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Genome Regulation by Polycomb and Trithorax Proteins

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Polycomb group (PcG) and trithorax group (trxG) proteins are critical regulators of numerous developmental genes. To silence or activate gene expression, respectively, PcG and trxG proteins bind to specific regions of DNA and direct the posttranslational modification of histones. Recent work suggests that PcG proteins regulate the nuclear organization of their target genes and that PcG-mediated gene silencing involves noncoding RNAs and the RNAi machinery.

Epigenetic regulation of gene expression is necessary for the correct deployment of developmental programs and for the maintenance of cell fates. Polycomb group (PcG) and trithorax group (trxG) genes were discovered in *Drosophila melanogaster* as repressors and activators of Hox genes, a set of transcription factors that specify cell identity along the anteroposterior axis of segmented animals. Subsequent work has shown that PcG and trxG proteins form multimeric complexes that are not required to initiate the regulation of Hox genes, but rather to maintain their expression state after the initial transcriptional regulators disappear from the embryo. Subsequent work in *Drosophila* led to the identification of DNA regulatory elements that recruit PcG and trxG factors to chromatin in vivo. These elements, called PcG and trxG response elements (PREs and TREs), respectively, mediate epigenetic inheritance of silent and active chromatin states throughout development (reviewed in Muller and Kassis, 2006; Schwartz and Pirrotta, 2007). PcG and trxG genes have also been identified in vertebrates, where they also regulate Hox genes. In addition, PcG and trxG proteins are implicated in cell proliferation (reviewed in Martinez and Cavalli, 2006), stem cell identity and cancer (reviewed in Sparmann and van Lohuizen, 2006; also see review by Jones & Baylin, in this issue), genomic imprinting in plants and mammals (reviewed in Delaval and Feil, 2004; Guitton and Berger, 2005; Bernstein et al., in this issue) and X inactivation (reviewed in Heard, 2005 and Yang and Kuroda, this issue). An appreciation for the extensive biological roles for PcG and trxG proteins has motivated efforts to determine their mechanisms of action.

Some trxG and PcG components possess methyltransferase activities directed toward specific lysines of histone H3, whereas other trxG and PcG proteins interpret these histone marks. Recent work has established the genome-wide distribution of PcG proteins, and considerable progress has been made toward understanding how PcG and trxG proteins are recruited to chromatin and how they regulate their target genes. Here, we discuss the molecular mechanisms of action of PcG and trxG proteins, their roles in regulating cell fate during development in eukaryotes, and analyze their functions from an evolutionary perspective.

Recruitment of PcG and trxG Proteins to Their Chromatin Targets

PcG proteins form three different classes of complexes (Table 1). Polycomb repressive complex 2 (PRC2) contains the four core components: E(z) (Enhancer of zeste), Esc (Extra sex combs), Su(z)12 (Suppressor of zeste 12) and Nurf-55 (in humans, EZH2, EED, SUZ12 and RbAp46/48). The SET domain-containing E(z) subunit trimethylates lysine 27 of histone H3 (H3K27me3) (reviewed in Cao and Zhang, 2004). This mark is specifically recognized by the chromodomain of Polycomb (Pc) (Cao and Zhang, 2004), a subunit of PRC1-type complexes. PRC1 contains Pc, Polyhomeotic (Ph), Posterior sex combs (Psc) and dRing, in addition to several other components, including TBP-associated factors (Saurin et al., 2001). Recently, a third complex involved in homeotic gene silencing, PhoRC, has been identified (Klymenko et al., 2006). PhoRC includes the sequence specific DNA binding protein Pleiohomeotic (Pho) as well as the dSfmbt protein [*Scm*-related gene containing four malignant brain tumor (MBT) domains], which binds specifically to mono- and dimethylated H3K9 and H4K20 via its MBT repeats.

Neither PRC2 nor PRC1 core complexes contain sequence specific DNA binding proteins, but Pho has been shown to bind to PRC2 subunits and to induce PRC2 recruitment at the *bxd* PRE of the *Ubx* gene in *Drosophila* (Wang et al., 2004b). A simple pathway for PcG protein recruitment has been suggested based on the stepwise

Table 1. PcG and trxG Complexes

	Drosophila melanogaster	Human
PcG complexes		
PhoRC	dSfmbt	?
	Pho	?
PRC2	E(z)	EZH2
	Esc	EED
	Su(z)12	SUZ12
	N55	RpAp48
		RpAp46
PRC1	dRing	RING1A
	Pc	HPC1-3
	Ph	HPH1-3
	Psc	BMI1
	Scm	SCMH1-2
	TBP-associated factors	
trxG complexes		
SWI/SNF	Brm	BRM
	Osa	BAF250
	Moira	BAF170
	Snr1	BAF47
NURF	Iswi	SNF2L
	N38	?
	N301	BPTF
	N55	RpAp46 RpAp48
TAC1	Trx[a]	
	dCBP	
	Sbf1	
Ash1	Ash1	
	dCBP	
MLL1-3		MLL1-3[a]
		WDR5
		ASH2L
		RbBP5
		CFP1

Only the core components of each complex are shown.
[a] Trx is an ortholog of MLL proteins, but the TAC1 complex isolated in *Drosophila* is composed of proteins that differ from the subunits of the MLL complex. However, orthologs of the mammalian MLL core-complex subunits are present in the *Drosophila* genome, and therefore MLL-like complexes may exist in flies. Question mark indicates that the protein is present in human, but it is not known whether it forms the same complex as in flies.

