Polycomb silencing mechanisms and the management of genomic programmes

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Abstract | Polycomb group complexes, which are known to regulate homeotic genes, have now been found to control hundreds of other genes in mammals and insects. First believed to progressively assemble and package chromatin, they are now thought to be localized, but induce a methylation mark on histone H3 over a broad chromatin domain. Recent progress has changed our view of how these complexes are recruited, and how they affect chromatin and repress gene activity. Polycomb complexes function as global enforcers of epigenetically repressed states, balanced by an antagonistic state that is mediated by Trithorax. These epigenetic states must be reprogrammed when cells become committed to differentiation.

Segmentation gene
One of a group of genes that specify the segmental pattern within the anterior–posterior body axis of *Drosophila melanogaster* and other arthropods.

Gastrulation
A morphogenetic process in early embryogenesis, during which the endoderm, mesoderm and ectoderm germ layers are formed.

Position-effect variegation
A phenomenon that was discovered in *Drosophila melanogaster*, which occurs when genes that are placed close to large heterochromatic regions are repressed. Typically, silencing occurs stochastically in some cells and their clonal descendants.

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The ability to partition the genome into sets of active and quiescent genes, and to subsequently maintain this partitioning through many cell divisions, underlies the process of cellular differentiation, which is essential for all but the simplest multicellular life forms. Recent studies in flies and mammals provide evidence that Polycomb and Trithorax proteins are key components of the 'maintenance' part of this genome-programming system.

Polycomb group (PcG) proteins were first discovered in *Drosophila melanogaster* as the products of genes that are required to prevent inappropriate expression of homeotic (Hox) genes[1,2]. Genetic and molecular analyses of this system have produced a basic understanding of the underlying mechanism, although many specific aspects are still unclear or controversial. In *D. melanogaster*, the role of PcG proteins in the control of the homeotic genes begins in the 3-hour-old embryo, shortly after the homeotic genes have been turned on and their characteristic domains of expression have been shaped by transient expression of segmentation gene products, which work as activators and repressors. The action of PcG proteins first becomes detectable at gastrulation, during which they prevent gene reactivation at the time when the early repressors begin to disappear. This maintenance of the repressed state of target genes wherever they had not previously been active in the early embryo is characteristic of PcG silencing of homeotic genes (FIG. 1). As a consequence, a target gene is competent to be activated at later stages only in the progeny of cells in which it was active in the early embryo. This dependence on the history of the gene implies that a kind of cellular memory marks a previously silenced gene, so that it continues to be repressed after every cell cycle. Genetic analysis has shown that an antagonistic system that involves the Trithorax (TRX) protein functions to set a mark for the active state of a PcG target gene[3,4] (TABLE 1). In the absence of TRX, a homeotic gene can become repressed by the PcG-mediated mechanism even in cells in which it had been active in the early embryo. A similar antagonistic relationship is thought to be involved in mammalian PcG mechanisms but much less direct evidence is available.

Many features of PcG-mediated silencing, including evidence that the PcG proteins work together and seem to be sensitive to dosage, resemble the processes of heterochromatic silencing and position-effect variegation (PEV). For PcG silencing, as for heterochromatic PEV, the decision of whether or not to silence a particular gene is made early in development, resulting in variegated expression — silencing of the gene in some cells but not in others, producing mosaic tissues. The assumption of similarity, although prescient in many ways, has tended to condition our view of PcG silencing in ways that are now seen to be inconsistent with current results. Here we review recent developments in our understanding of PcG mechanisms, and their implications for the programming of gene expression in development.

Many other aspects of PcG mechanisms will not be discussed, although they offer tantalizing glimpses into

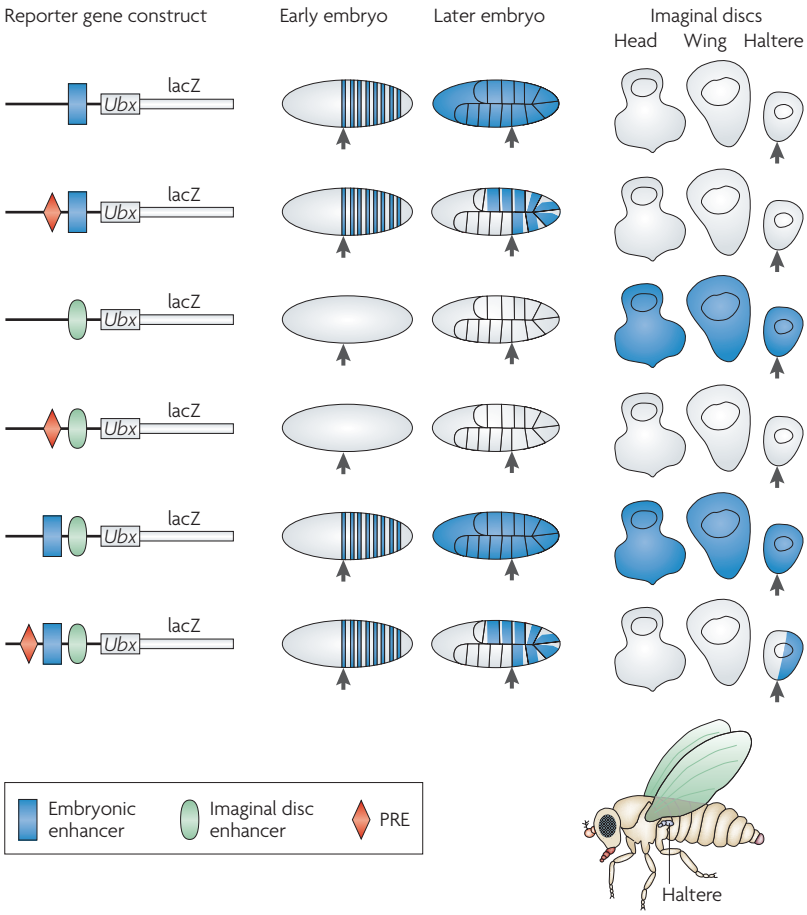

Figure 1 | **How Polycomb group complexes regulate a homeotic gene.** The correct domain of expression of the *Ultrabithorax* (*Ubx*) gene is posterior to parasegment 6 (indicated by an arrowhead). *Ubx* regulation is recapitulated by a series of reporter constructs, using the *Ubx* promoter and different combinations of the *Ubx* embryonic enhancer, Polycomb response element (PRE) and imaginal disc enhancer. The expression pattern that is produced by the different combinations is shown as blue-coloured regions of the early embryo, later embryo or the imaginal disc precursors of adult head, wing or haltere. The early enhancer initially produces the correct pattern of expression, confined to the posterior half of the embryo (posterior to parasegment 6). Shortly after, however, the early repressors in the anterior region disappear and the reporter gene is expressed in all segments. If the PRE is added, repression is maintained in the anterior region. Imaginal disc enhancers are active only at later stages in the head, wing and haltere discs. If combined with the PRE, they remain in the silent state that is established in the embryo. However, when the three elements are combined, the early enhancer sets the early pattern, the PRE maintains the repression anterior to parasegment 6, and the imaginal disc enhancer now remains active posterior to parasegment 6; that is, in the haltere, but not in the wing or head.

the management of chromatin in the nucleus. These include: where PcG proteins and their target genes are localized within the nucleus[5,6]; the effects of PcG action on genes in their spatial proximity, even if they are not on linked DNA (transvection-like effects)[7,8,9]; the role of PcG mechanisms in mammalian X inactivation and genomic imprinting (for reviews, see REFS 10,11); and the possible involvement of structural RNAs or RNAi mechanisms in PcG silencing[12]. Here we concentrate on aspects of PcG silencing that seem most closely related to the question of genomic programming.

Parasegment
Regions of the *Drosophila melanogaster* embryo that contain the posterior part of a hemisegment and the anterior part of its neighbour. They are patterned by segmentation genes. Mesodermal thickenings and ectodermal grooves demarcate parasegment borders.

PcG complexes

PcG silencing involves at least three kinds of multiprotein complex that work together. These are now referred to as the PRC1, PRC2 and PhoRC complexes; they are described below and a summary of their structure is shown in FIG. 2.

