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# Polycomb complexes and silencing mechanisms

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Advances in the past couple of years have brought important new knowledge on the mechanisms by which Polycomb-group proteins regulate gene expression and on the consequences of their actions. The discovery of histone methylation imprints specific for Polycomb and Trithorax complexes has provided mechanistic insight on how this ancient epigenetic memory system acts to repress and indicates that it may share mechanistic aspects with other silencing and genome-protective processes, such as RNA interference.

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## Abbreviations

<b>CMM</b>	cellular memory module
<b>GAF</b>	GAGA factor
<b>HMTase</b>	histone methyltransferase
<b>HP1</b>	heterochromatin protein 1
<b>HDAC</b>	histone deacetylase
<b>MLL</b>	mixed lineage leukaemia
<b>PcG</b>	Polycomb group
<b>PRC</b>	Polycomb repressive complex
<b>PRE</b>	Polycomb response element
<b>RNAi</b>	RNA interference
<b>TRE</b>	Trithorax response element
<b>TrxG</b>	Trithorax group

## Introduction

In the past few years Polycomb group (PcG) proteins have been assigned a multitude of biological functions such as stem-cell fate decisions, differentiation and neoplastic development. Individual PcG members have been identified in many different nuclear protein complexes, further broadening the functional range and complexity of Polycomb biology. In addition, elucidation of the role of PcG-mediated gene regulation in the development of both flowering plants and *Caenorhabditis elegans* has further underlined the importance of this ancient family of chromatin modifiers. In this review we shall limit ourselves to an update on the emerging mechanisms underlying how PcG complexes assemble and mediate

target gene repression. For recent broader reviews see [1–4].

## PcG complexes and their recruitment to target sites

Polycomb proteins separate biochemically and functionally into two major multiprotein complexes, although increasing evidence suggests the existence of many sub-complexes (reviewed in [3]). In both humans and flies the initiation core complex, also known as PRC2, purifies as a 400–600 kDa complex consisting of Esc, E(z), Su(z)12 and RbAp48/Nurf-55 [5•–7•] (see Table 1 for names of *Drosophila* and mammalian PcG homologues). The core of the polycomb repressive complex (PRC)1 consists of Pc, Ph, Psc and dRing, but many additional proteins have been found to co-purify with PRC1 [8]. During early *Drosophila* larval development one large PcG complex is transiently formed, consisting of members from both PRC1 and PRC2 [9]. This meta-complex represents the only currently known physical association between PRC1 and PRC2. Notably, Psc is lacking from this complex and is at this stage predominantly found at core promoters [1,9]. At later stages in fly development, PRC1 and PRC2 separate into discrete complexes and Psc associates to PRC1, suggesting that these proteins are regulated in a highly dynamic fashion during development and differentiation. Whereas previous models for PcG function depicted complex nucleation at a PRE (Polycomb response element) followed by spreading of repressed chromatin to nearby promoters, current data favors a looping model in close analogy to classical enhancer–promoter models. It is currently unclear whether stable complexes are formed between PREs and promoter regions or whether more transient ‘hit and run’ interactions are responsible for conferring a repressive imprint from the PRE to the promoter. The fact that *Drosophila* PRC1 co-purifies with multiple general transcription factors is evidence in favor of a stable interaction [8], whereas (GA)<sub>n</sub>-binding proteins such as GAGA factor (GAF) and Pipsqueak were recently suggested to facilitate looping [10]. In addition, biochemical evidence using recombinant mouse PRC1 components indicates that PcG complexes assembled on an artificial template have the inherent capacity to recruit and repress another chromatinized DNA strand, thereby further substantiating a looping model [11]. The looping model is in accordance with the presence of Pc at core promoters at later stages of fly development and with the absence of PRC1 binding to chromatin areas between PRE and promoter regions [12]. Interestingly, dynamic long range interactions have recently been described for the mouse Hox D cluster genes, which represent the main PcG target genes [13••].