recruitment of PRC2 proteins by Pho (and Pho-like, a protein that binds to the same DNA motifs), followed by PRC1 recruitment to the H3K27me3 mark deposited by PRC2. However, PcG recruitment is much more complex than this. Firstly, Pho is not only able to recruit PRC2, but it also interacts directly with the Pc and Ph subunits of PRC1 in vitro (Mohd-Sarip et al., 2002). The presence of Pho enables the core complex of PRC1 (PCC) to bind specifically, and without the need of PRC2, to a short sequence motif that is present at natural PREs close to Pho sites (Mohd-Sarip et al., 2005). Secondly, core PREs might be depleted of nucleosomes. Mohd-Sarip and colleagues studied the architecture of the ternary complex of PRE DNA, Pho and PCC that had been reconstituted in vitro. This complex wraps DNA around the protein component and, in the presence of 6 Pho binding sites and juxtaposed PC binding elements, it includes over 400 bp of DNA in this interaction. This argues against a nucleosomal structure for this PRE in vivo (Mohd-Sarip et al., 2006). The absence of core histones at the *Ubx* PRE is also supported by in vivo studies using chromatin immunoprecipitation (ChIP) (Kahn et al., 2006; Mohd-Sarip et al., 2006; Papp and Muller, 2006), which suggest that the H3K27me3 chromatin mark might not be the recruiter of PcG proteins at core PREs. Finally, Pho binding sites alone are insufficient to tether PcG proteins to DNA in vivo, even when multimerized or when the number of sites and the spacing between them is the same as in a natural PRE (Brown et al., 1998; Dejardin et al., 2005). Indeed, Pho can form a second complex with components of the INO80 nucleosome remodeling complex, and may play other roles in addition to recruitment of PcG proteins, which may be mediated by a subset of the genomic Pho binding sites (Klymenko et al., 2006). Moreover, a *Drosophila* mutant lacking both Pho and Pho-like is lethal at a late developmental stage and, in mutant salivary glands, most PcG sites are stained normally in polytene chromosomes despite lack of detectable Pho protein (Brown et al., 2003), suggesting that other proteins can recruit PcG factors in the absence of Pho and Pho-like. GAGA factor (GAF), Pipsqueak, Dsp1, Grainyhead and members of the Sp1/KLF family have all been suggested to be involved in PcG recruitment (reviewed in Muller and Kassis, 2006). Mutations in these genes do not have a clear PcG phenotype and, intriguingly, all seem to be involved in activation as well as in silencing. One possibility is that a combination of several DNA binding factors, including as yet unknown components, could lead to tethering of PcG proteins to DNA in vivo.

To date, PREs have only been characterized in *Drosophila*. In general, PREs can be simply defined as DNA elements necessary and sufficient for recruitment of PcG complexes and for PcG-dependent silencing of flanking promoters. Many of the PcG binding sites identified by chromatin immunoprecipitation in vertebrates might fit this criterion, and this prediction will be tested by transgenic assays. Their DNA sequences are likely to differ from fly PREs, because three of the DNA binding factors

involved in PcG recruitment, GAF, Pipsqueak and Zeste, are not conserved in vertebrates.

In addition to a "DNA code" and, possibly, the H3K27me3 mark, small RNAs and proteins of the RNAi machinery might be involved in PcG recruitment. It was shown that silencing mediated by a 3.6 kilobase DNA element from the *Fab-7* regulatory region of the *Abd-B* Hox gene was relieved by mutations in the RNAi machinery (Grimaud et al., 2006). Although the recruitment of PcG proteins was only slightly affected (suggesting that RNAi-independent mechanisms are sufficient to anchor PcG complexes at a majority of their endogenous target genes) a recent report shows that the human AGO1 homolog can drive transcriptional gene silencing of promoters targeted by specific small interfering RNAs (siRNAs) via recruitment of the PcG protein EZH2 (Kim et al., 2006). However, the reported phenotypes caused by mutations in genes of the RNAi machinery are not similar to those seen in PcG mutants. Thus, RNAi components might act redundantly with DNA binding proteins at a subset of the PcG targets.

Recruitment of trxG proteins is even more mysterious. TrxG proteins are a somewhat heterogeneous group (Table 1). One class of trxG members is composed of SET domain factors like *Drosophila* Trx and Ash1 and vertebrate MLL, as well as their associated proteins. A second class of trxG factors includes components of ATP-dependent chromatin remodeling complexes like the SWI/SNF or the NURF complexes. Vertebrate complexes containing homologs of *Drosophila* Trx and Ash1 proteins are recruited at Hox genes, but the mechanisms are unknown (Hughes et al., 2004; Wysocka et al., 2003). In *Drosophila*, Trx binds a minimal *Fab-7* element in salivary glands in the absence of transcriptional activation (Dejardin and Cavalli, 2004). Other work suggests that a second DNA element overlapping the *bxd* PRE upstream of *Ubx* is involved in Trx-dependent maintenance of *Ubx* activation (Tillib et al., 1999). Furthermore, Trx is reported to bind at this element irrespective of the state of *Ubx* expression in imaginal discs of *Drosophila* larvae (Papp and Muller, 2006), suggesting that specific DNA tethers recruit Trx independent of the action of transcription factors. In *Drosophila* embryos, Trx is observed to constitutively bind to the promoter regions of the Ubx gene and of the *bxd* element. Interestingly however, this paper also reports recruitment of Trx to transcribed *Ubx* regions, but only upon activation (Petruk et al., 2006). Thus, between the two papers there is a discrepancy in Trx location. However, the first study used antibodies directed against the C-terminal part of the protein, whereas in the latter study the antibody was directed against the N-terminal part. The Trx protein is proteolytically cleaved by the Taspase enzyme (Hsieh et al., 2003), and the two moieties might target different chromatin regions upon cleavage.

Other trxG components seem to be recruited to chromatin in an activation-dependent manner. For instance, upon *Ubx* activation Ash1 is recruited to the region immediately downstream the transcription start site (Papp and Muller, 2006). The SWI/SNF component Brm is also recruited to polytene chromosomes upon activation of a transgene carrying a minimal *Fab-7* element (Dejardin and Cavalli, 2004). Interestingly, mutation of Zeste sites in the *Fab-7* element prevents recruitment of Brm, but not of Trx. Thus, multiple DNA tethers cooperate for recruitment of trxG proteins needed for gene activation.