The PRC1 complex. The PRC1 complex biochemically purified from flies[13,14] contains a core quartet of PcG proteins: Polycomb (PC), Posterior sex combs (PSC), polyhomeotic (PH) and RING, as well as lower amounts of Sex comb on midleg (SCM). Many additional proteins were also co-purified with these core components, including ZESTE, TBP (TATA-box binding protein)-associated factors TAFII250, TAFII110, TAFII85, and TAFII62, and elements of other multiprotein complexes, such as MI2, SIN3A, SMRTER[14]. The observed association with many of these proteins might be artefactual, but the presence of the TAF promoter factors is intriguing, indicating the possibility of a direct interaction between PcG complexes and promoter complexes; this putative interaction awaits further examination.

Some *D. melanogaster* PcG genes have closely related homologues that are thought to function as alternatives in different tissues, developmental stages or even at different target genes in the same cell. For example, there are two *ph* genes, encoding Polyhomeotic proximal (PHP) and Polyhomeotic distal (PHD) proteins, the functions of which have not been clearly differentiated[15]. Similarly, PSC and Suppressor of *zeste* 2 (SU(Z)2) are closely related and are thought to have partially homologous functions[16,17]. Pleiohomeotic (PHO) and Pleiohomeotic-like (PHOL)[18], and Extra sex combs (ESC) and Extra sex combs like (ESCL)[19], are two other pairs of PcG genes with wholly or partially overlapping functions.

The mammalian PRC1 complex has been isolated from HeLa cells using exogenously expressed tagged protein components and affinity purification[20]. The core components are similar to those of the *D. melanogaster* PRC1 complex, although no TAFs have been detected in association with it. In mice and humans, to a greater extent than in *D. melanogaster*, several of these proteins have other homologues that presumably function as alternatives at different targets or in different tissues. The purified complex(es) include HPC1, 2 and 3, HPH1, 2 and 3, RING1A and RING1B, BMI1 and, potentially, its homologue MEL18. These are, correspondingly, homologous to the fly PC, PH, RING and PSC. The presence of several homologues and the dependence of the stoichiometry on the type of tagging that is used for purification strengthen the impression that the purified PRC1 is a mixture of slightly different complexes.

Functional features that are associated with the PRC1 type of complex include the chromodomain of PC, which binds specifically to trimethylated lysine 27 of histone H3 (H3K27)[21]. RING in flies and RING1A and B in mammals all contain RING domains, and have been shown to function as E3 ubiquitin ligases that mono-ubiquitylate lysine 119 of histone H2A[22–24].

Haltere
A balancing organ that is located on the third thoracic segment in Diptera and is an evolutionary modification of a wing.

Transvection
A phenomenon whereby homologous chromosomes are synapsed in somatic cells, and as a result some enhancers and/or silencers can function in *trans*.

The presence of BMI1, also a RING-domain protein and the homologue of PSC in flies, enhances the catalytic activity of RING1A[25,26]. The role of this ubiquitylation is unknown, but, in its absence, PcG-dependent silencing is said to be abrogated.

The PRC2 complex. The key component of PRC2 is the SET domain H3 methyltransferase protein Enhancer of *zeste* (E(Z)). Biochemical purification of PRC2 from *D. melanogaster* showed, along with the presence of SU(Z)12 and two WD40-domain proteins, ESC or its homologue ESCL and P55 (RBAP46/RBAP48)[27–30], a histone-binding protein that is also associated with the chromatin assembly factor CAF1, and other remodelling complexes[31,32]. Each of these components contributes to the ability of the complex to bind and methylate nucleosomes[33]. E(Z) has no histone methyltransferase activity when alone, but when assembled in the complex it methylates H3K27 (REFS 27,28). *In vivo*, trimethylation of H3K27 is characteristic of PcG target genes[34], but E(Z) is also responsible for widespread mono- and dimethylation of more than 50% of H3K27 in the *D. melanogaster* genome[35]. While H3K27 trimethylation is specifically recognized by the PC chromodomain, the role of H3K27 monomethylation and H3K27 dimethylation remains unknown. *In vitro*, the PRC2 complex also methylates H3K9 (REFS 27,30), and H3K9 trimethylation has been reported *in vivo* at PcG target genes[27,36,37]. Nevertheless, it remains unclear whether this is due to crossreactivity of the anti-H3K9-trimethylation antibody with H3K27 trimethylation, as different antibodies have given conflicting results.

Several molecular species of the E(Z) complex have been detected. In *D. melanogaster*, as in mammals, the most prevalent species is a 600 kD complex, universally

Table 1 | **Main components of the Polycomb/Trithorax maintenance system**

Drosophila protein	Complex	Protein domains	Biochemical activity	Mouse protein homologues
Polycomb group				
PC	PRC1	Chromodomain	Binding to trimethyl H3K27	NPCD, M33 (CBX2), CBX4, CBX6, CBX7, CBX8
PH	PRC1	SAM	?	PHC1, PHC2, PHC3
PSC	PRC1	RING	Cofactor for SCE	BMI1, MEL18
SCE (RING)	PRC1	RING	E3 ubiquitin ligase specific to H2AK119	RING1A, RING1B
SCM	PRC1?	SAM, MBT, Zn-finger	?	SCMH1, SCML2
E(Z)	PRC2	SET	Methylation of H3K9, H3K27	EZH2, EZH1
ESC	PRC2	WD40	Cofactor for E(Z)	EED
ESCL	PRC2	WD40	Cofactor for E(Z)	EED
SU(Z)12	PRC2	Zn-finger	?	SUZ12
PCL	PRC2	PHD, Tudor	?	PHF19, MTF2 (M96)
PHO	PhoRC	Zn-finger	DNA binding	YY1, YY2,
PHOL	?	Zn-finger	DNA binding	YY1, YY2
CG16975 (SFMBT)	PhoRC	MBT, SAM	Binding to mono- and dimethyl H3K9, H4K20	L3MBTL2, MBTD1
SU(Z)2	?	RING,	?	
SXC	?	?	?	?
ASX	?	PHD	?	ASXL1, ASXL2,
MXC	?	LA, RRM	?	Q9CUQ5
E(PC)	?	?	?	EPC1, EPC2
Trithorax group				
TRX	TAC1	PHD, SET,	Methylation of H3K4	WBP7, MLL1
ASH1	?	SET, PHD, BAH	Methylation of H3K4, H3K9, H4K20	ASH1L
ASH2	?	PHD, SPRY	?	ASH2L
BRM	SWI/SNF	SNF2, HELICc, Bromo	ATP-dependent nucleosome sliding	SMARCA4
MOR	SWI/SNF	SWIRM, SAINT	Cofactor for BRM	SMARCC1, SMARCC2
OSA	SWI/SNF?	BRIGHT	?	ARID1B

ARID1B, AT-rich interactive domain 1B; ASH, absent, small, or homeotic discs; ASX, Additional sex combs; BRM, brahma; CBX, chromobox homologue; EED, embryonic ectoderm development; E(PC), Enhancer of *Polycomb*; ESC, extra sex combs; ESCL, extra sex combs like; E(Z), Enhancer of *zeste*; MLL1, myeloid/lymphoid or mixed lineage leukaemia; MOR, moira; MTF, metal response element-binding transcription factor; MXC, multi sex combs; NPCD, neuronal pentraxin with chromodomain; PC, Polycomb; PCL, Polycomb-like; PH, polyhomeotic; PHC, polyhomeotic-like; PHF19, PHD-finger protein 19; PHO, pleiohomeotic; PHOL, pleiohomeotic-like; SCE, Sex combs extra; SCM, Sex comb on midleg; SFMBT, *Scm*-related gene containing four MBT domains; SU(Z), Supressor of *zeste* ; SXC, super sex combs; TRX, Trithorax; WBP7, WW-domain binding protein 7; YY, Yin-Yang transcription factor.

hailed as the PcG complex[38,27–30]. It is possible, however, that this complex is responsible for the genomic mono- and dimethylation of H3K27, rather than its trimethylation. Because the E(Z) complex is not found to be non-specifically associated with chromatin, this methylation activity is probably due to a transient interaction. A second complex of ~1 MD contains the PcG protein Polycomb-like (PCL) in flies[39], and a mammalian counterpart contains the mammalian PCL homologue, PHF (PHD-finger protein) (K. Sarma and D. Reinberg, personal communication). In flies, PCL is required for Polycomb silencing of homeotic genes, and is found at Polycomb sites on polytene chromosomes[37,39]. This indicates that the larger complex is present at PcG target sites and is likely to be responsible for trimethylation of H3K27. Other larger and less well-understood complexes appear at later developmental stages in the fly and contain SIR2, the homologue of the yeast SIR2 NAD$^+$-dependent histone deacetylase[40].