**Table 1**

<i>Drosophila</i> proteins	Human proteins <sup>a</sup>	Mouse proteins <sup>a</sup>
<b>Esc-E(z)/PRC2 initiation complex</b>		
Esc	EED	Eed
E(z)	<b>EZH1</b>	<b>Ezh1/Enx2</b>
	<b>EZH2</b>	<b>Ezh2/Enx1</b>
Su(z)12	<b>SUZ12</b>	
<b>PRC1/maintenance complex</b>		
Pc	<b>CBX2/HPC1</b>	<b>Cbx2/M33</b>
	<b>CBX4/HPC2</b>	<b>Cbx4/Mpc2</b>
	<b>CBX8/HPC3</b>	
Ph	<b>EDR1/HPH1</b>	<b>Edr1/Mph1/Rae28</b>
	<b>EDR2/HPH2</b>	<b>Edr2/Mph2</b>
	<b>EDR3/HPH3</b>	
dRing	<b>RING1/RNF1/RING1A</b>	<b>Ring1/Ring1a</b>
	<b>RNF2/RING1B</b>	<b>Rnf2/Ring1b</b>
Esc	BMI1	Bmi1
	<b>RNF110/ZFP144</b>	<b>Rnf110/Zfp144/Mel18</b>
	ZNF134	<b>Znf134/Mblr</b>
Pho	YY1	Yy1
Pho-like		
Scm	SCML1	Scmh1
	SCML2	Scml2
Pcl	PHF1	

<sup>a</sup>Official gene names according to HUGO Gene Nomenclature Committee and the Mouse Genomic Nomenclature Committee are shown in bold.

PcG and Trithorax-group (TrxG) complexes have long been thought to have antagonistic effects on target genes: PcG proteins act as stable repressors whereas TrxG proteins promote maintenance of gene activity. However, recent results favor a more complex interplay. Polytene chromosome stainings have revealed >100 PREs in *Drosophila*, but hitherto only a few have been thoroughly analyzed. PcG response elements can overlap with TrxG response elements (TREs) and binding of PcG and TrxG complexes is not mutually exclusive. On the contrary, TrxG proteins have been found at PREs associated with repressed genes and PcG proteins have been identified at core promoters of transcriptionally active genes. Furthermore, PRC1 members co-immunoprecipitate with the TrxG protein GAF [9], an interaction possibly facilitated by Corto [14], suggesting that PcG and TrxG proteins may function in a concerted fashion. Acknowledging the functional overlap, the *cis* elements have been named cellular memory modules (CMMs) [15]. Tethering of PcG complexes to PREs is facilitated by DNA binding proteins, of which four have been described in the fly so far: Pho, Pho-like, Zeste and GAF, the latter two also being involved in sequestration of Trx [16]. Another protein likely to be involved in PcG targeting is Pipsqueak. Similar to GAF, Pipsqueak is needed for PcG-mediated silencing of homeotic genes [10], binds to (GA)<sub>n</sub> sequences and was recently found to co-purify with epitope-tagged PcG complexes from *Drosophila* S2 cells [17]. Interestingly, the binding of GAF to (GA)<sub>n</sub> sequences, in concert with functionally related proteins

Pipsqueak and Batman, may mediate nucleosome displacement, thereby creating nucleosome-free regions [10]. This is in accordance with data from the laboratory of Pirrotta suggesting that PREs may be devoid of a classical nucleosomal structure (V Pirrotta, personal communication).

Using an *in silico* approach, Paro and coworkers predicted 167 PRE/TREs in *Drosophila* on the basis of the clustered presence of binding sites for Pho, GAF and Zeste, thereby covering >50% of the immunologically detected PcG binding sites [18<sup>\*</sup>]. This study revealed three novel sequence motifs, which may be binding sites for PcG-tethering factors yet to be identified. Pho, the *Drosophila* homologue of the mammalian transcription factor YY1 [19], can form ternary complexes with DNA and Pc [20], thereby facilitating tethering of the PRC1 complex, but Pho alone is insufficient for functional targeting of PcG complexes [21]. Interestingly, in contrast to findings from experimental set-ups using naked DNA templates, Pho tethering to chromatinized DNA is dependent upon the TrxG protein GAF [22], further enhancing the significance of TrxG and PcG interactions at target genes. Flies harboring both Pho and Pho-like mutations display loss of PRC1 attachment to a few chromosomal locations only, which is indicative of a role for Pho and Pho-like in tethering to a subset of PREs [23] but also indicates that there must be additional DNA-binding proteins involved in PcG recruitment. In mammalian model systems, YY1 has been found associated with members of both PRC1 and PRC2 [24,25]. YY1 knock-out mice display early embryonic lethality prohibiting further analyses of homeotic phenotypes [26]; however, morpholino knock-down experiments using the *Xenopus* YY1 homologue resulted in antero-posterior axial patterning defects [27<sup>\*</sup>], a typical phenotypic readout for PcG mutations. Importantly, human YY1 was found to repress PcG target genes in *Drosophila* embryos and to partially rescue *Drosophila* Pho mutation phenotypic defects [28], thus establishing YY1 as a *bone fide* PcG member. In mammalian cells the transcription factor E2F6 has been shown to interact with PcG members and to target them to promoter regions [29,30]; however, *E2f6*<sup>-/-</sup> mice display only a mild homeotic phenotype incompatible with an essential role in PcG targeting [31].