In summary, recruitment of PcG and trxG proteins involves combinatorial signals from multiple DNA motifs. The simultaneous binding of multiple silencing and activating factors at PREs/TREs suggests that they build switchable regulatory platforms (Figure 1), which may be able to read early developmental cues and transform them into heritable states of gene expression or transcriptional silencing.

Posttranslational Chromatin Marks Linked to PcG and trxG Proteins

PRC2-type complexes possess H3K27-specific trimethylase activity (Cao and Zhang, 2004) whereas several trxG complexes have H3K4 trimethylase activity (Figure 2A). (Byrd and Shearn, 2003; Dou et al., 2005; Wysocka et al., 2005). Do these two histone trimethylation marks mediate PcG-dependent silencing and trxG-dependent activation as part of a Yin and Yang relationship?

Recent genome-wide analysis of the distribution of both marks reveals insight into their epigenetic roles. The components of the PRC2 complex in flies, mouse and human are typically found in the regions that are trimethylated at H3K27 (Boyer et al., 2006; Lee et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006). In contrast, H3K4me3 is present at most active promoters in the genome (Kim et al., 2005 and see review by Li et al. in this issue).

Papp and Müller analyzed the relation between H3K4me3 and H3K27me3 at the *Drosophila Ubx* gene by ChIP analysis of tissues in which the gene is in an active or a repressed state (Papp and Muller, 2006). Both Trx and PcG proteins bind at the *Ubx* PREs in either state without extensive coating of the remainder of *Ubx* chromatin. Yet, in the repressed state, the whole *Ubx* gene is trimethylated at H3K27. In contrast, in the active state, H3K27me3 is still present in the upstream region of the gene, but is virtually absent at the promoter and the coding region of the gene. The absence of H3K27 trimethylation in part of the gene correlates with the binding of Ash1 immediately downstream to the promoter, which induces trimethylation of H3K4. Trx binds constitutively at the PRE in the absence of detectable H3K4me3 around the PRE region (as revealed by an antibody directed against the C-terminal portion of the protein). The mammalian Trx homolog MLL1 is also responsible for H3K4 trimethylation at the human *HOXA9* locus (Dou et al., 2005), but a knockout of the SET domain of mouse Mll results in the specific depletion of monomethylated H3K4 (Terranova et al., 2006) at the *Hoxd4* and *Hoxc8* genes. Thus, the role of Trx and MLL1 in histone methylation might be gene specific and might be assisted by additional histone methylase

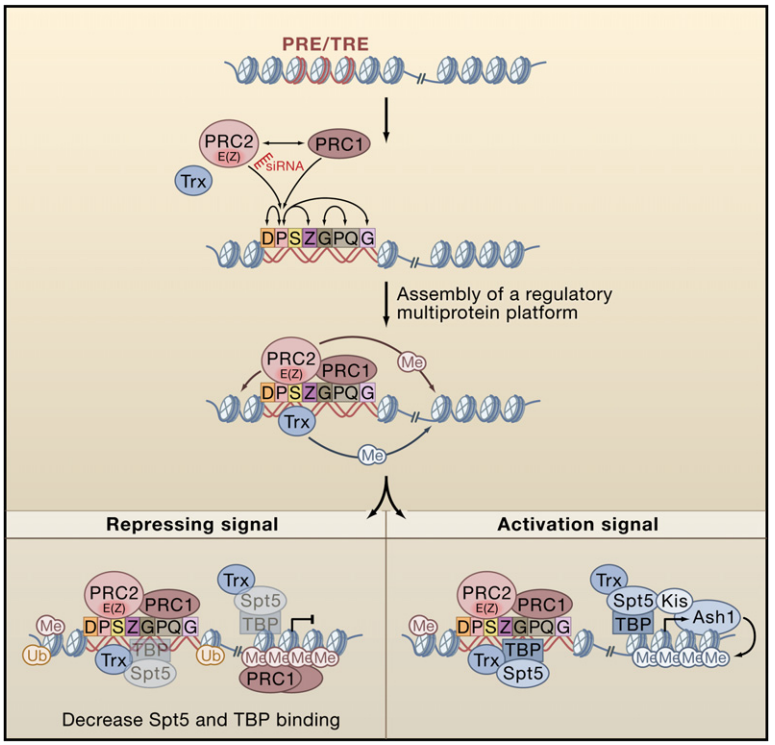


Figure 1. PREs and TREs as Molecular Binding Platforms

Multiple DNA binding proteins like Pho (P), Dsp1 (D), SP1/KLF (S), Zeste (Z), GAGA factor (G), Pipsqueak (PQ), and Grainyhead (G) recruit PcG complexes to PREs. The recruitment of either PcG or TrxG proteins to the PRE sequence does not depend on the activation status of the gene. Moreover, general transcription and elongation factors, such as TBP and Spt5, are constitutively bound to the PRE. A developmental signal then determines whether the PRE mediates gene activation or gene repression, which are accompanied by trimethylation (Me) of histone H3 on lysine 4 or lysine 27, respectively. Upon activation, Kismet (Kis), a protein facilitating elongation, and Ash1, leading to local H3K4 methylation, are recruited to the promoter region. Small RNAs (red ladder) may also contribute to PcG protein recruitment.

activities to produce the H3K4me3 mark associated with gene expression.

