nature structural & molecular biology

REVIEW

Transcriptional regulation by Polycomb group proteins

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Polycomb group (PcG) proteins are epigenetic regulators of transcription that have key roles in stem-cell identity, differentiation and disease. Mechanistically, they function within multiprotein complexes, called Polycomb repressive complexes (PRCs), which modify histones (and other proteins) and silence target genes. The dynamics of PRC1 and PRC2 components has been the focus of recent research. Here we discuss our current knowledge of the PRC complexes, how they are targeted to chromatin and how the high diversity of the PcG proteins allows these complexes to influence cell identity.

The DNA of eukaryotic cells is organized in chromatin fibers, with the nucleosome forming the basic repeating unit. Each nucleosome comprises 145–147 bp of DNA wrapped in 1.8 helical turns around an octamer of four highly evolutionarily conserved histone proteins—H2A, H2B, H3 and H4. Histone H1 binds the linker DNA between two adjacent nucleosomes, causing further compaction of the chromatin fibers into higher-order structures, often referred to as solenoids. Analysis of the crystal structure of the nucleosome revealed that N-terminal histone tails are flexible and protrude outward from the nucleosome core[1]. Histone tails undergo numerous post-translational modifications (PTMs) that influence a large number of nuclear processes, including transcription, replication, DNA repair, chromosome compaction and localization. Two main functions have been ascribed to histone PTMs so far. First, lysine acetylation neutralizes the positive charge of histone tails and can thereby alter histone-DNA interactions and/or decrease interactions between different histones in adjacent nucleosomes. Second, several PTMs can generate docking sites or modulate the affinity of nuclear proteins for chromatin. The specific recognition of histone PTMs is achieved by a dozen protein domains, which are present in a large number of chromatin-associated proteins[2]. In turn, these adaptor proteins are usually part of large protein complexes implicated in chromatin remodeling, transcription and/or further modification of histone tails.

The functions of Trithorax group (TrxG) and PcG proteins exemplify how histone PTMs are involved in transcriptional regulation. These proteins were identified in *Drosophila melanogaster* almost 40 years ago[3] as activators and repressors of homeotic (Hox) genes, respectively, during early embryonic development. Mechanistically, proteins of both families modify histone tails: TrxG catalyzes the deposition of a trimethyl group on K4 of histone H3 (forming the H3K4me3 mark), and PcG is responsible for both di- and trimethylation of H3K27. PcG and TrxG appear to be required for propagation of the repressed or activated transcriptional state during the cell-division cycle[4,5].

TrxG and PcG proteins are involved in maintaining cellular identity and are required for normal differentiation[6,7]. Moreover, in accordance with the notion that cancer is a disease of stem cells and differentiation, TrxG and PcG genes are frequently found to be mutated and/or deregulated in cancer[8–10]. In this Review, we will discuss recent advances in understanding of the role of PcG proteins in gene regulation, specifically in controlling self-renewal of embryonic stem cells (ESCs), and their subsequent role in lineage choice and development. Moreover, we will discuss the mechanisms involved in regulating the targeting of Polycomb complexes to specific genomic loci.

Polycomb complexes: composition and evolution

Most PcG proteins are part of transcriptional-repressive complexes, termed PRCs[11], in most metazoan species. Two major complexes were identified more than 15 years ago, PRC1 and PRC2 (**Fig. 1** and **Table 1**). However, recent data suggest that the diversity of PRC complexes is greater than anticipated, as discussed below. In mammals, PRC2 consists of three core PcG components: enhancer of zeste 2 (EZH2) or its close homolog EZH1, embryonic ectoderm development (EED), and suppressor of zeste 12 (SUZ12). As components of PRC2, EZH2 and EZH1 can catalyze mono-, di- and trimethylation of H3K27 (refs. 12,13).

H3K27me3 can act as a docking site for the chromobox-domain (CBX) protein subunits of PRC1, thus providing a mechanism for the orderly recruitment of PRC2 and PRC1 to target genes. CBX proteins form the core of PRC1 together with one member of the PCGF family (PCGF1–PCGF6), of the RING1 family (RING1a and RING1b) and of the HPH family (HPH1–HPH3) (**Fig. 1**). These complexes catalyze the monoubiquitination of H2A on K119 (H2AK119ub1) through the E3 ligases RING1a and RING1b[14,15].

Sequence alignment of PcG proteins has revealed a high conservation across species, particularly within key functional domains, such as SANT, SET, WD40 and zinc-finger motifs, thus underscoring the essential function of PcG in transcriptional control and development.

Diversity of PcG complexes. The number of PcG genes found in vertebrates is roughly twice that found in other species: the *Drosophila* genome contains 15 Polycomb genes, whereas mammals have 37.

