatin templates *in trans* (Lavigne et al. 2004). Alternatively, the sequence-specific DNA-binding protein Zeste has been shown to be a component of PRC1 (Saurin et al. 2001), raising the possibility that Zeste may mediate loop formation.

Evolutionary Conservation of PcG Gene Silencing

As listed in Table 1, PcG proteins have been structurally and functionally conserved during evolution. In addition, the core components of the ESC-E(Z)/EED-EZH2 and the PRC1 complexes are conserved from *Drosophila* to human (Francis et al. 2001; Cao et al. 2002; Levine et al. 2002; Muller et al. 2002). One of the conserved functions of PcG proteins is their involvement in Hox gene silencing. For example, PcG mutations in *Drosophila* or mice result in homeotic transformation because of derepression of Hox genes (Kmita and Duboule 2003). Data presented above illustrate the importance of ESC-E(Z)-mediated H3-K27 methylation in *Ubx* gene silencing. To examine whether the function of H3-K27 methylation is conserved in mammalian cells, we reconstituted the human EED-EZH2 complex and demonstrated that the HMTase activity requires a minimum of three components, including EZH2, EED, and SUZ12. Addition of RbAp48 and AEBP2 stimulated the enzymatic activity (Cao and Zhang 2004b).

To evaluate the role of SUZ12 in H3-K27 methylation in vivo, we generated a stable SUZ12 knockdown cell line that expresses ~25% of the normal levels of SUZ12 protein and ~35% of the normal levels of SUZ12 mRNA (Fig. 7a). Compared with the control empty vector knockdown cells, SUZ12-targeted knockdown resulted in a significant decrease on the trimethyl-K27 level but had little effect on the trimethyl-K9 level (Fig. 7b, third and fourth panels). Interestingly, an increase in monomethyl-K27 and a moderate decrease in dimethyl-K27 were also observed (Fig. 7b, top two panels). The fact that SUZ12 knockdown does not affect EZH2 level (Fig. 7a) in combination with the requirement of SUZ12 for H3-K27 methyltransferase activity in vitro (Fig. 7b) allows us to conclude that SUZ12 directly contributes to H3-K27 methylation in vivo.

Previous studies in *Drosophila* have established a critical role for Su(z)12 in Hox gene silencing (Birve et al. 2001). The fact that SUZ12 is required for H3-K27 methylation in combination with the fact that H3-K27 methylation is critical in Hox gene silencing (Cao et al. 2002; Muller et al. 2002) predict that SUZ12 knockdown will result in derepression of at least some Hox genes. Analysis of HoxC6, HoxC8, and HoxA9 in the knockdown cells and the parallel control cells revealed derepression of HoxC8 and HoxA9 genes in the knockdown cells (Fig. 7c). These data support the notion that, like most other PcG proteins, the function of SUZ12/Su(z)12 in Hox gene silencing is conserved from human to *Drosophila*.

CONCLUSIONS AND FUTURE DIRECTIONS

As a result of these and other studies, we can now begin to assign molecular/biochemical activities to more than half of the known PcG proteins. We propose that PcG proteins may be placed in either of two categories: Recruiters or Effectors. Proteins such as Pho, Phol, or their mammalian homolog YY1 and components of the Esc-E(z)/EED-EZH2 complex primarily function as Recruiters. The sequence-specific DNA-binding Pho and Phol bind to sites within PREs and directly recruit ESC-E(Z) complexes, which in turn methylates H3 at K27 in the immediate vicinity of the PRE. The PC chromodomain binds to the methylated H3-K27 tag, facilitating recruitment of PRC1, or related complexes (Fig. 6). Thus,