Additional components might also be involved in H3K4 trimethylation. In yeast, H3K4 trimethylation requires monoubiquitylation of histone H2B at lysine 123 by Rad6/Bre1 (Sun and Allis, 2002). Although *Drosophila* dBre1 has no known involvement in trxG-mediated activation, a complex between the proteins USP7 and GMP synthetase (GMPS) has been shown to contribute to PcG-mediated gene silencing via deubiquitylation of histone H2B (van der Knaap et al., 2005). This suggests a trans-histone interplay between activating H3K4 trimethylation, stimulated by ubiquitylation of H2B, and silencing H3K27 trimethylation, stimulated by deubiquitylation of H2B (Figure 2B).

In addition to H3K27 trimethylation, PRC2 containing a specific isoform of the EED protein is thought to catalyze trimethylation of lysine 26 of histone H1 (H1K26me3) (Kuzmichev et al., 2004). Surprisingly, similar experiments performed with recombinant complexes and di- or oligonucleosomes could not confirm whether it has the ability to methylate histone H1 (Martin et al., 2006), suggesting that differences in assay conditions can affect the histone substrate specificity of PRC2-type complexes.

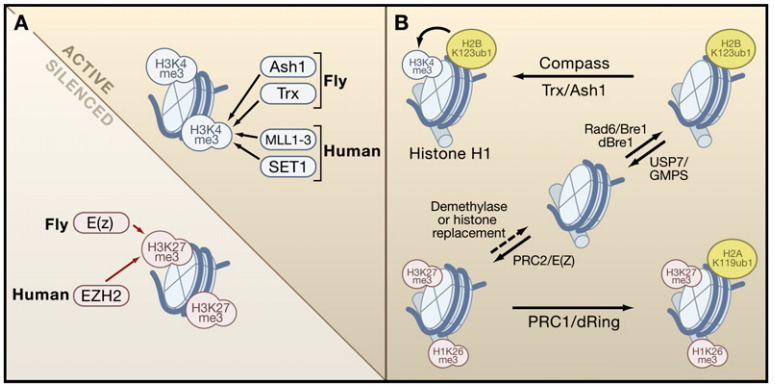


Figure 2. Histone Marks in PcG and trxG Protein Function

(A) PcG and trxG complexes deposit histone marks that play complementary roles in silencing and activation of their target chromatin. The enzymatic subunits of PcG and trxG complexes responsible for H3K27me3 and H3K4me3, respectively, in flies and humans are shown.

(B) Ubiquitylation of histone H2B on lysine K123 (H2BK123ub1) by the yeast complex Rad6/Bre1 stimulates histone methylation of histone H3 on lysine 4 by COMPASS, a trxG complex, resulting in gene activation. In contrast, deubiquitylation of histone H2B by the *Drosophila* USP7/GMPS complex may be essential for histone methylation of histone H3 on lysine 27 (H3K27me3). Moreover, this methyl mark might help to recruit dRing, a protein with E3 ligase activity leading to ubiquitylation of histone H2A on lysine 119 (H2AK119ub1).

PcG complexes of the PRC1-type also contain an evolutionarily conserved histone modification activity leading to ubiquitylation of lysine 119 of histone H2A (H2AK119ub1, see Figure 2B) (de Napoles et al., 2004; Wang et al., 2004a), which is required for PcG-mediated silencing of the *Drosophila Ubx* gene (Wang et al., 2004a) as well as of the mouse HoxC13 gene (Cao et al., 2005). A putative "reader" of this histone mark remains to be identified. Other histone modifications are associated with PcG and trxG proteins, although their role is not well understood. For instance, Papp and colleagues reported that trimethyhlation of H3K9 and H4K20 accompanies the H3K27me3 mark (Papp and Muller, 2006).

Mechanisms of trxG-Mediated Activation and PcG-Mediated Silencing

What are the roles of all these histone modifications and are they sufficient to explain PcG-mediated silencing and trxG-mediated activation? H3K4me3 is recognized by the PHD finger domain of the Nurf-301 protein (Li et al., 2006; Wysocka et al., 2006). The NURF complex tethered to trxG responsive promoters might facilitate the recruitment of the transcriptional machinery via ATP-dependent nucleosome remodeling. H3K4me3 might also stimulate transcriptional elongation. In particular, H3K4me3 and Ash1 are found downstream to the *Ubx* promoter (Papp and Muller, 2006). Trx has also been shown to facilitate transcriptional elongation at heat shock genes (Smith et al., 2004) and, more recently, the *Drosophila* Trithorax complex TAC1 has been proposed to play a global role in transcriptional elongation (Petruk et al., 2006). Mll, the mouse counterpart of Trx, is also distributed all along the coding part of its Hox target genes, and Mll mutations affect the distribution of elongating RNA pol II (Milne et al., 2005).

What then is the role of H3K27me3? PRC2-type complexes are conserved throughout the eukaryotic kingdoms, including in those organisms with no trace of PRC1, such as plants. A plant homolog of E(z) deposits the H3K27me3 mark on large domains spanning its target genes leading to their silencing (Schubert et al., 2006). It is not clear how silencing in the absence of PRC1 is achieved. One possibility is that PRC1 is replaced by other factors. For instance, the LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) is necessary for the maintenance of the epigenetically repressed state of some euchromatic genes (Sung et al., 2006). An alternative possibility is that H3K27me3 represses transcription directly, for instance by inhibiting some step involved in transcriptional activation or by preventing the deposition of histone marks associated with gene activation, such as acetylation, ubiquitylation of histone H2B or trimethylation of H3K4 (Figure 3A).