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Received 9 May; accepted 12 August; published online 4 October 2013; doi:10.1038/nsmb.2669

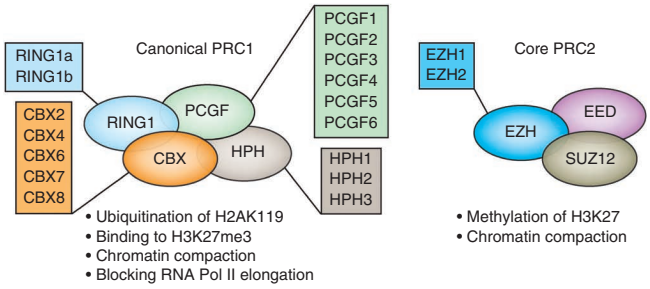

Figure 1 Composition and function of the main Polycomb complexes. Core components of mammalian PRC1 and PRC2 are shown. The diversity of Polycomb complexes is achieved by the incorporation of homologous proteins. Here, we show the canonical PRC1 complex but not the recently reported variations of it (described in the main text). Additional protein components of PRC1 and PRC2 that are present depending on cell type and isolation procedure are not depicted.

For example, the *Drosophila* Pc protein has five paralogous CBX genes in mouse and human cells. Similarly, six members of the PCGF protein family are the mammalian counterparts of Psc in *Drosophila* (**Fig. 1** and **Table 1**). Thus, a total of 180 different PRC1 complexes could exist in mammals if all combinations of the homologous PRC1 proteins were able to form. A recent study, focusing on the PCGF component of PRC1, reported that at least six different PRC1-like complexes could form[16]. Interestingly, consistent with a number of other results[17–23], this analysis showed that the PCGF proteins form part of PRC1-like complexes (i.e., noncanonical PRC1 complexes) that do not contain a CBX protein.

The functional and physiological roles of the many different PRC1 complexes are not clear. As discussed below, the different complexes might have different biochemical activities and therefore different functional roles, and they might form at different times during differentiation and development. For instance, two recent studies showed that the PRC1 protein-complex composition and the corresponding target genes vary dynamically in pluripotent ESCs compared to differentiated cells[24,25]. In self-renewing mouse ESCs, Cbx7 is the only mammalian paralog of *Drosophila* Pc found within the PRC1 complex. Cbx7 binding to the H3K27me3 mark is required for the proper targeting of this PRC1 complex to genomic loci[24]. In differentiating cells and fibroblasts, Cbx7 is replaced by Cbx2 and Cbx4. These variations of the PRC1 complex facilitate the repression of pluripotency genes and the repression of the Cbx7 promoter. Although the switch in PRC1 composition is necessary for the three germ layers to be correctly established, it is unclear how this process is regulated.

Functional differences between PRC1 complexes were shown in another recent study on a noncanonical PRC1 complex that contains RYBP but is devoid of any Cbx proteins[16,22]. Interestingly, the classes of genes regulated by the canonical (Cbx7-containing) and the noncanonical (RYBP-containing) PRC1 complexes (denoted Cbx7-PRC1 and RYBP-PRC1) are substantially different. Cbx7-PRC1 mainly represses early lineage-commitment genes, whereas RYBP-PRC1 is mostly implicated in modulating the expression of metabolic and cell cycle–progression genes[26]. That Cbx7-PRC1 acts as a repressor, whereas RYBP-PRC1 allows promoter activity, corroborates the observations that Cbx7-PRC1 is more efficient in compacting chromatin *in vitro*[16], whereas target genes of RYBP-PRC1 are co-occupied by the elongating RNA polymerase II (RNA Pol II)[26]. The global contribution of PRC1 to compacting chromatin in intact cells still remains to be shown[27]. Moreover, it is not known whether RYBP-PRC1 and RNA Pol II colocalize on the same promoters or on different alleles.

Polycomb proteins in other complexes. In addition to the PRC complexes, PcG proteins are also present in several other multiprotein complexes (**Table 2**). Biochemical purification of the dRING-associated proteins in *Drosophila* led to the identification of dRAF[19], a new protein complex, comprising the PcG proteins dRING and Psc together with dKDM2, an H3K36me2 demethylase enzyme. This complex can couple the removal of dimethylated H3K36, which is normally associated with transcriptional elongation, with mono-ubiquitination of H2AK119. In *Drosophila*, the TrxG protein ASH1 contributes to the establishment of H3K36 methylation, thus demonstrating a further antagonistic action between Polycomb and Trithorax proteins. A similar coordination of histone modification is provided by one of the MLL complexes, which contains the H3K27me3 demethylase UTX[28] and MLL3 or MLL4 (homologs of TrxG). This complex

Table 1 Polycomb repressive complexes[a]

Homo sapiens	*D. melanogaster*	Reported or potential function	Domain
PRC1			
RING1A (RNF1)	dRing	Ubiquitinates H2AK119	RING finger
RING1B (RNF2)			RING finger
CBX2	Pc	Can bind H3K27me3, H3K9me3 and RNA. CBX4 is reported to be a SUMO E3 ligase.	Chromodomain; AT hook
CBX4			Chromodomain
CBX6			Chromodomain
CBX7			Chromodomain
CBX8			Chromodomain
PCGF1 (NSPC1)	Psc	Enhancer of RING1a and RING1b activity	RING finger
PCGF2 (MEL18)			RING finger; proline/serine rich
PCGF3			RING finger
PCGF4 (BMI1)			RING finger
PCGF5			RING finger
PCGF6 (MBLR)			RING finger; proline rich; glutamate rich
SCMH1	SCM		MBTs; SAM; DUF3588
RYBP	dRYBP	PRC1 recruitment (?)	Zinc finger; proline rich; lysine rich; serine rich
YAF2			Zinc finger
PRC2			
EZH1	E(z)	H3K27 di- and trimethylation	SANT; CXC; SET
EZH2			SANT; CXC; SET
EED	ESC	H3K27me3 binder, required for catalytic activity of PRC2	WD40
SUZ12	Su(z)12	Complex stability, required for catalytic activity of PRC2	RING finger; VEFS box; glycine rich; alanine rich
RBBP7 (RBAP46)	Nurf55	Nucleosome binding	WD40
RBBP4 (RBAP48)		Nucleosome binding	WD40

[a]Alternative protein names are shown in parentheses.