In mammals, the story is more complicated. PRC2, PRC3 and PRC4 complexes have been biochemically characterized, and they differ by the presence of different isoforms of EED, the homologue of the fly ESC[41,42]. In the presence of histone H1, PRC2, but not PRC3, preferentially methylates H1K26, which has an amino acid context similar to that of H3K27, although it is present in only one of the mammalian H1 variants and absent in *D.melanogaster* H1. PRC4 accumulates when EZH2, the mammalian E(Z), is overexpressed in cultured cells. It contains an EED isoform that is expressed only in undifferentiated ES cells, and SIRT1, a mammalian SIR2 homologue. In mammals, the knockout of *SUZ12* also decreases the level of EZH2 protein, indicating that formation of the complex stabilizes EZH2. The result is loss of di- and trimethyl K27 (REF. 43). By contrast, EED is required for all H3K27 methylation, implying that a different EED-containing complex monomethylates H3K27 (REF. 44). The PRC2 complex and its components seem to be the functional core and the most ancient part of the PcG mechanism (BOX 1).

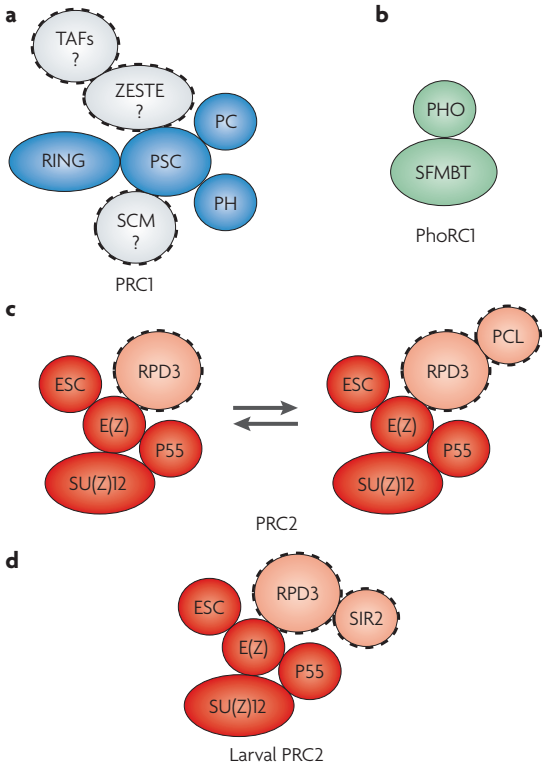

Figure 2 | ***Drosophila* Polycomb group complexes.** Throughout the figure, the core proteins are shown as solid coloured spheres, which have contacts that reflect known interactions. Proteins that are known to associate with the complex but for which direct interaction partners are not known are depicted as coloured spheres with dashed borders. **a** | The PRC1 complex consists of a quaternary core that includes Polycomb (PC), polyhomeotic (PH) and RING held together by the Posterior sex combs (PSC) protein[140,80,25] and probably a number of other proteins, the identities of which are not well established. A more comprehensive review of PRC1 biochemistry can be found in REF. 141. **b** | The characterization of PhoRC1 is still in its infancy. So far, only two directly interacting components, SFMBT (*Scm*-related gene containing four MBT domains) and PHO/PHOL (pleiohomeotic/pleiohomeotic-like), have been described[51]. **c** | Several isoforms of PRC2 have been reported to date (for review, see REF. 142). All of them have a core that consists of four proteins that are crucially important for histone methyltransferase activity (ESC, E(2), P55 and SU(Z)12)[143,144]. In embryos, the PRC2 600 kDa complex is the most abundant and contains the histone deacetylase RPD3, in addition to the core components[27]. Another, larger 1 MDa embryonic complex includes both RPD3 and PCL (Polycomb-like)[39]. Theses two proteins seem to interact with each other directly, but how they bind to the rest of the complex has not been described. The relationship between the two embryonic PRC2 complexes is unclear, and it is possible that they are in a dynamic equilibrium. **d** | In larvae, another species of PRC2 was found to contain the NAD$^+$-dependent histone deacetylase SIR2 (REF. 40).

Polytene chromosomes
Chromosomes in many larval tissues that have replicated repeatedly without cell division and without separating the daughter DNA molecules, which remain aligned with one another.

The PhoRC complex. PHO and its closely related homologue, PHOL, are the only PcG proteins that are known to bind directly to DNA[45–47]. These two proteins are the *D. melanogaster* homologues of the mammalian factor Yin-Yang 1 (YY1), so named because it has both activating and repressive functions. Although it has been reported to interact molecularly with both PRC1 (REF. 20) and PRC2 (REFS 48–50) complexes in flies and mammals, PHO is not an important constituent of either of these two purified complexes. Instead, PHO has been found in two other kinds of complex. In one of these complexes it is associated with the chromatin remodelling machine INO80, which is not known to be involved in PcG mechanisms[51].

The second *D. melanogaster* PHO-containing complex, PhoRC, is involved in homeotic gene silencing, and includes an MBT-domain protein, SFMBT. The MBT domain is found in many mammalian homologues, one of which is the mouse SFMBT (*Scm*-related gene containing four MBT domains). Although not previously known in *D. melanogaster*, the fly SFMBT homologue functions as a bona fide PcG protein and is required for PcG silencing. Its MBT repeats bind specifically to mono- and dimethylated H3K9 and H4K20 (REF. 51).

Vernalization
The mechanism that makes normal flowering dependent on the exposure of the plant to cold.

Chromatin immunoprecipitation
A technique that isolates sequences from soluble DNA chromatin extracts (complexes of DNA and protein) by using antibodies that recognize specific chromosomal proteins.

Tiling arrays
Microarrays containing evenly spaced genomic sequences representing the non-repetitive parts of the genome, often at high resolution.

The Polycomb response element

Functional analysis of the regulatory regions of several PcG target genes allowed the identification of specific elements that are necessary and sufficient for PcG-mediated repression in *D. melanogaster*. All three PcG complexes, as well as TRX, bind to such sequences, called Polycomb response elements (PREs). Although several PREs have been identified in *D. melanogaster*, much of the information about their structure and function comes from work on a few specific PREs: the *engrailed* PRE, the *Fab7* PRE and, pre-eminently, the *bxd* PRE. These are compound elements of several hundred base pairs, different parts of which, by themselves, have weaker PRE-like activity than the whole and contribute to the overall function[52].

PREs are not defined by a conserved sequence. Instead, like many complex enhancers, PREs include many conserved short motifs, several of which are recognized by known DNA-binding proteins. *Drosophila melanogaster* PREs often contain clusters of GAGAG motifs, which bind GAGA factor (GAF) and Pipsqueak (PSQ), both BTP/POZ proteins that reportedly associate with PcG complexes[52,53]. PREs also often contain binding sites for PHO and PHOL. SP1/KLF protein binding is important for the function of the *engrailed* PRE[54]. Binding of the high mobility group (HMG)-like dorsal switch protein 1 (DSP1) is also involved in the function of at least some PREs[55]. A more comprehensive discussion of these factors can be found in REF. 56.

The role of these factors in PRE function is poorly understood. Some might function as recruiters of one or more of the components of the PRC1 or 2 complexes. Alternatively, like GAF, they might serve to mediate the displacement of nucleosomes to allow the binding of other proteins[57,58]. PHO has been reported to bend DNA and to promote the binding of PRC1 complexes to PRE *in vitro*[59]. On the basis of *in vitro* binding experiments with recombinant proteins, PHO has been found to bind to PREs in a highly cooperative fashion with a core PRC1 complex[59]. However, the number and, in some cases, even the presence of the consensus motifs for these proteins varies among PREs, and their sequence context and relationship to one another are not conserved from one PRE to another. A phylogenetic comparison of the *bxd* PRE of the *Ubx* gene from several *Drosophila* species is shown in FIG. 3.