Another notable feature of H3K27 trimethylation is that it is distributed over large chromosomal domains, sometimes covering several hundreds of kilobases. This might provide the basis for epigenetic inheritance of PcG-dependent silencing during cell division. Even if PcG proteins

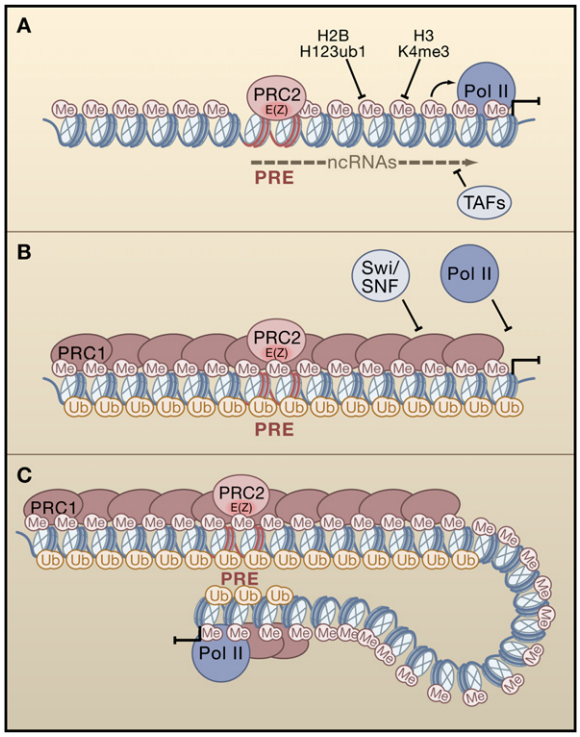


Figure 3. Different Layers of PcG-Mediated Gene Silencing

(A) PRC2-mediated histone H3 methylation on lysine 27 (Me) might directly interfere with transcriptional activation and/or inhibit ubiquitylation of histone H2B or trimethylation of H3 on lysine 4. Transcription of noncoding RNAs may mediate repression of a downstream gene by transcriptional interference. TAFs, TBP-associated factors.

(B) H3K27me3 and PRC1 complexes spread from the PRE to a promoter located close to the PRE, interfering with ATP-dependent nucleosome remodeling activities (SWI/SNF) and RNA Pol II recruitment. The E3 ligase activity of dRing leads to H2A ubiquitylation, contributing to silencing by unknown mechanisms.

(C) RNA Pol II can be recruited to a subset of PcG-silenced genes, suggesting a role for PRC1 in gene silencing downstream of RNA Pol II assembly at the promoter region. For promoters located far away from PRE sequences, PRC2 complexes bound at PREs may loop out and contact neighboring nucleosomes. E(z) activity may then generate a large repressive domain of H3K27me3. Moreover, PRE looping may allow PcG proteins to contact distant promoters.

are lost from their targets during DNA replication or mitosis (Buchenau et al., 1998), they would rapidly gain access to the originally silenced chromatin via specific interactions with PRE DNA, assisted by interaction of the Pc chromodomain with H3K27me3. Meanwhile, the same mark might prevent the local deposition of activating marks and inappropriate gene reactivation. Binding of PRC2 components to PREs would rapidly restore the trimethylation of H3K27 that is lost upon DNA replication.

Although histone marks may be directly responsible for PcG-mediated repression, it is important to note that some PcG target genes must be strongly and reliably repressed throughout many cell divisions. This robust silencing might require the contribution of other

mechanisms in addition to the marking of histones. PRC1 can repress ATP-dependent nucleosome remodeling by the SWI/SNF complex in vitro (Shao et al., 1999), and the PCC complex is able to condense chromatin in a Psc-dependent manner and in the absence of histone modifications (Figure 3B) (Francis et al., 2004). Moreover, native PRC1 in *Drosophila* contains TBP-associated factors (Breiling et al., 2001; Saurin et al., 2001), suggesting that PcG proteins might contact promoters. Consistent with this notion, PRE-mediated silencing does not necessarily prohibit recruitment of RNA pol II, but may interfere with DNA-melting at the promoter during initiation of transcription (Dellino et al., 2004).

The position of PREs relative to their target genes is variable. Sometimes they overlap the promoter, whereas in other cases they are located tens of kilobases away (Negre et al., 2006). One possible explanation for silencing of distant promoters is that PRE-bound E(z) establishes a large domain of H3K27me3 via transient chromatin contacts mediated by the looping of PREs. This mark might then silence promoters located within its realm. Alternatively, PcG proteins bound to a PRE might establish specific contacts with promoter-bound components of the transcription machinery upon PRE looping (Figure 3C). Contact between distal domains by PRE looping has been demonstrated by the tethering of Dam methyltransferase to the *Drosophila Fab-7* region (Cleard et al., 2006). Moreover, a recent study (in this case, using a transgenic construct containing the PRE upstream of *Ubx*) indicates that PRE looping can drive promoter silencing (Comet et al., 2006). This is also consistent with the weak but significant binding of PcG members to the *Ubx* promoter in *Drosophila* embryos or cultured cells (Comet et al., 2006; Kahn et al., 2006).

Noncoding RNAs (ncRNAs) may also play a role in the function of PcG and trxG proteins, but studies have produced contrasting results. It had been suggested from earlier work that ncRNAs produced from the regulatory regions of Hox genes may counteract PcG-dependent silencing (reviewed in Schmitt and Paro, 2006). Further evidence for an activating role of ncRNAs came from a study of the *bxd* regulatory region of the *Ubx* gene, in which *bxd* transcripts are shown to recruit the Ash1 protein to this region inducing *Ubx* transcription in larval tissues (Sanchez-Elsner et al., 2006). However, these results contrast with more recent work showing that in embryos, *Ubx* is not transcribed in the same cells as *bxd*, and that embryonic *bxd* transcripts may participate in PcG-mediated silencing rather than activation of *Ubx* (Petruk et al., 2006). In particular, the authors did not observe ectopic activation of *Ubx* by overexpression of *bxd* transcripts in larval tissues. They further showed that repression of *Ubx* by *bxd* transcription is mediated in *cis* by transcriptional interference (Figure 3A), and does not involve siRNA or miRNA-based mechanisms. Also, *bxd* ncRNAs were not detected in larval stages (Petruk et al., 2006), making it unlikely that they are involved in the maintenance of repression. It will be essential to examine the distribution of PcG proteins at the Ubx gene with or without *bxd* transcription to clarify whether transcription at the *bxd* region displaces PcG components. In summary, ncRNAs are likely to play a role in regulating PcG silencing at a subset of the target genes, but more work is required in order to clarify their function and understand their molecular mechanisms of action.