Understanding the dynamics and functions of PcG complexes in mammals is further complicated by the fact that many PcG and TrxG genes have been duplicated and diversified over the course of evolution (Table 1). Despite the fact that several PcG targets including hox genes have been found, the first mammalian PRE is yet to be described. Whereas no physical associations have been demonstrated between PRC1 and PRC2 complexes in mammals, both complexes are essential for mouse development, as is evident from the early embryonic lethality observed in mice deficient for Eed, Ezh2 and Rnf2

[32–34]. Hence, PcG complexes are likely to act in a concerted manner as an integrated silencing machinery, and, like chromatin remodeling complexes, may function in a certain temporal order ([35] and references therein).

### A role for RNA in PcG silencing?

The difficulties in pinpointing crucial mediators of PcG recruitment could indicate that PcG targeting is not an ability inherent to a few DNA-binding proteins only, but may reside in transient interactions with a broad range of transcription factors acting to deliver target gene specificity. Also, in analogy with the findings on the HP1/SUV39H silencing system, the involvement of non-protein components, such as RNA, cannot be ruled out [36]. This could in theory provide excellent gene specificity akin to the involvement of short dsRNA in RNA interference (RNAi). In support of this possibility, genetic interactions between PcG mutants and RNAi machinery components have been found in *C. elegans* and *Drosophila* [37,38]. An important challenge will be to substantiate these results with biochemical and functional data as well as to extend these observations to vertebrates. Interestingly, in *C. elegans* and *Drosophila*, PcG homologues play important roles in maintaining genome stability through silencing of repetitive sequence elements, such as pericentromeric heterochromatin repeats and transposons [37,39]. Likewise, in the fly, PcG proteins have been found to bind telomeric sequences consisting of transposable elements [40,41<sup>\*</sup>]. This raises the appealing possibility that in higher metazoans as well PcG complexes may be involved in telomere stabilization and serve as a genome defense system through repeat suppression. Of interest in this respect, the only currently known PcG binding sites in mammals consist of a special subset of pericentromeric heterochromatin repeats [42,43]. Another example implicating RNA is the emerging role of PcG proteins in X chromosome inactivation. This well-studied silencing process involves a series of epigenetic events involving, amongst other features, binding of the PRC2 complex members EED and EZH2 and the large non-coding RNA transcript Xist (for recent reviews see [44] and the review by Heard in this issue).

In the balance between PcG and TrxG complexes at CMMs, silencing appears to be the default mechanism. This can be inferred from the fact that PcG complexes are sequestered to PREs in a body-segment-independent manner and from recent experiments showing that lowering the amount of Trx protein results in inappropriate gene repression [16]. Understanding spatially and temporally how CMMs can switch from repression to a heritable state of transcriptional activation is a key question in the field. Ectopic expression of Trx stimulates transcription of de-repressed target genes but cannot autonomously overcome established repression patterns [16]. An emerging model suggests that transcription through a PRE interferes with target gene silencing

and promotes the switch from repression to a heritable state of activation in a segment-specific manner [45–48]. It has been known for more than a decade that the intragenic regulatory regions of the bithorax complex are transcribed in a manner dependent on segmental position and developmental timing [49]. Transcription through the PRE precedes target gene activation and some of the transcripts have distinct cellular localizations, indicating a potential role for the actual transcripts (possibly in targeting), aside from the CMM-switching function of transcriptional read-through. It is in this context interesting to note that RNA polymerase II in yeast associates with histone methyltransferases (HMTases), resulting in the formation of an epigenetic mark for transcriptional history [50].