The idea that clusters of such motifs are characteristic features of PREs was exploited by Ringrose *et al.*[60] to produce an algorithm with which to search the *D. melanogaster* genome for putative PREs. The motifs they considered to be typical of PREs are the consensus binding sequences for GAF and PHO, as well as the ZESTE binding sequence, which is often found at known PREs, although rarely in the core region. Both GAF and ZESTE require clusters of binding sites for effective binding, and the algorithm accordingly rewarded the presence of multiple sites within the presumptive PRE. Using this algorithm, more than 100 presumptive PRE sequences have been catalogued in the *D. melanogaster* genome[60]. Recent genome-wide mapping of PcG distribution using chromatin immunoprecipitation (ChIP) or the DamID approach (which involves tagging PcG proteins by fusion with the bacterial DNA methylase Dam and detecting the binding sites by the resulting DNA methylation), and analysis on genomic tiling microarrays, have now tested these predictions directly[34,61,62]. The results indicate that, although about 20% of the detected PcG binding peaks are predicted by the algorithm, the majority are not. Conversely, a similar majority of the predicted PREs fail to bind PcG proteins in these experiments. It is likely that GAF, PHO and ZESTE are not the only DNA-binding proteins that are associated with PREs, and that their presence is not sufficient to define a functional PRE. Other proteins might also be required, or might take the place of this trio in subsets of PREs.

No mammalian PRE has been identified to date, although it is clear that mammalian homeotic genes, and many other genes, are under PcG control, bind PcG proteins and bear methylated H3K27. So far, it is not known whether mammalian PREs exist but are more extensive or diffuse, and therefore harder to identify, or whether, in mammals, PcG proteins are recruited by a different mechanism (see below).

Recruitment of PcG complexes

Early work indicated that the *esc* gene had a special role in the earliest stages of the establishment of PcG silencing in the *D. melanogaster* embryo. The ESC protein component of the PRC2 complex was apparently needed only in the first 4 hours of development[63]. This mysterious

Box 1 | Polycomb group mechanisms in plants

The PRC2 complex is, phylogenetically, far more ancient than PRC1. Genes that encode PRC2 components have been discovered as regulators of different developmental processes in plants. The first to be identified, *CURLY LEAF* (*CLF*), encoded an Enhancer of *zeste* (E(Z)) homologue that is required to regulate homeotic genes in flower development[133]. Although they determine the identity of floral organs, these homeotic genes are entirely unrelated to animal Hox genes. Other PRC2 genes were discovered because of their role in maternal effects that control seed development. The *FIS* (*FERTILIZATION INDEPENDENT SEED*) genes repress development of the seed and endosperm until fertilization[134]. A third set of PRC2 genes was found through mutations that disrupt the normal regulation of flowering, or vernalization[135]. As in animals, the genes that are repressed by these PRC2 mechanisms become methylated at histone H3 on lysine 27 (H3K27).

The genetic and molecular analyses show that plants such as *Arabidopsis thaliana* contain multiple genes for PRC2 components. Three different genes, *CURLY LEAF*, *SWINGER* (*SWN*) and *MEDEA* (*MEA*), encode homologues of E(Z), three genes encode Suppressor of *zeste* 12 (SU(Z)12) proteins and there are five different genes for the P55 homologue, but, at least in *A. thaliana*, there is only one extra sex combs (ESC) homologue[136,137]. Different combinations of these components could, in principle, produce many variant PRC2 complexes, of which at least three are known. An example of this is the SU(Z)12 homologue that is encoded by the vernalization gene *VRN2*, which can interact with the E(Z) homologues CLF and SWN, at least in yeast two-hybrid assays. The E(Z) homologue MEA and SU(Z)12 homologue FIS2 are instead involved in the complex that regulates seed development. Recent work indicates that complexes that contain the three E(Z) homologues regulate the same target genes, but at different stages of development and possibly in different tissues[138].

Despite the multiplicity of PRC2 complexes, plants have no homologues of PRC1 components, including Polycomb itself. Lower metazoans, such as nematodes, also lack PRC1; their PRC2 complex includes an E(Z) and an ESC homologue, and has an important role in controlling gene expression in the germ line[139]. It is not known whether the plant and nematode PRC2 complexes utilize some surrogate to take on the role of PRC1, or whether the PRC2 complex is capable of carrying out all the functions that are necessary for silencing.

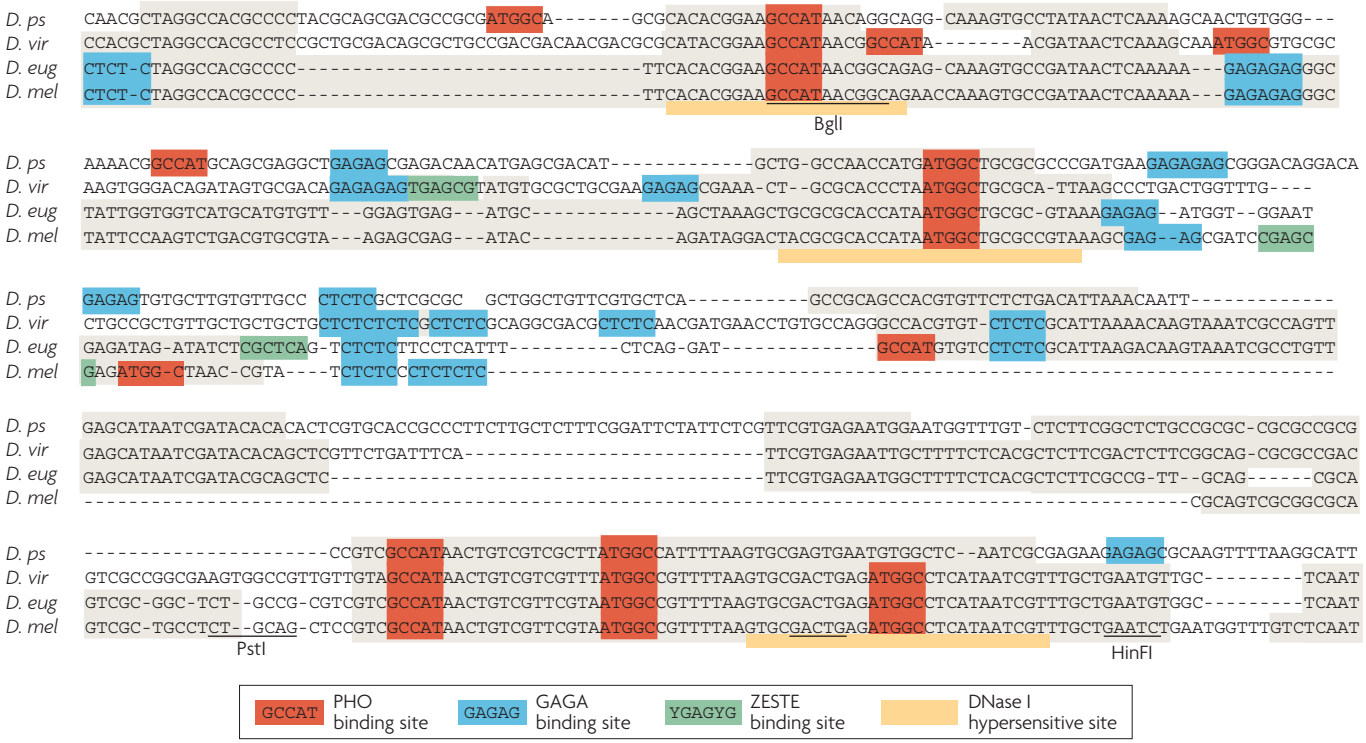

Figure 3 | **Phylogenetic comparison of the *bxd* PRE of the *Ubx* gene from several *Drosophila* species.** Although much of the 1.5 kb sequence of the *Ultrabithorax* (*Ubx*) Polycomb response element (PRE) is well conserved, and, in some stretches, especially highly conserved (99 bp of 100 bp), it also contains sequences of unknown significance. The figure shows the core region of this PRE, compiled in part from REF. 145, with the addition of the corresponding sequence from *Drosophila pseudoobscura* (*D. ps*) from REF. 146. Binding motifs for GAF (GAGA factor), ZESTE and PHO (pleiohomeotic) are highlighted and sequence blocks with high homology are shaded. GAF and PHO motifs are in many cases conserved in their context, but often they are mutated and a new canonical consensus is recreated nearby, indicating that the number of sites, but not their exact relationship, is important. Furthermore, intrusions of extraneous sequences, often repetitive runs of simple motifs, can occur, and a sequence of about 132 bp in the functional core of the PRE that was present ancestrally has been deleted in the phylogenetic branch that includes *Drosophila melanogaster* (*D. mel*)[145]. These features are reminiscent of the sequence conservation that has been reported for enhancer elements[147,148] and support the idea that, although multiple motifs (only a few of which are known at present) are important, they are not equally important in different PREs, and their precise relationship to one another is flexible. *D. vir*, *Drosophila virilus*; *D. eug*, *Drosophila eugracilus*.

early dependence fueled speculations that a different mechanism might be involved in the establishment, but not in the maintenance, of PcG silencing. Recent work has now put these ideas to rest. There is indeed a strong dependence on maternally deposited ESC for the establishment of PcG silencing. However, after the discovery of the homologue ESC-like (ESCL)[19], it now seems that either ESC or ESCL is required at all stages, and that the two proteins are interchangeable for the functioning of the PRC2 complex (REF. 19 and K. Ohno, D. McCabe and V.P., unpublished observations).