The formation of subnuclear silencing compartments might also contribute to the stable repression of transcription. *Drosophila* PcG proteins have a speckled nuclear distribution (Grimaud et al., 2006) and the number of "PcG bodies" is progressively reduced during development, and is smaller than the number of genomic binding sites detected by combining ChIP with DNA microarrays (ChIP on chip) (Schwartz et al., 2006; Tolhuis et al., 2006). A combination of immunofluorescence staining with DNA FISH has shown that multiple target PcG elements can associate in the nucleus to enhance the strength of PcG-mediated silencing (Bantignies et al., 2003). Clustering of PcG target genes into PcG bodies might facilitate silencing by exclusion of RNA polymerase. Interestingly, the association of PcG target elements requires nuclear components of the RNAi machinery that colocalize with PcG proteins (Grimaud et al., 2006).

In mammalian cells PcG-mediated repression and DNA methylation might be coordinated in order to stabilize silencing at PcG target genes. EZH2 can directly recruit DNA methyltransferases (DNMTs) to target genes (Reynolds et al., 2006; Vire et al., 2005), and it collaborates with DNMT1 to recruit Bmi-1 to PcG bodies (Hernandez-Munoz et al., 2005b). Two recent studies show that Polycomb-marked genes are major targets for DNA methyltransferases, leading to de novo methylation of PcG target genes and to aberrant and permanent silencing in cancer cells (Schlesinger et al., 2006; Widschwendter et al., 2006). Additional work is needed to understand what triggers PRC2-mediated recruitment of DNA methyltransferases during tumorigenesis.

These data indicate that the balance between gene silencing and transcriptional activation at PcG/trxG target genes is regulated by direct interactions with the transcriptional machinery, the deposition of specific epigenetic marks on histones and DNA, the transcription of noncoding RNA, and the regulation of nuclear organization.

Genome-Wide Distribution and Biological Functions of PcG Proteins

The genome-wide distributions of PcG proteins have been described recently in mouse and human cells and in *Drosophila*. Although the comparison is not straightforward because different cell types and PcG proteins were analyzed, these studies clearly indicate important similarities as well as differences between vertebrates and flies. In all species, binding of PcG proteins is highly correlated with the distribution of the H3K27me3 mark, which is sometimes localized to restricted genomic regions, whereas in other cases it forms domains that are hundreds of kilobases in size, the largest ones including Hox gene

clusters (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006; Negre et al., 2006; Schwartz et al., 2006; Squazzo et al., 2006; Tolhuis et al., 2006). PcG binding negatively correlates with the presence of RNA pol II, suggesting that RNA pol II is excluded from many PcG target genes as a consequence of silencing. A striking observation common to all reports is that PcG proteins bind preferentially to genes encoding transcription factors, including many homeodomain-containing genes. This suggests that the main function of PcG proteins is to regulate transcription pathways. Meta-analysis of the putative target genes from these studies reveals that many of the target genes are common in the three species analyzed. As an example, 98 of the 260 target genes identified by Schwartz and coworkers have clear mouse and human homologs (identified by HomoloGene). Only 26 of them are unique targets in flies, whereas the 72 others are targets in human and/or mouse (Figure S1A in the Supplemental Data available with this article online). Strikingly, 63 of these 72 conserved target genes (87.4%) encode transcription factors, while only 38.5% of the nonconserved targets do (Figures S1B and S1C; Tables S1 and S2). Clearly, genes encoding transcription factors represent the most highly conserved class of PcG targets. This indicates that regulation of transcriptional pathways is a major raison d'être of PcG genes.

Many of the PcG target genes are involved in developmental patterning, morphogenesis, and organogenesis. Some of these pathways are highly enriched in PcG targets. For instance, 55% of the transcription factors known to be involved in segmentation of the fly embryo were scored as PcG targets in several independent mapping studies (Figure S2A; Table S3). Similarly, most of the master transcription regulatory genes involved in eye and limb development are bound by PcG proteins (Figures S2B, S2C, and S3). This suggests that PcG proteins might play a global role to orchestrate these pathways. Future work should show how many of these targets are bound in each cell type and how many bound genes are indeed regulated by these proteins.