### PcG repression and histone code modifications

A fundamental question has been how PcG complexes exert their repressive function and how the information on which genes to repress is carried from the PRC2 complex to the PRC1 complex. Important answers came from landmark work on SUV39H demonstrating that the SET domain of SUV39H exhibits methyltransferase activity towards histone proteins [51]. Many chromatin modifiers carry a SET domain, a protein motif originally identified in the *Drosophila* proteins Suvar39, Enhancer of zeste and Trithorax [52]. This finding boosted the field of chromatin research by suggesting that other SET domain proteins also could exert their effects through histone methylation imprints. Indeed, four research groups independently demonstrated that the PRC2 complex containing E(z)/EZH2 is indeed an active enzyme capable of methylating the histone H3 tail at lysine-9 (K9) and more prominently at K27 [5<sup>\*</sup>–7<sup>\*</sup>,41<sup>\*</sup>]. Using temperature-sensitive *Drosophila* mutants of E(z), it was shown that loss of functional E(z) caused a loss of PcG protein binding to polytene chromosomes [41<sup>\*</sup>], stripped *Drosophila* histones of K27 methylation and furthermore reduced the binding of PC to a PRE, resulting in de-repression [5<sup>\*</sup>]. Likewise, HMTase-negative SET-domain-mutant E(z) proteins failed to repress HOX genes *in vivo* [6<sup>\*</sup>]. Another module found in many chromatin-associated proteins, including the HP1 proteins and homologues of the Polycomb protein, is the chromo domain. Previously, methylated K9 of histone H3 was shown to act as a binding site for members of the HP1 family [53,54] and several laboratories subsequently demonstrated that the trimethylated K27 forms a binding interface for the chromo domain residing in the PC protein [5<sup>\*</sup>,7<sup>\*</sup>,41<sup>\*</sup>]. This was further substantiated by colocalization studies showing a near-perfect overlap between methyl-K27 and PcG proteins Pc and Psc. Conversely, methyl-K9 and HP1 is present mainly in heterochromatic regions devoid of PcG proteins [55–57]. Thus, the K27 methylation marks imposed by E(z) facilitate recruitment of Pc and may thereby mediate targeting of the PRC1 complex to PcG targets.

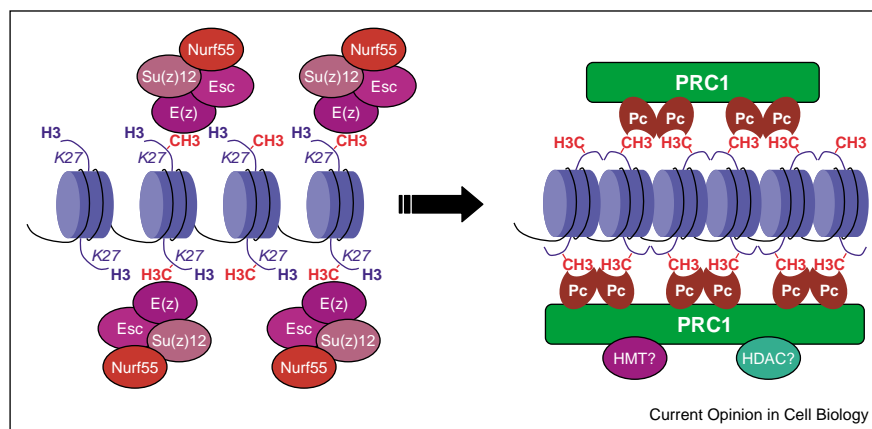
Lysine residues can be mono-, di- or trimethylated at the  $\epsilon$  amino group, thereby potentially vastly expanding the complexity of the histone code. Whereas the chromo domain of Pc will bind with high affinity to trimethylated K27 only, HP1 appears to be less discriminative in binding [56]. The methylated K9 and K27 residues are both present within a highly related ARKS sequence motif, posing the question of how the structurally similar chromo domains of HP1 and Pc can discriminate between the two functionally different histone codes. Structural analyses have demonstrated that the preference of the Pc chromo domain for methylated K27 over K9 resides in an extended binding groove, allowing additional specific contact points to the histone tail [56,58\*\*]. Intriguingly, the crystal structure of the Pc chromo domain in complex with a K27 trimethylated histone peptide also revealed the capacity of the Pc protein to dimerize [58\*\*]. As steric hindrance probably prohibits the binding of a Pc dimer to two methyl-K9 tails from the same nucleosome, Xu and coworkers proposed the appealing model that Pc dimers may bridge two adjacent nucleosomes. Upon sequestration of the remaining PRC1 complex, molecular linkage of neighboring nucleosomes may serve to create a compact and repressive higher order chromatin structure [58\*\*] (Figure 1). This work sheds light on the mechanism by which the PRC2 complex imposes target gene repression, and provides evidence that the information on which loci to repress is transferred from PRC2 to PRC1 via a histone code. How then does the PRC1 complex maintain transcriptional repression? Currently, three not mutually exclusive mechanisms have been suggested: first, that PRC1 blocks the access to DNA for transcription factors and chromatin remodeling factors; second, that PRC1 associates with chromatin-modifying enzymes such as histone deacetylases (HDACs) and HMTases; and third,