Table 2 Multiprotein complexes containing Polycomb proteins[a]

H. sapiens	*D. melanogaster*	Reported or potential function	Domain
E2F6-containing complexes (E2F6.com)			
RING1a and RING1b		H2AK119 ubiquitination	RING finger
RYBP and YAF2		Recruitment (?)	Zinc finger; proline rich; lysine rich; serine rich
CBX5 (HP1γ)		Binds H3K9me3	
PCGF6 (MBLR)			RING finger; proline rich; glutamate rich
L3MBTL2			MBT; Zinc finger
E2F6		Transcription factor	E2F DNA-binding domain
DP1		Heteromeric partner of E2F	E2F DNA-binding domain
MAX		Binds E boxes	bHLH
MGA		Binds E boxes	bHLH; T Box
KMT1D (Eu-HMTase1)		H3K9me1 and H3K9me2 methyltransferase	Ankyrin repeat: Pre-SET; SET
KMT1C (G9a)		H3K9me1 and H3K9me2 methyltransferase	Ankyrin repeat: Pre-SET; SET; Post-SET
Polycomb repressive deubiquitinase (PR-DUB)			
BAP1	Calypso	H2AK119 deubiquitination	Peptidase C12
ASXL1–ASXLASXL3	ASX		Atypical zinc finger
dRING-associated factors (dRAF)			
	dRING	H2AK119 ubiquitination	RING finger
	PSC	Enhancer of dRing activity	RING finger
	dKDM2	H3K36me3 demethylation	JmjC; F Box; CXXC
BCL6 co-repressor complex (BCOR)			
RING1a and RING1b		H2AK119 ubiquitination	RING finger
PCGF1 (NSPC1)		Enhancer of Ring1 activity	RING finger
PCGF4 (BMI1)			RING finger
KDM2B (FBLX10)		H3K36me2 demethylation, binding to GC-rich areas	JmjC; CXXC; PHD; FBOX; leucine-rich repeats
RYBP and YAF2		Recruitment (?)	Zinc finger; proline rich; lysine rich; serine rich
CK2a			Serine/threonine protein kinases
SKP1			
CBX5 (HP1γ)		Binds H3K9me3	Chromodomain; chromo shadow
CBX8		Binds H3K27me3	Chromodomain

[a]Alternative protein names are shown in parentheses.

can therefore potentially couple the removal of a repressive mark with the establishment of the H3K4me3 active mark.

RING1a and RING1b are also part of the BCOR complex[18,21], which is homologous to the *Drosophila* dRAF complex. The following proteins are associated with BCOR: RING1a or RING1b, PCGF4 (BMI1), PCGF1 (NSPC1), KDM2B (FBXL10), RYBP or YAF2, CK2a, Skp1, CBX5 (HP1γ), CBX8 and the BCL6 co-repressor, BcoR, which lends its name to the complex. Both PCGF1 and PCGF4 enhance the catalytic activity of RING1b *in vitro*[18] and *in vivo*[21]. Downregulation of PCGF1 in HeLa cells leads to a dramatic reduction of H2AK119ub1 levels at the Polycomb-target gene *HOXA7* (ref. 29). As mentioned above, KDM2B is a Jumonji C (JmjC) domain–containing histone demethylase for methylated H3K36. Thus, in the mammalian version of the dRAF complex, histone-demethylase activity is also coupled with the deposition of H2AK119ub1. Although some of the characteristics of dRAF and BCOR complexes have been unveiled, it is not yet known whether there is a functional interaction with the canonical PRC1 complexes.

The purification of E2F6-associated proteins in quiescent cells led to the identification of yet other RING1a- and RING1b-containing complexes, termed E2F6.com, that comprises E2F6, DP1, MGA, MAX, L3MBTL2, PCGF6, RING1a, RING1b, HP1Y, YAF2, KMT1C and KMT1D[20]. This complex methylates K9 on histone H3 and probably regulates promoters containing an E2F-binding site and Myc-response elements. Neither the function of the Polycomb proteins within this complex nor whether it possesses H2A ubiquitination activity has been elucidated. A variation of this complex was also isolated in proliferating cells[30]. Interestingly, genetic deletion of E2F6 in mice causes homeotic transformation of the axial skeleton[31], similar to the skeletal transformations observed in Polycomb-knockout mice, thus suggesting that E2F6 has an essential role in the transcriptional repression of a subset of Polycomb-target genes.