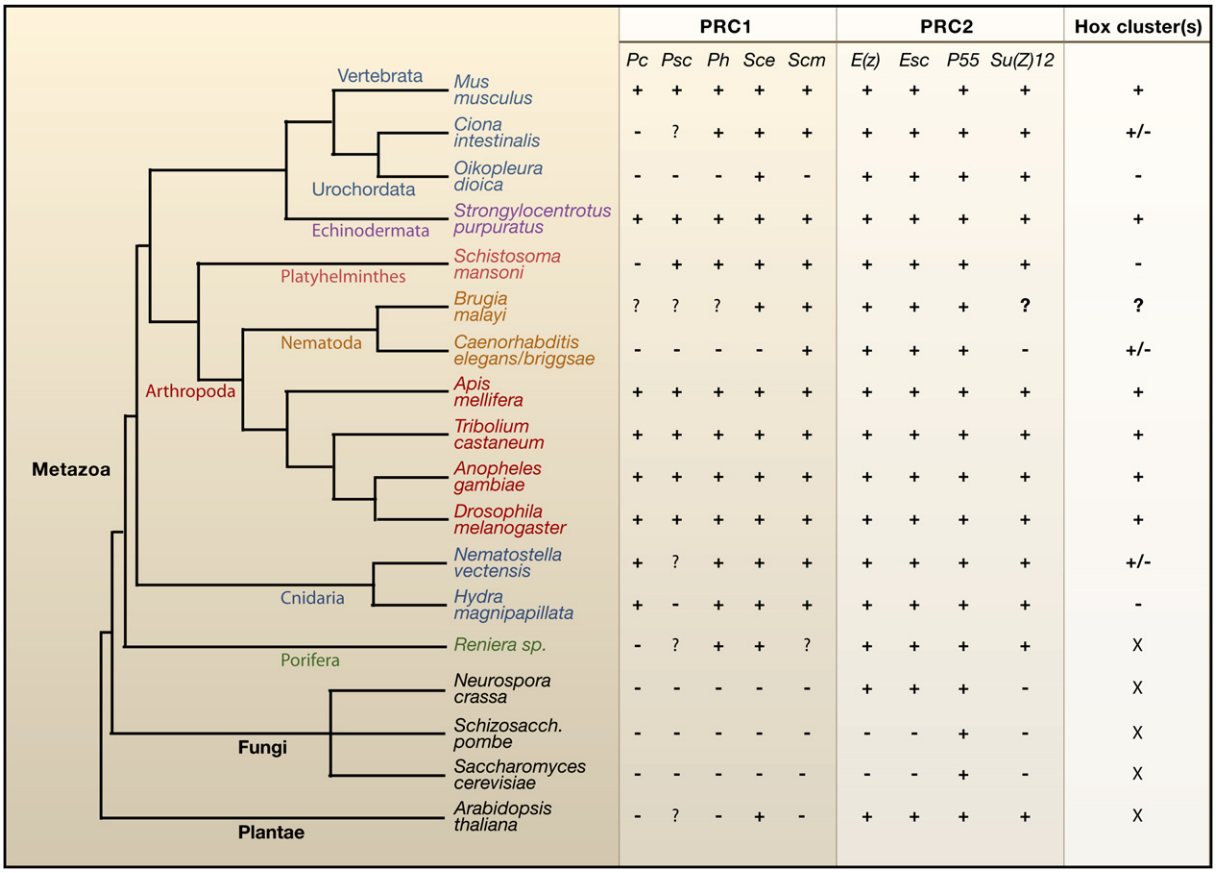
Despite similarities in the biochemistry of PcG complexes and in the identity of target genes, striking differences in the distribution of PcG components were also found. Mouse and human PRC2 components bind throughout the H3K27me3 regions (Bracken et al., 2006; Lee et al., 2006; Squazzo et al., 2006), whereas *Drosophila* PRC2 members bind to restricted regions, presumably PREs, even though H3K27me3 covers large domains (Papp and Muller, 2006; Schwartz et al., 2006). Thus, the molecular mechanisms by which H3K27me3 is deposited on chromatin might differ between flies and vertebrates. Furthermore, over 90% of the mammalian PcG binding sites are located close to proximal gene promoter elements (Boyer et al., 2006; Lee et al., 2006), which is much higher than in *Drosophila* (Negre et al., 2006), suggesting that fly PcG proteins frequently act over a longer range than in mammals. Finally, although the binding of fly PcG proteins shows some degree of developmental dynamics, many fly target sites are constitutively bound throughout development (Negre et al., 2006). Binding of PcG proteins to their target genes in vertebrates appears more dynamic, and many of the PcG targets in embryonic stem cells are activated at later stages, concomitant with loss of PcG proteins (Boyer et al., 2006; Lee et al., 2006). This suggests that, although fly PcG proteins may be used for epigenetic maintenance of transcriptional states at many targets, mammalian PcG factors are often involved in reversible gene repression, and other systems such as DNA methylation might stably lock in transcriptional silencing in these organisms. This is consistent with the high levels of PcG gene expression and a prominent role of PcG proteins in maintenance of stem cell identity in mammals and it indicates that PcG (and probably trxG) factors do not only serve to maintain long term memory of transcriptional states.

Evolution of PcG and trxG Genes

These observations put into question some preconceived views of the biological role of PcG and trxG proteins and suggest that the analysis of these factors from an evolutionary perspective might give useful insight into their function. PcG and trxG proteins are often said to be evolutionarily conserved. Indeed, most trxG components are found in fungi, plants and animals (Supplemental Experimental Procedures; Table S4), consistent with a conserved role in the regulation of global gene transcription.

The components of the PRC2 complex are found in plants and animals, but not in the distantly-related fungi *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Figure 4). However, their ancient origin is confirmed by their presence in another fungus, *Neurospora crassa*, which also possesses the H3K27me3 mark (Eric Selker, personal communication). Thus, PRC2 genes might have an ancient function in transcriptional repression.

The picture is much more complex for components of PRC1. First, there is no trace of the core PRC1 genes in fungi and plants (Figure 4) (Springer et al., 2002). Blast analysis of several recently sequenced animal genomes (Supplemental Experimental Procedures) revealed that PRC1 genes originated early in animal evolution. They are present in 'basal' animals: two different cnidarian species (*Hydra magnipapillata* and *Nematostella vectensis*) and, at least to some extent, in the sponge *Reniera sp.* (Figure 4). The PRC1 gene set is complete in several insect and vertebrate species, as well as in the echinoderm *Strongylocentrotus purpuratus*, but a varying number of PRC1 genes are missing in species from other phyla (Figure 4). For instance, all PRC1 core genes (except *Scm*) are absent in two *Caenorhabditis* species, and at least three PRC1 subunits are not found in the urochordate *Oikopleura dioica*. Finally, Polycomb itself, the "reader" of the H3K27me3 histone mark, is missing in many species though present in both cnidarians (Figure 4). This is a strong indication that PRC1 genes have been repeatedly lost during evolution of the animal kingdom.