Nevertheless, it is clear that H3K27 methylation has a crucial role in the stable binding of PcG complexes. The discovery of the specific binding of chromodomains to methylated histones immediately indicated that methylation might recruit the chromodomain-containing complexes. The idea is that, if PRE-binding proteins can recruit the PRC2 complex, the ensuing methylation would then recruit the PRC1 complex. Wang *et al.*[50] presented evidence suggesting this order of events. This idea is also supported by the fact that, when E(Z) is inactivated by a temperature-sensitive mutation during larval development, binding of PRC1 components is eventually lost from polytene chromosomes[64,27]. Furthermore, PC binding to polytenes can be specifically competed away from at least some sites by incubating the cells with the H3K27 trimethylated peptide[36]. Contradictory to these arguments is the fact that the dissociation constant for PC binding to trimethylated H3K27, as measured *in vitro*, is of the order of 10^{-6} M — respectable binding, but still several orders of magnitude weaker than the interaction of most sequence-specific DNA-binding proteins.

More conclusive are the results of recent chromatin mapping experiments by ChIP and related techniques. It should be noted that ChIP, although it has proven extremely valuable, is a complex technique with many variables that affect the execution and the evaluation of the results. Some of these have recently been discussed[37,65–67]. ChIP experiments, using quantitative PCR or microarray approaches, detect PcG proteins of both PRC1 and PRC2 complexes, peaking sharply at known

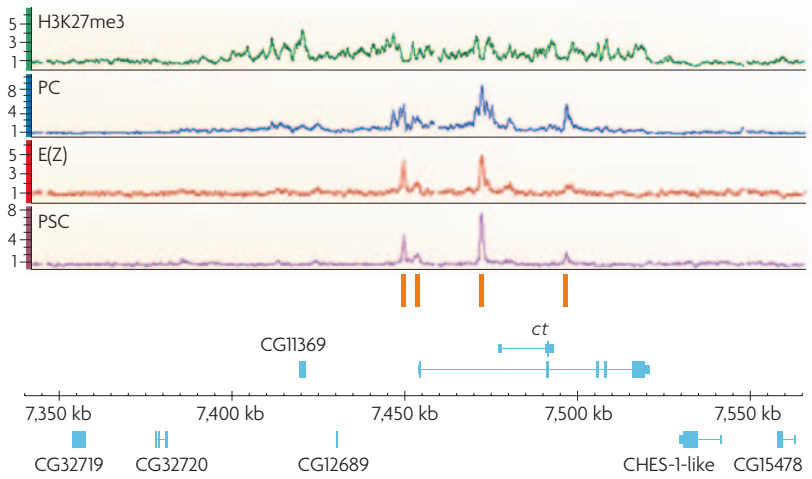

Figure 4 | **PcG proteins at a typical genomic site.** The plots represent the distribution of PcG (Polycomb group) proteins E(Z) (Enhancer of *zeste*), PSC (Posterior sex combs) and PC (Polycomb), as well as of histone H3 trimethylated at K27 (H3K27me3), which were obtained by chromatin immunoprecipitation and analysis on *Drosophila melanogaster* genomic tiling microarrays[34]. The vertical axis represents relative enrichment. The region that surrounds the *cut* locus (*ct*) is shown. Genes shown above the scale are transcribed from left to right, genes below the scale are transcribed from right to left. The vertical orange bars indicate the positions of presumptive Polycomb response elements (PREs). Although PSC and E(Z) are tightly localized at the position of the PREs, the distribution of PC is broader and tails gradually. By contrast, the methylation domain covers nearly 150 kb. The *cut* gene has a complex regulatory unit with multiple enhancers, some as far as 100 kb upstream of the promoter.

or presumptive PREs in *D. melanogaster*. By contrast, the distribution of trimethylated H3K27 at a silenced gene extends over the entire transcription unit and the upstream regulatory region, frequently involving many tens of kilobases[34,37,67]. Although the distribution of PC itself is generally broader than that of the other PcG proteins, and tails gradually from the PRE peak, it certainly does not parallel the distribution of methylation. On the contrary, the known and presumptive PREs seem to be depleted of H3 methylation, probably because they are depleted of nucleosomes[34,37,67,68]. Indeed, a recent study using scanning force microscopy concluded that the PHO complex cooperates with the core PRC1 complex to wrap the PRE DNA around its own surface, rather than around a histone core[68]. The most reasonable conclusion would be that, although K27 methylation is important for stable binding of PC, it is not sufficient. The fact that the PRE is undermethylated also indicates that the manner of PC binding at the PRE itself is not dependent on methylation, and might be different from the binding that is seen at methylated regions that flank the PRE.

In similar experiments in mouse or human cells, PRC1, PRC2 proteins and H3K27 methylation have also been found together at target genes, but in most cases the proteins bind over broader regions than in *D. melanogaster* and their distributions are often co-extensive with the methylation domain[69–72]. This has frustrated attempts to identify mammalian PREs. Are mammalian PREs constituted of much more widely scattered sequence elements, or is the difference simply due to a lower resolution attained with mammalian chromatin? Functional tests that involve placing PcG-binding sequences next to reporter genes might help to clarify this.

Scanning force microscopy
A microscopy technique that works by detecting the vertical position of a probe, which is in physical contact with the sample, while horizontally moving the probe relative to the sample.

PRE binding versus spreading

The discovery some 15 years ago that PC and the heterochromatin protein HP1 share a domain that is now known as the chromodomain[73], together with the condensed appearance of PcG-binding regions in *D. melanogaster* polytene chromosomes, suggested that PcG and heterochromatic silencing mechanisms might be closely related. In both cases, a strong dependence on gene dosage suggested that silencing involved binding of a large number of protein monomers that would spread along the chromosome and colonize extensive chromatin domains[74,75]. In yeast, the SIR complexes that are recruited by the binding of repressor–activator protein RAP1 at telomeric repeats are thought to spread progressively for several kilobases[76]. By analogy, PcG complexes have also been supposed to spread along the chromatin and coat or package it in a condensed, impervious form. Certainly, PREs can work at distances of several tens of kilobases. PREs could be thought of as the recruitment sites of PcG complexes that subsequently spread through cycles of histone deacetylation, methylation and binding of more PcG complexes. However, the localization of PcG proteins at *D. melanogaster* PREs does not support this model[34,37,67]. Although the PC distribution has significant tails that taper slowly in the flanking regions, other PRC1 and PRC2 components seem to be localized at the PRE (FIG. 4). This low-level, extended presence of PC alone beyond the PRE could represent a different mode of binding, mediated by the interaction of the PC chromodomain with methylated nucleosomes: a 'cloud' of PC, weakly bound but held in the vicinity of the methylated domain. This is a moot point in relation to mammalian PcG-binding sites because PcG binding seems to be nearly co-extensive with methylation.

An alternative interpretation is indicated by the extent of the methylation domain in *D. melanogaster*. If the E(Z) complex remains localized at the PRE, the simplest way to account for this widespread methylation is by a looping action of the PRE with its associated complexes, to interact with nucleosomes over a large distance (FIG. 5). Such looping could be mediated by transient interactions of the PC chromodomain with trimethylated H3K27, and could also explain the apparent presence of PC beyond the PRE. Because the chromodomain is the interacting element, PC would be more likely to be crosslinked to these more distant nucleosomes, with which it transiently interacts, than would be other components of the PRE-bound complexes.