Recently, the Polycomb repressive deubiquitinase (PR-DUB) complex has been identified in *Drosophila*[32]. This complex consists of Calypso, a deubiquitinating enzyme (a homolog of human BRCA1-associated protein 1 (BAP1)) and of additional sex combs (ASX; a homolog of ASXL1, ASXL2 and ASXL3 in mammals). PR-DUB catalyzes the deubiquitination of H2AK119ub1. Paradoxically, this complex is bound at PcG targets and is essential for promoter silencing, a result suggesting that a balance between H2AK119ub1 deposition by PRC1 and/or dRAF and deubiquitination by PR-DUB is required for Polycomb-mediated repression. Recent results in human hematopoietic cells show that ASXL1 binds PRC2 and is required for H3K27 methylation[33]. The exact mechanism by which PR-DUB contributes to the regulation of cell differentiation and proliferation is not known, and this is further complicated by the presence of additional components found in the mammalian complex[34,35]. However, due to the frequent mutations of *BAP1* and of *ASXL1* in several human diseases[36], intense research to elucidate the function of PR-DUB is ongoing.

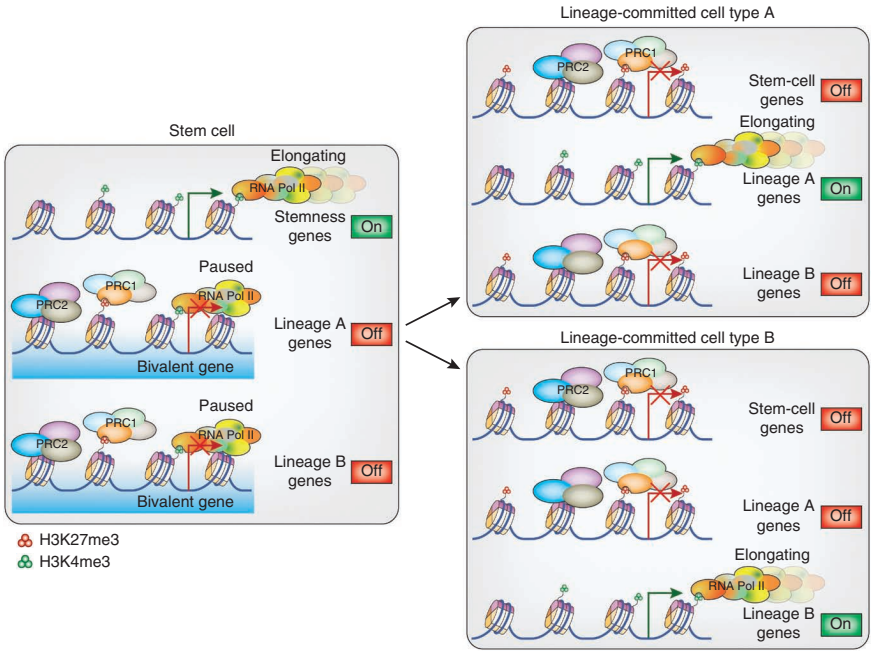

Figure 2 Role of PcG proteins in determination of stem-cell fate. Stem cells and progenitor cells have the potential to differentiate into multiple lineages (here simplified as cell type A or B). Before differentiation stimuli act, genes that determine 'stemness' are transcribed by the RNA Pol II machinery, whereas bivalent genes implicated in lineage specification are silenced, in part also by PRCs. Of note, bivalent genes are also found in other cell types of restricted potency. During differentiation, PRCs are displaced from a specific set of lineage-specific genes, depending on the differentiation stimulus received. A switch in the composition of the PRCs then allows them to target and silence the stemness genes. In a differentiated cell, the bivalent domains are resolved into activated or repressed promoters, marked by RNA Pol II or PRCs, respectively.

The role of Polycomb in gene regulation

PcG proteins are present on repressed genes. Whereas PRC2 is enriched at CpG islands close to transcriptional start sites, H3K27me3 and PRC1 cover parts of the gene bodies as well. Paradoxically, in pluripotent ESCs, H3K27me3 is often found at developmentally regulated genes together with the TrxG-dependent mark H3K4me3. The presence of both active and repressive marks at the same genomic loci, often referred to as bivalent domains, has been suggested to poise genes for subsequent activation during cell-fate decision (**Fig. 2**).

The presence of PRC1 and PRC2 at chromatin leads to chromatin compaction, which is believed to be mediated by the E3 ligase activity of the Ring1 component of PRC1 (refs. 37,38) (**Fig. 3**). This compaction is often observed within nuclear foci called PcG bodies[39]. In *Drosophila*, Polycomb proteins are additionally involved in longer-range chromatin contacts, and this adds a degree of complexity to PcG-mediated higher-order chromatin organization[40]. The compact state of chromatin reduces the accessibility both of transcription factors and ATP-dependent chromatin-remodeling machineries, such as SWI/SNF[41] (**Fig. 3**). Interestingly, the nucleosome remodeling and deacetylase co-repressor complex (NuRD) seems to promote PRC2 activity because its deletion impairs PRC2 recruitment and proper gene silencing[42,43]. The mechanism might involve NuRD-mediated deacetylation of the acetyl-H3K27 mark, a modification that blocks PRC2 activity[43].