	PRC1					PRC2				Hox cluster(s)
	Pc	Psc	Ph	Sce	Scm	E(z)	Esc	P55	Su(Z)12	
Mus musculus	+	+	+	+	+	+	+	+	+	+
Ciona intestinalis	-	?	+	+	+	+	+	+	+	+/-
Oikopleura dioica	-	-	-	+	-	+	+	+	+	-
Strongylocentrotus purpuratus	+	+	+	+	+	+	+	+	+	+
Schistosoma mansoni	-	+	+	+	+	+	+	+	+	-
Brugia malayi	?	?	?	+	+	+	+	+	?	?
Caenorhabditis elegans/briggsae	-	-	-	-	+	+	+	+	-	+/-
Apis mellifera	+	+	+	+	+	+	+	+	+	+
Tribolium castaneum	+	+	+	+	+	+	+	+	+	+
Anopheles gambiae	+	+	+	+	+	+	+	+	+	+
Drosophila melanogaster	+	+	+	+	+	+	+	+	+	+
Nematostella vectensis	+	?	+	+	+	+	+	+	+	+/-
Hydra magnipapillata	+	-	+	+	+	+	+	+	+	-
Reniera sp.	-	?	+	+	?	+	+	+	+	X
Neurospora crassa	-	-	-	-	-	+	+	+	-	X
Schizosacch. pombe	-	-	-	-	-	-	-	+	-	X
Saccharomyces cerevisiae	-	-	-	-	-	-	-	+	-	X
Arabidopsis thaliana	-	?	-	+	-	+	+	+	+	X

Figure 4. Phylogenetic Distribution of the PRC1, PRC2, and Hox Gene Clusters

Depicted is a current view of the phylogenetic relationships among a broad spectrum of eukaryotes (Adoutte et al., 2000; Delsuc et al., 2006; Kurtzman and Robnett, 2003). Phylogenetic groups are indicated either on the left of the nodes that define each group or below some of the terminal branches. For each species, + indicates the presence and – the absence of the proteins that constitute the PRC1 and PRC2 complexes. The existence of Hox gene clusters in the different species is also indicated. + indicates the presence of one or more "bona fide" Hox clusters, +/– indicates the existence of partial Hox clusters, – indicates that Hox genes exist, but are not clustered, and X indicates the absence of Hox genes.

The phenotypes of PcG mutants and the strong binding of PRC1 to Hox gene clusters in flies and vertebrates suggest that these clusters are important PRC1 targets. Thus, one hypothesis might be that PRC1 genes can be lost as a consequence of the disintegration of the Hox gene cluster, which occurred repeatedly during evolution (Chourrout et al., 2006; Pierce et al., 2005; Seo et al., 2004). Indeed, most PRC1 genes are absent in the urochordate *Oikopleura dioica*, which is an extreme example due to its nine unlinked Hox genes (Seo et al., 2004). PRC1 genes are also absent in both *Caenorhabditis* species, which have profoundly rearranged Hox clusters (Aboobaker and Blaxter, 2003). However, the integrity of Hox gene clusters does not strictly correlate with the presence of a full set of PRC1 genes (Figure 4). Indeed, most or all PRC1 genes are found in several species with degenerated clusters, including both cnidarians *Nematostella vectensis* and *Hydra magnipapillata* (Chourrout et al., 2006) the platyhelminth *Schistosoma mansonii* (Pierce et al., 2005) and the urochordate *Ciona intestinalis* (Spagnuolo et al., 2003). It is important to stress that the function of Hox genes is essentially unknown in most animal species apart from arthropods/vertebrates. In other species, these genes may not necessarily specify the anteroposterior axis of the body plan. However, diminution of the PRC1 complement may accompany breakdown of the Hox cluster without being caused by it. Elucidating how PcG has coevolved with the Hox cluster could illuminate the contribution of epigenetic mechanisms to the evolution of animal development.

An interesting hypothesis is that the role of PRC1 proteins in mammalian stem cells might reflect an evolutionarily conserved function for these factors in regulating cell plasticity and/or switching between pluripotent and differentiated cell states. PRC1 genes may be absolutely required in species showing strong cellular plasticity and a developmental regulation including the ability to regenerate up to the adult stage, as observed in cnidarians. In these species, PRC1 might cooperate with PRC2 and trxG complexes to reinforce and fine-tune silencing of

master developmental genes involved in these functions. Conversely, PRC1 genes—in particular Pc—would not be required in animals displaying highly determinate development with invariant cell lineage such as *Caenorhabditis* and the urochordates. In these cases the differential rates of genome evolution might have resulted in variable levels of PRC1 gene loss and breakdown of Hox gene clusters within each major taxon.

Perspectives

Thanks to recent fundamental discoveries and the development of analytical tools, we are likely to witness great progress in the coming years toward understanding the biological roles of PcG and trxG proteins. This will include clarifying the role of histone marks in PcG and trxG regulation. Furthermore, the activities of PcG and trxG complexes toward nonhistone substrates have not been investigated in detail. For instance, human Pc2/CBX4 has been shown to have SUMO E3 activity directed toward CtBP, a transcriptional corepressor (Kagey et al., 2005). PcG and trxG proteins are themselves among the putative nonhistone targets. For example, BMI1 can be ubiquitylated by the CULLIN3/SPOP E3 ligase to contribute to female X-inactivation (Hernandez-Munoz et al., 2005a). The set of rules that lead to the targeting of specific genes by PcG and trxG proteins should also be decrypted. In particular, we need to learn more about the contribution of nuclear organization and RNAi components. Another exciting area of investigation will be to determine how these proteins transmit memory of gene expression states during the process of cell division. To date this long-standing question has not been approached directly and addressing it might require new technology. Finally, genomic and proteomic studies, evolutionary analysis using bioinformatics, and experimental approaches in nonmodel organisms will provide new insights into the biological roles of these proteins.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three figures, and four tables and can be found with this article online at http://www.cell.com/cgi/content/full/128/4/735/DC1/.

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