In addition to H3K27 methylation, widespread H3K9 and H4K20 methylation has been reported at the repressed *Ubx* gene[37]. The genome-wide distributions of these two histone modifications have not yet been determined in mammals, although K9 methylation has been reported in association with active, as well as repressed, genes. The protein(s) that are responsible for H4K20 methylation at PcG target genes have not been

identified. Although the significance of this modification remains to be assessed, like H3K27 methylation, it might provide other interactions to stabilize the binding of PcG proteins. For example, MBT domains, such as those found in the SFMBT component of the PhoRC complex, and PHD fingers and tudor domains, which are both present in PCL, have been reported to bind methylated histones, including H4K20 (REFS 51,77–79). Therefore, the complexes that are bound at the PRE could engage in multiple interactions with the domain of methylated nucleosomes in the repressed gene. These interactions would provide a mechanism for the spread of methylation from the vicinity of the PRE, by allowing the PRE-bound complexes to interact with methylated nucleosomes and methylate any neighbouring nucleosomes that lack methyl marks. The same principle would account for the maintenance of the methylation domain after each round of DNA replication and nucleosome deposition that dilutes the methyl marks.

The PcG mechanisms of transcriptional repression

Although the repression of transcription is the principal outcome of PcG function, we still know little about how it is achieved. Despite its appealing simplicity and wide acceptance, there is little direct evidence to support the chromatin-condensation model.

Both purified and reconstituted PRC1 complexes from flies and humans inhibit chromatin remodelling and transcription *in vitro*[13,20,80,81]. Recently, a complex that was reconstituted from the PRC1 core components PC, PSC, RING and PH was also shown *in vitro* to cause compaction of a nucleosome array, indicating that this could account for its repressive activity[82]. Unfortunately, the extent to which these findings reflect the situation *in vivo* is difficult to evaluate. As none of the PRC1 core components have sequence-specific DNA-binding activity, PRC1 had to be present at high concentrations in these experiments in order to achieve association with chromatin. Whether a PRE-based

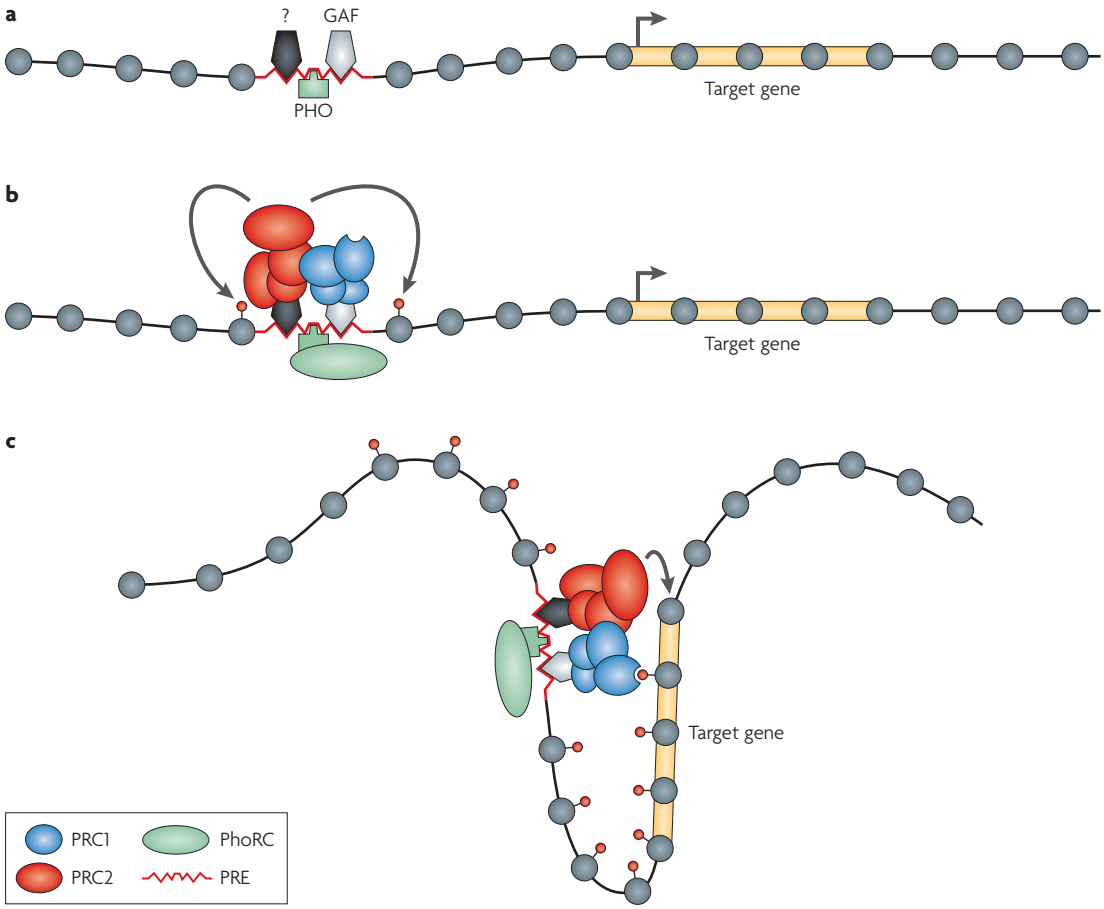

Figure 5 | **Methylation of histone H3 by the PRE complexes. a** | The model depicts DNA-binding proteins, of which only pleiohomeotic (PHO) and GAGA factor (GAF) are shown, binding to the Polycomb response element (PRE), which is shown free of nucleosomes (grey spheres), possibly with the help of a chromatin remodelling complex, and cooperatively recruiting the PRC1 and PRC2 complexes. **b** | The PRE-bound complexes methylate flanking nucleosomes (indicated by red pinballs on the nucleosomes) on either side of the PRE. **c** | The methylation domain is extended by looping of the PRE-bound complexes to contact nucleosomes over a broad region. The looping is mediated by interactions of the PC chromodomain and possibly other methyl-binding domains, such as PHD fingers, MBD, Tudor and SET domains in other PcG proteins, creating and maintaining a broad methylation domain. The PRE also constitutively binds Trithorax (TRX), which would be carried along in the looping contacts and could, in principle, also deposit the histone H3 methylation of lysine 4 (H3K4) mark (not shown).

complex would inhibit remodelling at physiological concentrations remains an open question.

The same applies to the *in vitro* system that was used by Francis *et al.*[82] to monitor chromatin compaction. There is no strong evidence for the idea that PcG complexes induce chromatin compaction *in vivo*. The condensed appearance of major PcG sites in *D. melanogaster* polytene chromosomes is largely attributable to the underreplication of these regions[83,84]. Furthermore, unlike HP1, which in the nuclei of diploid cells mainly localizes with the condensed pericentromeric region (the so called pericentromeric heterochromatin), none of the PcG proteins seem to co-localize with DNA-dense regions in diploid cells[5,85]. In any case, it remains to be proven whether chromatin condensation causes silencing, or whether transcriptional inactivity results in condensation. Several attempts to detect a reduction in chromatin accessibility in genes that have been silenced by PcG mechanisms have given conflicting results, indicating that the effect, if any, is unlikely to constitute the primary repression mechanism[86–89]. The production of a stable condensed chromatin state by PcG complexes would also be difficult to reconcile with *in vivo* photobleaching experiments, which show a dynamic binding of PC and PH proteins to chromatin, exchanging rapidly within a few minutes[85].

If PcG proteins do not compact chromatin and do not prevent access of the transcriptional machinery to the silenced genes, how do they silence? Experiments with reporter constructs indicate that the effects are likely to involve the primary chromatin fibre rather than higher order structures. For example, when the well-defined PRE from the *D. melanogaster Ubx* gene was placed in front of the *lacZ* reporter driven by the heat shock-inducible *hsp26* promoter, the silencing did not significantly interfere with the binding of RNA polymerase II (RNA POL II), TBP or heat shock factor, but it blocked initiation of RNA synthesis by POL II (REF. 90). Heat shock promoters belong to a class of promoters that are said to be pre-set, in the sense that they are constitutively configured to bind RNA POL II. Similar experiments with other kinds of promoters and, in particular, with mammalian systems would help to clarify the generality of these results.