PcG-mediated gene repression. H2AK119ub1 decorates about 10% of endogenous H2A, and original reports indicated that it is present at transcribed regions[44]. The major enzyme responsible for the deposition of the ubiquitin moiety at H2A is RING1b[15]. Studies have proposed that H2AK119ub1 at bivalent promoters restrains RNA Pol II activity[45] and that it prevents the eviction of the H2A–H2B dimers from nucleosomes that is necessary for transcription elongation[46]. Moreover, a direct connection between H2AK119ub1 and methylated H3K4 has been documented: *in vitro* experiments suggest that the presence of H2AK119ub1 specifically prevents H3K4 methylation[47]. A recent report has further highlighted the implication of H2AK119ub1 in gene silencing and chromatin compaction. Ablation of the catalytic activity of both Ring1a and Ring1b in mouse ESCs is dispensable for PRC1 occupancy and for compaction of the Hox loci, but it is indispensable for efficient repression of target genes and for ESC identity[48]. However, during reactivation of Polycomb-silenced genes H2AK119ub1 serves as a binding platform for the transcription factor ZRF1, whose occupancy causes displacement of PRC1 from promoters[49].

PcG proteins and poised RNA Pol II. Recent data have highlighted an unexpected link between the general transcription machinery and PcG proteins. Studies in *Drosophila* have suggested that the occupancy of PcG proteins to repress transcription does not exclude TATA-binding protein (TBP) and TBP-associated factors (TAFs) from promoters[50,51]. Similarly, results in mouse ESCs have suggested that many bivalent promoters are occupied by RNA Pol II, probably with the TAF3 component of TFIID docked at H3K4me3 (ref. 52) and PRC1 at H3K27me3 (**Fig. 2**). The phosphorylation state of the engaged RNA Pol II (phosphorylated S5 of the C-terminal

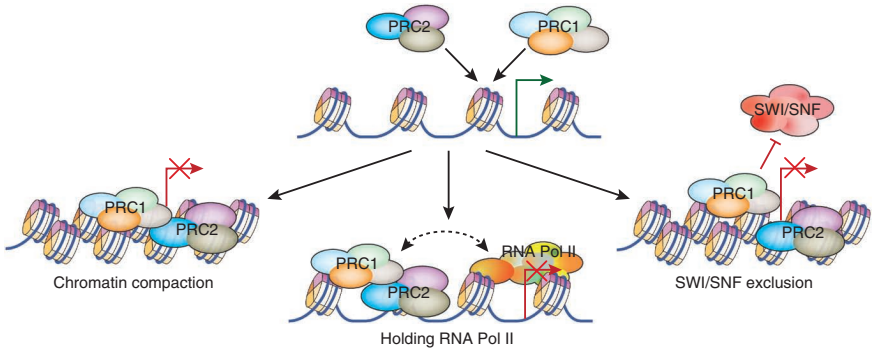

Figure 3 Polycomb-mediated gene repression is a multilayer process. Polycomb-complex binding contributes to gene silencing in numerous ways: it induces chromatin compaction, as observed both *in vitro* and *in vivo* (bottom left), and it interferes with transcription by preventing RNA Pol II activity (bottom middle) or SWI-SNF accessibility to promoters (bottom right).

domain of the Rpb1 subunit) indicates that the polymerase is paused[53], and low levels of short transcripts (50–200 nucleotides in length) are detected[54]. These promoters are also decorated with H2AK119ub1 (ref. 45). Interestingly, deletion of RING1a and RING1b leads to promoter activation and to a switch in the phosphorylation state of RNA Pol II at S2, which correlates with the elongation, splicing and polyadenylation processes. Thus, it seems that the presence of PcG at bivalent promoters interferes with transcription by 'holding' the RNA Pol II over the transcription start site, thereby preventing promoter escape and/or elongation. Therefore, a possible scenario is that TFIID marks a subset of PcG-regulated genes poised for rapid expression during ESC differentiation (**Fig. 2**); however, the importance of such a regulatory mechanism remains to be determined.

Role of PcG proteins in ESC self-renewal and differentiation

The PcG proteins are essential for embryonic development, and early studies suggested that Ezh2 and Eed are also required for self-renewal and pluripotency of mouse ESCs[55,56]. However, the latter observation appears to be a result of the specific growth conditions used because several laboratories have reported that ESCs lacking Ezh2, Eed or Suz12, with self-renewal capacities similar to that of wild-type ESCs, could be successfully generated and maintained[13,57,58]. Moreover, *Eed*-knockout (KO) cells, which are devoid of detectable H3K27 methylation, have been shown to contribute to all tissues in chimeric embryos, and therefore they appear to retain pluripotency[57]. This conclusion, however, might be confounded by a potential contribution of wild-type cells in the chimeric setting, and it is therefore not clear whether *Eed*-KO cells, strictly speaking, are pluripotent. Nevertheless, PRC2 activity is not required for ESC self-renewal, and the differentiation defects observed *in vitro* are consistent with reports demonstrating that early lineage commitment is not affected in mice lacking Ezh2, Eed and Suz12 but that the mice die during[56,58,59] and after implantation.