that PRC1 and K27 methylation blocks positively-acting imprints such as histone H3 K4 methylation, in analogy to the mutually exclusive phosphorylation of H3 serine 10 and methylation of lysine 9 [51]. Using a biochemical approach, Kingston and colleagues have demonstrated that reconstituted PRC1 complexes can block the access of chromatin remodeling factors to an artificial chromatin template [59]; however, members of the transcription machinery, such as TBP and RNA polymerase II, appear capable of interacting with a repressed promoter, suggesting that physical shielding does not play a major role *in vivo* (V Pirrotta, personal communication).

To date, little evidence exists suggesting that enzymatic activities such as histone deacetylase and methyltransferase activity are associated with PRC1, although interactions have been demonstrated between SUV39H1 and the mammalian Pc homologue HPC2 in overexpression studies [60]. Individual PRC1 PcG proteins have been co-isolated with large protein complexes that also contain methyltransferases such as Eu-HMTase1 and G9a [30,61]. Interestingly, the G9a HMTase is capable of methylating both K9 and K27 of histone H3 [62,63]. Its euchromatic-region-specific activity made G9a an interesting candidate for a PRC1-associated HMTase. However, recent assessments revealed little impact of G9a on K27 methylation patterns *in vivo* [64,65].

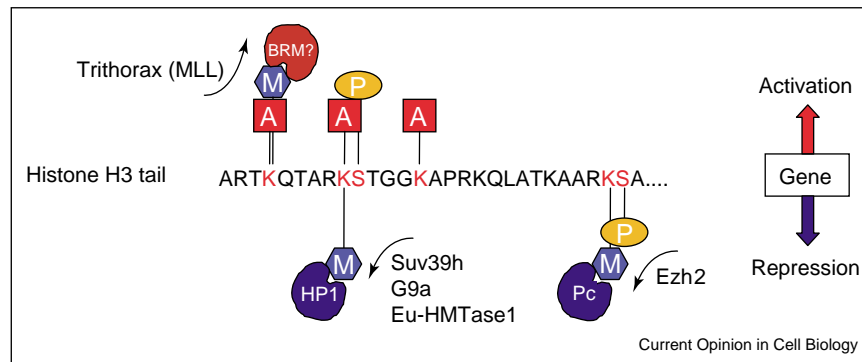
The counteracting effects of TrxG and PcG complexes appear to be directly reflected in the histone codes deposited by these complexes (Figure 2). Methylation of H3 K4 correlates with transcriptional activity [66] and the human Trx homologue MLL/ALL was recently demonstrated to be a H3-K4-specific HMTase [67\*,68\*]. In addition, the TrxG protein Ash1 encodes an active

Figure 1



Model for PcG complex composition and function. In early stages of development (and upon cell division?), the PRC2 complex is recruited to PcG targets to impose a methyl imprint on histone H3 K9 and K27. Methylated K27 serves as an anchorage point for the Pc proteins and recruits in collaboration with sequence-specific DNA-binding proteins the PRC1 complex. Also depicted in this cartoon is the possible anchoring of neighboring nucleosomes within the array by Pc, potentially serving to further condense the chromatin. HMT, histone methyltransferase.