Another insight comes from the observation that, like enhancer action, PcG silencing can be blocked by chromatin insulators[7,67,91,92]. As in the enhancer case, one insulator between the PRE and promoter blocks silencing, but two insulators in tandem allow bypass of the block and lead to promoter silencing[92,93]. This indicates that continuous DNA linkage is not necessary for silencing, and that the PRE-bound PcG complexes are likely to loop out to contact the target promoter. The observation that the insulator prevents the spreading of H3K27 trimethylation from the PRE concomitant with the block that is imposed on silencing suggests that this chromatin mark might be directly involved in repression, or result from the same process that produces repression[67].

In summary, the current evidence, much of which is derived from *D. melanogaster*, indicates that the PcG complex at the PRE must contact the promoter complexes to interfere with transcription initiation and perhaps other aspects of transcription. Histone methylation might stabilize the PRE complexes, but might also provide a means for the long-distance interaction between PRE and promoter. The mechanism of transcriptional interference remains unknown, but could take many forms, including covalent modification of promoter factors, for example, by methylation or ubiquitylation mediated by PRC2 or PRC1.

The role of Trithorax proteins

Several other important aspects must be considered in building a mechanistic model of PcG silencing. To begin with, the repression that is imposed is not 'all-or-nothing'. Low but measurable transcription levels have been detected at silenced *D.melanogaster* Hox genes[34]. Two *D. melanogaster* PcG loci: *ph* and *Psc–Su(z)2* are known to be PcG targets themselves, and are associated with PcG proteins[34,94]. Although transcription levels of the repressed Hox genes are well below physiologically relevant levels, binding of PcG proteins to PcG genes and H3K27 trimethylation are clearly compatible with functional levels of expression.

All PcG target genes that have been studied are also known to be positively regulated by Trithorax and other proteins including ASH1 and ASH2, which seem to work antagonistically to the PcG proteins[3,4]. In *trx* or *ash1* fly mutants, expression of target genes is depressed in a way that is dependent on PcG regulation. If both PcG silencing and *trx* are impaired, expression returns to near normal. Careful analysis shows that the loss of *trx* causes PcG target genes to be silenced, even in regions in which they should remain derepressed. Therefore, it seems that ASH1 and TRX function as anti-repressors rather than typical activators. The molecular basis for this antagonistic activity is not obvious. Most or all known or presumptive PREs also bind TRX constitutively, whether or not the target gene is active or silenced[37,67,95], and thereby function as Trithorax response elements (TREs). The same DNA sequence can therefore behave as a PRE or a TRE, depending on the early events that set the epigenetic state of the gene.

The mammalian TRX homologue, MLL (or ALL) was first discovered because translocations that fuse MLL to various transcriptional regulators are involved in certain human leukaemias. In mice, as in *D. melanogaster*, loss of MLL function is lethal. Heterozygosity for MLL causes homeotic transformations by decreasing the expression of Hox genes and reducing the anterior boundary of Hox gene expression. Other phenotypes are growth retardation and haematopoietic abnormalities[96].

Both TRX and ASH1 are SET-domain proteins with H3K4 methyltransferase activity. Although TRX binds constitutively at PREs, both ASH1 and TRX bind specifically to active genes. TRX is found at the active *Ubx* promoter[37], but it is also recruited at active heat shock promoters and is necessary for their transcriptional activity[97]. This activity corresponds to that of SET1, a yeast TRX homologue that is responsible for the H3K4 methylation that is associated with transcriptional elongation[98–100]. Consistent with this, the mammalian

Chromatin insulators
Cis-acting DNA sequences that function as barriers to the effects of distal enhancers and silencers.

Epigenetic state
Chromatin state that can be inherited by progeny cells and dictates the functional competence of a gene.

Table 2 | **PcG target genes that are important for cell-fate decisions and development**

Drosophila gene	Function in *Drosophila*	Human and mouse orthologue
orthodenticle	gap gene TF	*OTX1*, *OTX2*
empty spiracles	gap gene TF	*EMX1*, *EMX2*
even-skipped	pair-rule TF	*EVX1*, *EVX2*
sloppy-paired	pair-rule TF	*FOXL1*
engrailed	segment polarity TF	*EN1*, *EN2*
gooseberry	segment polarity TF	*PAX3*
wingless	secreted morphogen	*Wnt*
hedgehog	secreted morphogen	*DHH*, *SHH*, *IHH*
decapentaplegic	secreted morphogen	*BMP2*, *BMP4*
forkhead	FOX transcription factor	*FOXA1*, *FOXA2*
Egfr	transmembrane receptor	*ERBB3*
H15	TF controlling muscle development	*TBX20*
Dichaete	SOX transcription factor	*SOX8*
Posterior sex combs	PcG gene	*BMI1*
Polyhomeotic distal and *Polyhomeotic proximal*	PcG gene	*PHC1*, *PHC2*, *PHC3*
ventral veins lacking	TF involved in wing and tracheal development	*POU3F/OCT6*
BarH1	TF with multiple developmental roles	*BARHL1*, *BARHL2*
eyeless	TF crucial for eye development	*PAX6*
cut	TF with multiple developmental roles	*CUTL1*, *CUTL2*
Distal-less	TF critical for morphogenesis of appendages	*DLX2*, *DLX3*, *DLX5*

BMP, bone morhogenetic protein; *Egfr*, Epidermal growth factor receptor; *ERBB*, erythroblastic leukemia viral oncogene homologue; *DHH*, desert hedgehog; *FOX*, forkhead box; *IHH*, Indian hedgehog; *OCT*, Octamer-binding transcription factor; *PAX*, Paired box gene; PcG, Polycomb group; *POU3F* , POU-domain transcription factor; *SHH*, sonic hedgehog; *SOX*, SRY-box containing gene; *TBX*, T-box family member; TF, transcription factor.

TRX homologue MLL is associated with the promoters of all expressed genes[101]. The MLL complex is closely related to the yeast COMPASS complex, which contains SET1 and binds to RNA POL II during transcriptional elongation. The MLL complex also contains ASH2L, the mammalian homologue of *D. melanogaster* ASH2, which is needed to proceed from K4 dimethylation to K4 trimethylation[102]. Because the TRX complex does not trimethylate H3K4 efficiently *in vitro*[27,103], the function of ASH1 might be necessary for adding the third methyl group to H3K4 after it has been dimethylated by the TRX complex. However, if TRX, ASH1 and ASH2 work in transcribed regions, the role of TRX at the PRE/TRE remains unclear.

Biological functions

It was long suspected that PcG proteins regulate many genes in addition to the Hox clusters, and more genes have been added to the list of targets over the years, in both fly and vertebrate systems. PcG mechanisms are required in processes that range from spermatogenesis[104] to the self-renewal of neural and haematopoietic stem cells[105–107], and possibly cell-cycle progression[108]. Recently, microarray studies and, in particular, the combination of ChIP with hybridization of precipitated DNA to genomic tiling arrays have begun to give a genome-wide picture[34,61,62,69–72,109]. These studies show that, in both mammals and *D. melanogaster*, a large number of genes are PcG targets. A high proportion of these genes encode transcriptional regulators, as well as morphogens, receptors and signalling proteins that are involved in all of the main developmental pathways (TABLE 2). Although these studies must be extended to more tissues and developmental stages, the overall picture is that, in any given cell type, most alternative genetic programmes are shut down by PcG mechanisms, except for the subset that is required in that cell type. What then is the PcG state of undifferentiated cells and, in particular, of the most undifferentiated cells: the pluripotent embryonic stem cells (ES cells)?

Not surprisingly, most developmental pathways are repressed in human or murine ES cells, and PcG proteins are found to be associated with the key genes that control these pathways. During differentiation, some of these pathways are induced, genes that encode corresponding developmental regulators are activated and PcG binding and the extent of H3K27 trimethylation at these genes decreases[69–71,109]. Consistent with the causal role of PcG silencing in maintaining pluripotency, mutations in *Ezh2* cannot be established in ES cells[110], and cells that lack EED, another component of PRC2, tend to differentiate spontaneously[69]. Analysis of the factors that are necessary for pluripotency and self-renewal[111,112] points to a small set of transcription factors such as OCT4, SOX2

and NANOG. A significant overlap between the genes that are repressed by OCT4 and NANOG and the PcG targets in ES cells indicates that PcG silencing might be recruited by and collaborates with these transcription factors to maintain the repressed state[70,109]. In a regulatory circuitry that is specific to ES cells, the pluripotency factors would set the stage, selecting sets of genes to be activated or repressed, whereas the PcG complexes would function as the enforcer, ensuring the maintenance of the repressed state. However, the distinguishing feature of ES cells is that they are poised to lift the repression and enter any one of the differentiation pathways. What is different about their repressed state?