The role of PRC1 in ESC self-renewal and differentiation is complicated by the number of homologous PcG proteins with overlapping functions that may compensate for each other. In agreement with this, knockout of single subunits of the PRC1 complexes, with the exception of Ring1b, produced relatively late developmental defects, whereas embryos lacking two homologous PRC1 proteins failed to pass midgestation[60,61]. This phenotype is similar to that observed in PRC2-null embryos and in embryos lacking Ring1b[62,63]. Whereas the role of the PRC1 members in ESC self-renewal and differentiation has not been studied as extensively as for PRC2, *Ring1b* (official symbol *Rnf2*)-KO ESCs, similarly to PRC2-KO ESCs, grow normally[64–66]. Interestingly, however, the co-deletion of *Ring1a* (official symbol *Ring1*) and *Ring1b* leads to spontaneous differentiation of ESCs[67]. Thus, the Ring1 proteins, in contrast to the PcG proteins of PRC2, are essential for ESC self-renewal. This observation could suggest that Ring1 complexes have regulatory functions, in addition to their functions within PRC1, that maintain the transcriptional program in ESCs. It is also consistent with the observation that Ring1 proteins form part of many noncanonical PRC1 complexes that are recruited to target genes independently of PRC2 and H3K27 methylation.

Taken together, the roles of PcG proteins in ESC self-renewal, differentiation and development are consistent with a model in which the PcG proteins contribute to maintaining the gene expression patterns in self-renewing ESCs and during ESC differentiation. PcG proteins are not required for ESC self-renewal (although this is confounded by the spontaneous differentiation observed in *Ring1a* and *Ring1b* double-KO ESCs), but they are required for maintaining a proper differentiation program during development.

Targeting Polycomb to chromatin

On the basis of studies of the Hox clusters in *Drosophila*, a dominant model for PcG regulation of transcription is that once cells are committed to differentiate and the transcriptional program is established, the PcG proteins bind to genes, which are switched off by transient signals and are maintained in a repressed state even after the signals are lost. In this model, the PcG proteins do not determine gene repression but rather maintain gene silencing. However, models have also been proposed in which the PcG proteins actively participate in setting up gene silencing in ESCs and during cell-fate transitions. For instance, the recent finding that PRC2-associated PHF1, MTF2 and PHF19 (also known as PCL1, PCL2 and PCL3) bind H3K36me2 and H3K36me3 through their Tudor domains has lent support to such a model[68–71]. Interestingly, the Tudor domain of *Drosophila* PCL cannot bind H3K36me3, and the mechanism by which *Drosophila* PCL contributes to PRC2 recruitment must therefore be different from that of its mammalian counterparts.

In *Drosophila*, the PcG proteins are recruited to their target genes, including the Hox loci, by binding Polycomb-responsive elements (PREs), which contain consensus sites for several different transcription factors[72–74]. However, the *Drosophila* transcription factors involved in this process are not conserved in mammalian cells, with the exception of YY1 (Pho in *Drosophila*) and GAF[74] (GAGA in *Drosophila*). Nevertheless, YY1 does not appear to have a major role in recruiting mammalian PcG proteins to their target genes in mammals[75–77]. The lack of conservation of the targeting transcription factors between *Drosophila* and mammals suggests that the mechanisms leading to PcG recruitment in mammals are also not conserved.

Genome-wide location analyses have shown that mammalian PcG proteins associate with several thousand genes in human and mouse ESCs and differentiated cells[55,78,79]. Strikingly, PcG proteins were found to associate with genes that determine every aspect of normal development, and it was therefore suggested that PcGs form part of an epigenetic blueprint for development and differentiation[78]. Because of the large number of PcG-target genes, it is difficult to envision a simple mechanism responsible for recruiting the PcG proteins to specific target genes, and the question of how PcG targets are specified in mammals is an area of intense investigation (**Fig. 4**).

Recruitment of PRC1 complexes. As mentioned earlier, recruitment of canonical PRC1 complexes depends on PRC2 activity and the presence of the H3K27me3 mark, and it could therefore be explained in part by a mechanism involving the binding of CBX chromodomain to H3K27me3 (**Fig. 4a**). In contrast, the noncanonical PRC1 complexes do not contain a chromodomain-containing subunit and, in agreement with this, are not dependent on H3K27 methylation for their recruitment. Instead, the noncanonical PRC1 complexes are associated with sequence-specific DNA-binding proteins, such as Fbxl10 (refs. 17,23) and E2F6 (ref. 20), which are required for their targeting to specific sites in the genome (**Fig. 4b**). Additionally, studies have shown that PRC1 associates with sequence-specific transcription factors, such as REST and RUNX1 (refs. 80–82). Thus, the recruitment of PRC1 appears to involve a combination of H3K27 methylation by PRC2 and sequence-specific DNA-binding proteins.