Figure 2



The epigenetic memory system composed of PcG and TrxG complexes is intrinsically linked to the histone code. Covalent post-translational modifications to the N-terminal tail of histone H3 are depicted along with the PcG and TrxG complexes involved in setting and recognizing the respective histone imprints. See text for details.

HMTase capable of methylating histone H3 K4, K9 and H4 K20 [69<sup>••</sup>]. Interestingly, the Ash1-mediated methylation imprint facilitates transcriptional activation of the otherwise silent *Ubx* gene in *Drosophila* and mediates displacement of the HP1 and Pc proteins normally bound to the repressed *Ubx* promoter. Transcriptional activation is mechanistically founded in histone H3 K4 and K9 double methylation, which serves as a binding site for the Brahma chromatin remodeling complex [69<sup>••</sup>]. Thus, the TrxG imprint facilitates transcriptional activation and appears to be incompatible with PcG binding.

### Conclusions and outlook

There is increasing evidence that PcG complexes are not static memory modules but rather dynamic molecular switches sensitive to environmental input and developmental regulation. Many different PcG complexes may exist within a cell, as is evident from biochemical purifications and from the discovery of individual PcG members in distinct protein complexes [3,8,30,59,61,70,71]. A major challenge for the future will be the development of tools to identify and analyze these separate complexes, study their real-time dynamics and uncover their temporal and spatial significance for cell fate decisions. Whereas PREs in the fly have been characterized, deciphering the way in which mammalian PcG complexes recognize target genes remains a challenge. Hopefully, recently developed techniques for chromatin exploration, such as chromatin profiling using tethered *E. coli* Dam-methylase [72] or chromosome conformation capturing [73], may provide new insights in the future.

Significant progress has been made towards understanding the molecular nature of PcG-mediated repression with the discovery of specific methylation imprints for PcG and TrxG complexes. Currently, methylation-specific antibodies are being developed in many laboratories that will enable more detailed dissections of the

combination of marks needed to create a heritable PcG- or TrxG-specific chromatin imprint. Despite these advances it remains an enigma how PcG-specific marks are gene-specifically delivered, recognized and re-set upon cell division. Recent biochemical data suggest that nucleosome imprints, like DNA methylation imprints, may be inherited in a semi-conservative manner during DNA replication [74<sup>••</sup>]. Importantly, this work suggests that new histone H3 and H4 proteins are deposited onto replicating DNA strands as heterodimers. This implies that the two existing H3/H4 heterodimers present within the histone octamer may be divided among the two daughter stands — carrying with them an epigenetic memory imprint. An alternative scenario (although the two are not mutually exclusive) could involve deposition of variant histone proteins as carriers of epigenetic information, as demonstrated in yeast, where H2A.Z prevents the binding of silencing protein to euchromatic regions [75<sup>••</sup>], and in mammals, where the presence of macroH2A (for instance on the inactive X chromosome) correlates with transcriptional repression though impeding the function of the SWI/SNF nucleosome remodeling complex [76<sup>••</sup>]. Interestingly, recent data indicate a more direct involvement of PcG proteins in DNA replication and chromatin assembly. Two PRC1 members, Mph1 and Scmh1, have been found to associate with the DNA replication inhibitor Geminin [77]. Geminin controls cell-cycle progression via binding and inhibition of Ctd1, a key component of the pre-replication complex. Moreover, Geminin was shown to bind and repress *hox* genes and to be involved in determining *hox* gene expression boundaries [77]. Furthermore, PcG mediated repression is in *Drosophila* genetically linked to the chromatin assembly and remodeling factor ACF/CHRAC [78]. Fly *acf1* mutants display de-repression of PRE reporters, and loss of *acf1* was found to enhance the homeotic phenotype of mutations in the *Pc* gene [78]. New mechanistic insights such as these, as well as the

investigation of possible roles of RNA in PcG silencing and the implications of this as a possible conserved genome defense system, will be prospering research areas for the coming years.

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