It has been proposed that, in ES cells, pluripotency is correlated with a 'bivalent' chromatin state. In these cells, PcG target genes simultaneously bear the 'repressive' trimethylated H3K27 chromatin mark and the 'activating' trimethylated H3K4 mark[109,113]. The mechanisms that result in the deposition of both kinds of chromatin marks remain to be determined. During differentiation, these bivalent domains are resolved to contain exclusively repressive or activating modifications, indicating that bivalency is an important feature of the target genes in ES cells[109]. Also, in *D. melanogaster* tissue culture, an active homeotic gene loses binding of PcG proteins to its PRE and the H3M27 methylation mark[34]. However, another study found that, in a larval imaginal disc in which the *Ubx* gene is active, the PcG proteins are still bound to the PRE and the regulatory region bears the trimethylated H3K27 mark, but the transcribed region lacks this repressive mark and acquires H3K4 methylation, which is consistent with the binding of TRX and ASH1 in the promoter region[37].

Furthermore, in mammals, PcG proteins and H3K27 methylation are found in active Hox genes in undifferentiated cells. During retinoic-acid-induced differentiation, they are displaced from anterior Hox genes, which become activated, but remain bound to posterior Hox genes, which become repressed.

Reprogramming

The realization that most differentiation pathways are regulated by PcG has made it clear that the concept of epigenetically stable silencing being set in the early embryo and perpetuated for the rest of development is not the general rule. There must be ways to bypass or overcome PcG silencing and switch target genes to the active state. In *D. melanogaster*, as we have seen, this can be accomplished by transcriptional activity before PcG silencing is established in the early embryo and the Trithorax function prevents silencing from encroaching. Far less is known about what sets the silenced or derepressed state in mammalian systems. It is clear that epigenetic states switch in the course of development, and in mouse ES cells many PcG target genes become derepressed during retinoic-acid-induced differentiation[69]. But, once established, how can a repressed state be reset?

Several recent studies have noted that transcription through the PRE interferes with PcG silencing, indicating that this is a mechanism for switching or resetting the epigenetic state[114–117]. According to this model, a silenced gene might be derepressed by activating a neighboring transcription unit, perhaps a non-coding RNA, that traverses the PRE. However, another study detected no accompanying transcription after resetting of the state that is mediated by a minimal Fab7 PRE/TRE[118]. A recent paper reported that non-coding TRE transcripts have a role in switching to the active state by directly recruiting the ASH1 histone methyltransferase to the TRE of the *Ubx* gene[119]. Although intriguing, this work does not explain how the TRE RNAs would be produced, and is in direct contradiction with another recent study[37], which finds ASH1 exclusively at the promoter of the active *Ubx* gene.

One way to overcome PcG silencing is to produce a flood of an activator that is targeted to the repressed gene. PcG silencing of a Gal4–UAS reporter construct in the *D. melanogaster* embryo could be lifted by expressing massive doses of Gal4[120,121]. Progeny cells inherited the derepressed state in a TRX-dependent way. These experiments showed that, during larval stages, it becomes difficult to derepress the gene by this method. As in ES cells, the chromatin of PcG-repressed genes in the embryo seems to be more plastic, and becomes progressively more committed as development proceeds. The structural basis for this remains unknown.

Evidence that, even in *D. melanogaster* homeotic genes, the epigenetic state can be reset dates back to the discovery of transdetermination[122]. Larval imaginal discs, which have a developmental identity that has been set in the embryo and maintained by PcG silencing, can be induced to change identity by cutting them and allowing them to regenerate. Recent analysis has shown that the cells that switch identity are subject to intense signalling by powerful morphogens: Wingless/WNT, Decapentaplegic/transforming growth factor-β (DPP/TGFB) and Hedgehog[123,124]. Why the activation of signalling pathways should weaken PcG silencing and favour switching of epigenetic state is not clear, although many interesting hints are accumulating. For example, Lee *et al.*[125] found that, during wound healing in *D. melanogaster*, the JNK pathway is activated, leading to a downregulation of PcG genes. This could favour resetting of the epigenetic state by altering the balance between TRX and PcG mechanisms.

It is likely that similar mechanisms to reprogramme epigenetic states operate in mammals. Several signalling pathways in mammalian cells have surprising effects on the PRC2 complex. Activation of integrins, T-cell receptor, platelet derived growth factor (PDGF) and similar pathways causes export of the PRC2 complex into the cytoplasm, where it seems to interact with the signal response pathway[126–128]. The functional integrity of PRC2 is required for the reorganization of the actin cytoskeleton in response to signalling[128]. At the same time that PRC2 has a role in the cytoplasm, this complex is also at least partly depleted from the nucleus. It is not known whether depletion is global or whether a subset of genes is specifically affected.

PcG proteins can also be relocalized within the nucleus. In *D. melanogaster* spermatogenesis, a set of specific genes that are repressed by PcG complexes in

Imaginal discs
An epithelial sheet that gives rise to external adult structures during insect metamorphosis, including the wings, eyes and antennae.

precursor cells are reactivated by the action of testis-specific TAFs, which bind to the promoters but also induce the relocalization of PcG proteins to the nucleolus[104]. Modifications of PcG proteins might be a way to evict them from target genes. Phosphorylation of PcG proteins induces their dissociation from chromatin[129]. Signalling pathways such as EGF activate ERK cascades leading to the activation of MAPKAP kinase 3, which binds to PcG complexes, phosphorylates their components and causes their dissociation from chromatin[130]. Another mechanism involves PI3K–AKT, which is activated by many signalling pathways. Activated AKT enters the nucleus and phosphorylates EZH2 (REF. 131). It is not known if these kinases are targeted to specific genes, thereby inducing local derepression rather than a global loss of silencing.

Conclusion

Polycomb mechanisms have proven to be highly conserved; in the case of the PRC2 complex, from plants through to humans. Their role in governing patterns of gene expression now seems to be pervasive, targeting key genes that activate all differentiation pathways. We have learned much about PcG involvement in the management of the genome, but many questions about PcG-mediated silencing remain to be resolved, including some of the most basic, such as the mechanism of repression. What seems evident is that the heterochromatin-derived ideas of PcG-complex recruitment by histone methylation, spreading, inducing chromatin compaction and reduced accessibility do not fit current observations. Instead, PRE-bound PcG complexes work at a distance. The promoter complex is clearly a direct target, but we do not know how transcription initiation is blocked. Nor is it clear whether PcG mechanisms also affect other stages of transcription, such as elongation. We have tended to assume that the effects of PcG complexes would be fully accounted for by the chromatin alterations, but it is entirely possible that the histone methylation pattern and the PcG-complex distribution are only devices to deliver the crucial function to the right places. For example, the real functional action in repression might be the ubiquitylation or methylation of some component of the transcriptional machinery by RING1 (a component of PRC1) or EZH2 (a component of PRC2). The report of the methylation of P53 by SET9 (REF. 132) or of the action of the PRC2 complex in the cytoplasm[128] should remind us that SET domain methyltransferases can target other proteins as well as histones.

The realization of the central importance of PcG silencing in genomic programming and cell fate determination makes it all the more important to gain a better understanding of the way PcG complexes are recruited to target genes. An account of what makes a PRE in *D. melanogaster* seems just around the corner. In mammals, in which PREs are still to be identified, the relationship between DNA-binding transcription factors and PcG recruitment might hold the key to the selection of target genes in different cell types and at different developmental stages. The recent genomic microarray analyses of PcG proteins in mouse and human cells could now help to identify such sites. Rapid advances being made in this area justify the hope that we will soon understand the nature of stem cells, their self renewal and their ability to maintain the pluripotent state. With that knowledge might come the ability to reprogramme differentiated cells to reacquire pluripotency or to enter alternative differentiation pathways.

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Competing interests statement
The authors declare no competing financial interests.

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