Recruitment of PRC2 complexes. Because PRC2 was believed for several years to be required for the subsequent recruitment of PRC1 to target genes, the mechanisms leading to sequence-specific targeting of PRC2 have received more attention than those of PRC1. Several groups have highlighted the almost-perfect overlap between PRC2-target genes and CpG islands[76,83], and studies have shown that

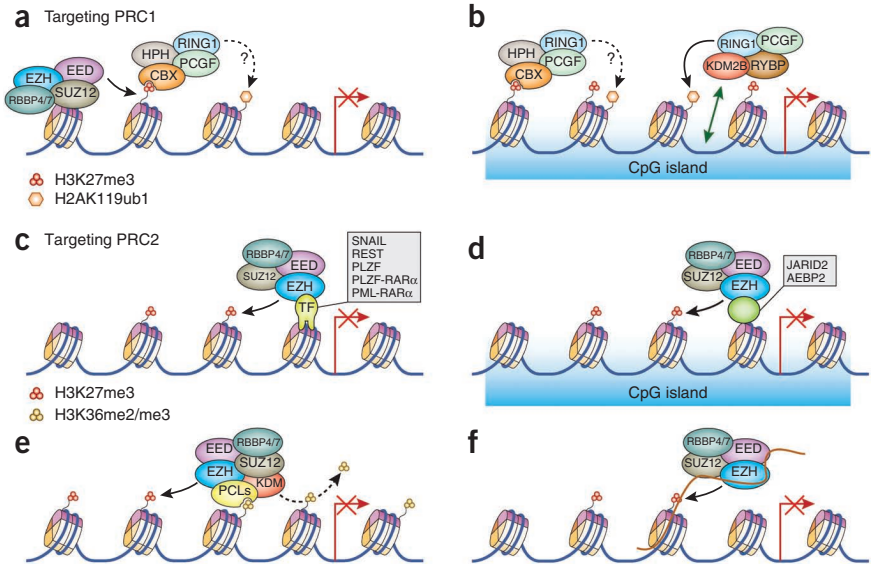

Figure 4 Targeting of PRCs. (**a**,**b**) Models of PRC1 binding. Binding of the canonical PRC1 to genomic loci requires its recognition of the H3K27me3 mark deposited by PRC2 (**a**), whereas the occupancy of noncanonical RYBP-PRC1 complex (**b**) relies on its affinity (through KDM2B (FBXL10)) for CpG islands. Of note, the noncanonical RYBP-PRC1 seems to be responsible for the majority of the H2Aub1 modifications present at PRC-target genes. (**c**–**f**) Several mechanisms described for the specific targeting of the PRC2 complex. Recognition of methylated H3K36me2 and H3K36me3 by the PCL family of proteins (**c**), affinity for CpG islands through JARID2 and AEBP2 (**d**) and interactions with transcription factors (**e**) or noncoding RNA (**f**) are possible mechanisms that can either act alone or in combination to allow PRC2 targeting to specific genomic loci. RBBP4/7, RBBP4 or RBBP7; H3K36me2/me3, di- or trimethylated H3K36.

GC-rich elements are sufficient to recruit PRC2 (refs. 75,84). These studies also suggest that DNA methylation and transcription prevent the binding of PRC2 to GC-rich stretches. So how is PRC2 recruited to the CpG islands? Three different (but not mutually exclusive) mechanisms have been suggested.

First, proteins that purify in almost stoichiometric levels with PRC2 show affinities for GC-rich stretches and for specific post-translational modifications of histones. For instance, the co-purified proteins JARID2 and AEBP2 have an affinity for GC-rich stretches[85–90] (**Fig. 4d**), PRC2 for H3K27me3 (refs. 4,91) and the PCL, and PHF proteins for H3K36me3 and me2 (as discussed above; **Fig. 4e**). JARID2 was shown to associate with PRC2 in ESCs, and loss of JARID2 leads to a significant decrease in PRC2 binding to target genes[86–90]. However, JARID2 has only a low affinity for GC-rich stretches, and this makes it difficult to imagine how JARID2 would provide the necessary specificity for PRC2 binding, unless PRC2 binds to all CpG islands with low affinity. Moreover, Jarid2-KO mice die later than do mice lacking the PRC2 core subunits, results showing that Jarid2 cannot provide the only mechanism leading to the correct targeting of PRC2. Although AEBP2 was also co-purified with PRC2, and recent results have shown that AEBP2 colocalizes with some PRC2-target genes[85], it is not clear whether AEBP2 has a major role in PRC2 binding to target genes. However, the three PCL proteins have been shown to be required for PRC2 targeting to a subset of genes as well as for the proper differentiation of mouse ESCs[68–71,92,93].

Second, sequence-specific transcription factors have been proposed to regulate the recruitment of PRC2 to target genes[10,94], thus implicating SNAIL, REST, PLZF, PLZF-RARα and PML-RARα (refs. 81,94–97). Because of the high affinity of transcription factors for specific sites, a model in which these transcription factors direct PRC2 to specific sites is very attractive (**Fig. 4c**). Unfortunately, however, it has been challenging to identify the transcription factors responsible for recruiting PRC2 to specific DNA sequences. This could be due to the difficulty in extracting unique transcription factor–binding sites from the GC-enriched Polycomb-binding sites. Moreover, transcription factors may only transiently associate with PcG proteins to recruit to specific sites, thus making it difficult to co-purify them with PcG proteins. If the interactions are indeed transient, the transcription factors might only be required for the initial recruitment of PRC2, and the proteins more stably associated in the PRCs might subsequently be sufficient for propagation and maintenance of histone modifications and therefore of the chromatin architecture.