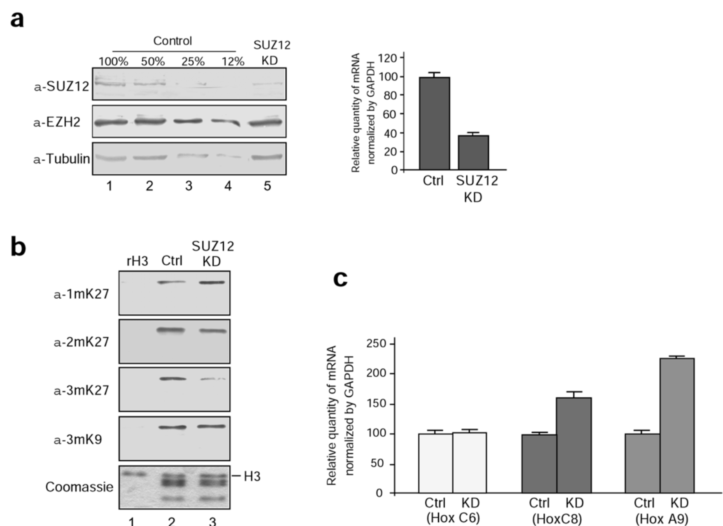

Figure 7. SUZ12 knockdown affects H3-K27 methylation and Hox gene expression. (*a*) Western blot (*left* panel) and quantitative RT-PCR (*right* panel) analysis of a SUZ12 stable knockdown cell line and a parallel mock knockdown cell line. Tubulin serves as a loading control for Western blotting. GAPDH serves as control for normalization in the quantitative RT-PCR. (*b*) Western blot analysis of histones extracted from control and knockdown HeLa cells with antibodies specific for mono-, di-, or trimethylated K27 and trimethylated K9. Equal loading of histone H3 was verified by Coomassie staining of a parallel gel (*bottom* panel). (*c*) Quantitative RT-PCR analysis of HoxC6, HoxC8, and HoxA9 expression in SUZ12 knockdown and mock knockdown cells. GAPDH was used as a control for normalization. Quantification is an average of two independent experiments with error bars. (Adapted from Cao and Zhang 2004b.)

the primary function of H3-K27 methylation in PcG silencing appears to be recruitment of PC-containing complexes. In vitro studies suggest that PRC1 may be classified as an Effector of transcriptional repression, which may inhibit transcription by any of several possible mechanisms. For example, PRC1 inhibits nucleosome remodeling by SWI/SNF complexes (Shao et al. 1999; Francis et al. 2001). Therefore, it may antagonize the nucleosome remodeling activity of the trxG BRM complex, thus interfering with activator binding or assembly of the preinitiation complex. In addition, PRC1 has been shown to be able to block transcription of chromatin or naked DNA templates by RNA polymerase II or T7 RNA polymerase (King et al. 2002). PRC1 does not appear to block activator binding in these assays, but instead seems to act upon the template to interfere with transcription initiation or elongation. These observations are consistent with in vivo studies in which RNA polymerase II and basal transcription factors were shown to be present at promoters under conditions of PcG repression (Dellino et al. 2004) and the presence of Pc- and E(z)-containing complexes at a discrete site just downstream of a silenced endogenous target gene, *Ubx*, in wing imaginal discs (Wang et al. 2004). In addition, our recent studies indicate that a PRC1-like complex possesses H2A ubiquitin ligase activity. Human Ring 2, a homolog of dRing/Sce, was identified as the catalytic subunit (data not shown). Although the mechanism by which this activity affects transcription has not been determined, it nevertheless suggests that PRC1 may interfere with transcription by multiple mechanisms.

Among the questions to be addressed in the near future is whether PRE-promoter loops actually form, what is the mechanistic basis for loop formation, and how are PcG complexes targeted to a site just downstream of the transcription start site. Once positioned downstream of the transcription start site, what is the mechanism by which transcription is prevented? Does PRC1 directly act upon the DNA template to prevent duplex melting, or might it interfere with some step in initiation such as RNA polymerase II CTD phosphorylation? What is the effect of H2A ubiquitination by dRing/hRing2? It is also important to point out that of the 15 genetically identified PcG genes, the products of only 9 have been identified either as sequence-specific DNA-binding proteins (Pho and Phol) or components of the PRC1 (Pc, Ph, Psc, dRing) or Esc-E(z) (Esc, E(z), Su(z)12) complexes. The remaining PcG proteins also play important roles in transcriptional silencing, but their activities are yet to be defined. In addition, other proteins, which may have pleiotropic functions and therefore are not easily classifiable as members of the PcG on the basis of genetic studies, also contribute to PcG silencing. Full understanding of this epigenetic gene regulation system will require an understanding of these other players in addition to those that have received the bulk of our attention to date.

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