Third, several recent results have suggested that noncoding RNAs have a role in gene silencing and PcG-protein recruitment (**Fig. 4f**). These are predominantly based on the observation that the PRC2 proteins and H3K27me3 accumulate on the inactive X chromosome during X inactivation and the subsequent demonstration that the long noncoding Xist transcript can interact with PRC2 (refs. 98–100). These studies have been extended to several other examples of noncoding RNA: HOTAIR has been reported to recruit PRC2 to the *HOXD* locus[101], whereas Kcnqot1 is involved in imprinting the *Kcnq1* cluster in a process that requires PRC2 (refs. 102,103). Moreover, RNA-immunoprecipitation techniques have identified several thousand RNAs associated with PRC2 (refs. 104,105). However, as critically discussed in a recent review[106], firm data to support the role of noncoding RNAs in Polycomb function are still lacking.

Taken together, several different modes of recruitment of PcG proteins have been proposed, including direct interaction with DNA or DNA-binding proteins, and post-translational modifications of histone H3 and noncoding RNAs, yet the respective roles of these interactions are not fully understood.

Conclusions

In the past decade, the PcG proteins have taken a center stage by serving as paradigmatic epigenetic regulators of transcription, with key roles in stem cells, differentiation and disease. Our knowledge regarding the cellular processes to which the PcG proteins contribute has increased tremendously, and, as outlined in this Review, there have been gains in the mechanistic understanding of how the PcG proteins exert their function. The overall picture is clear: the PcG proteins are present in multiprotein complexes that are endowed with enzymatic activities that modify histones (and other proteins). Histone modifications lead to the recruitment of other protein complexes that, together with chromatin compaction, leads to or retains gene silencing. In this way, the PcG proteins contribute to the maintenance of cell identity. However, PcG proteins associate with their target genes in a dynamic manner, thereby ensuring a tightly regulated differentiation processes. Owing to the key role of the PcG proteins in regulating the expression of developmental genes, mutation, inactivation or increased expression of the PcG proteins leads to a higher probability of misexpression

of the target genes and thereby to differentiation and developmental defects. Genetic alteration of the PcG genes predisposes somatic cells to various types of disease, including cancer.

However, despite this overall picture, a number of key features of Polycomb function and regulation remain to be understood, some of which we have discussed in this Review. Some of the most pertinent questions remain. What comes first, PcGs or gene silencing? In other words, does gene silencing lead to PcG-protein binding, or is the recruitment of the PcG proteins to specific genes the first step in silencing target genes? As discussed above, results have been presented for the *Drosophila* Hox cluster and a few mammalian genes, suggesting that PcG proteins are bound as a result of gene silencing, whereas other studies have suggested that PcG proteins are recruited to silence gene expression. Although it could be a combination of the two different mechanisms, the answer to this conceptually important question remains unknown.

How are the PcG proteins recruited to and dissociated from target genes? Are transcription factors essential for the initial recruitment, with the PcG proteins themselves sufficient to maintain gene silencing? What is the function of noncoding RNAs in this process? The past decade has led to a better biochemical characterization of the PcG-protein complexes and the identification of associated proteins, with several models proposed to explain recruitment (**Fig. 4**). Still, the field is far from having clear answers to these simple questions.

What are the respective functions of H3K27me3, H3K27me2 and H3K27me1, and how are these modifications regulated? In mouse ESCs, 60–80% of histone H3 is methylated on H3K27, with 7–10% of H3 trimethylated, 50–70% dimethylated and 4–10% monomethylated[107,108]. PRC2 is responsible for di- and trimethylation of H3K27, and it has been suggested to also be responsible for H3K27me1 deposition (ref. 13). PRC2 colocalizes with H3K27me3, and because the PRC2 conversion of H3K27me2 to H3K27me3 is much slower than that for mono- and dimethylation of H3K27 (ref. 109), the continuous presence of PRC2 on histones might therefore be required for catalysis of trimethylation. Despite being deposited by the same enzyme (although whether this includes H3K27me1 remains to be confirmed), the functions of H3K27me3, H3K27me2 and H3K27me1 appear to be different. H3K27me3 correlates with gene silencing[110], H3K27me2 does not correlate with transcription (probably owing to its abundance and widespread distribution throughout the genome), and H3K27me1 is associated with transcribed regions and enhancers (for example, as described in refs. 111,112). How can these observations be reconciled with a general role of the PcG proteins in gene silencing, and do different recruitment mechanisms (for example, residence times) of PRC2 lead to different levels of H3K27 methylation?

Answers to these questions will improve understanding of the function of the PcG proteins in developmental control and of how deregulation of PcG proteins contributes to diseases such as cancer.

ACKNOWLEDGMENTS

We thank V.A. Raker for help in preparing the manuscript. This work was supported by grants from the Spanish 'Ministerio de Educación y Ciencia' (BFU2010-18692) to L.D.C.; from European Commission's 7th Framework Program 4DCellFate (grant number 277899) to L.D.C. and K.H.; and from the Novo Nordisk Foundation, the Danish Cancer Society and the Danish Council for Strategic Research (12-110503) to K.H.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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