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Complexity beneath the silence

Myriam Calonje and Z Sung

Polycomb group (PcG)-mediated silencing by proteins that are conserved across plants and animals is a key feature of eukaryotic gene regulation. Investigation of PcG-mediated gene silencing has revealed a surprising degree of complexity in the molecular mechanisms that recruit the protein complexes, repress expression, and maintain the epigenetic silent state of target genes. This review summarizes our current understanding of the mechanism of PcG-mediated gene silencing in animals and higher plants.

Addresses
Department of Plant and Microbial Biology, University of California, Berkeley, California 94720, USA

Corresponding author: Sung, Z. Renee (zrsung@nature.berkeley.edu)

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Introduction

Chromatin remodeling plays a crucial role in the control of gene expression, especially in maintaining cellular memory. Recent studies have revealed epigenetic mechanisms involving histone modifications, DNA methylation and non-coding RNAs. Although the mechanistic relationship among these epigenetic marks is not clear, the demonstration that DNA methylation requires histone methylation provides compelling evidence that the mechanisms are intimately connected [1].

Two groups of proteins, the Trithorax group (TrxG) and the Polycomb group (PcG), remodel chromatin structure by covalently modifying histone tails or by altering nucleosome conformation, thereby maintaining appropriate levels of gene activity over many mitotic divisions. In general, PcG proteins are transcriptional repressors and TrxG proteins transcriptional activators that maintain the 'off' and 'on' state, respectively, of multiple genetic loci. The PcG/TrxG system is widely conserved across plants and animals, suggesting that it has an essential role in eukaryotic gene regulation.

In this review, we focus on PcG-protein-mediated epigenetic mechanisms. The PcG proteins have been divided into two types, PcG repression complex 1 (PRC1) proteins and PRC2 proteins, on the basis of their physical associations in distinct multiprotein complexes. PcG proteins have highly conserved structure and biochemical function. We review the current knowledge of PcG-mediated gene silencing and speculate on possible mechanisms by which PcG complexes regulate plant development.

PRC2s: labeling the targets

The PRC2 or Esc–E(z) complex isolated from *Drosophila* embryos contains four core proteins — Extra sex comb (Esc), Enhancer of Zeste (E[z]), Suppressor of Zeste 12 (Su[z]2) and p55 — and a small number of additional proteins [2–5]. Similar complexes have been isolated from mammalian cells (Table 1). The Esc–E(z) complex and its human counterpart Embryonic Ectoderm Development (EED)–Enhancer of Zeste homolog 2 (EZH2) have histone methyltransferase (HMTase) activity. Several lines of evidence indicate that the primary function of the histone methyl label in PcG silencing is recruiting other PcG proteins. The HMTase is specified by the SET (Su(var), E(z) and Trithorax) domain of E(z), but Esc and Su(z)12 are required for activity [4,6]. The *in vitro* targets of the complexes are histone 3 lysine 9 (H3–K9) and histone 3 lysine 27 (H3–K27) but *in vivo* experiments indicate that only H3–K27 is methylated at genomic PcG target sites. In *Caenorhabditis elegans*, H3–K27 is methylated by a PRC2-like complex composed of the *Drosophila* E(z) and Esc orthologs, Maternal-effect sterile protein 2 (MES–2) and MES–6, respectively, and the novel protein MES–3 instead of a Su(z)12 homolog ([7]; Figure 1a).

Arabidopsis has three PRC2s that have similar subunit composition ([8••,9]; Figure 1b). The Esc and p55 orthologs, FERTILIZATION INDEPENDENT ENDOSPERM (FIE) [10] and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) [11], respectively, are single copy genes and these proteins are probably present in all three complexes. Su(z)12 and E(z) each have more than one *Arabidopsis* homolog: EMBRYONIC FLOWER 2 (EMF2, [12]), FERTILIZATION INDEPENDENT SEED2 (FIS2) and VERNALIZATION2 (VRN2, [13]) are homologs of Su(z)12, whereas MEDEA (MEA; [14]), CURLY LEAF (CLF; [15]), and SWINGER (SWN; [8••]) are E(z) homologs (Table 1). Multiple homologs allow additional complexes with varying components. A protein complex consisting of MEA, FIE and MSI1 has been purified from plant cells, providing evidence for the existence of a FIS2–MEA–FIE–MSI1 complex [16]. The EMF2–CLF/SWN–FIE–MSI1 complex has been predicted on the basis of mutant phenotypes, gene

Table 1

Summary of the PcG proteins.

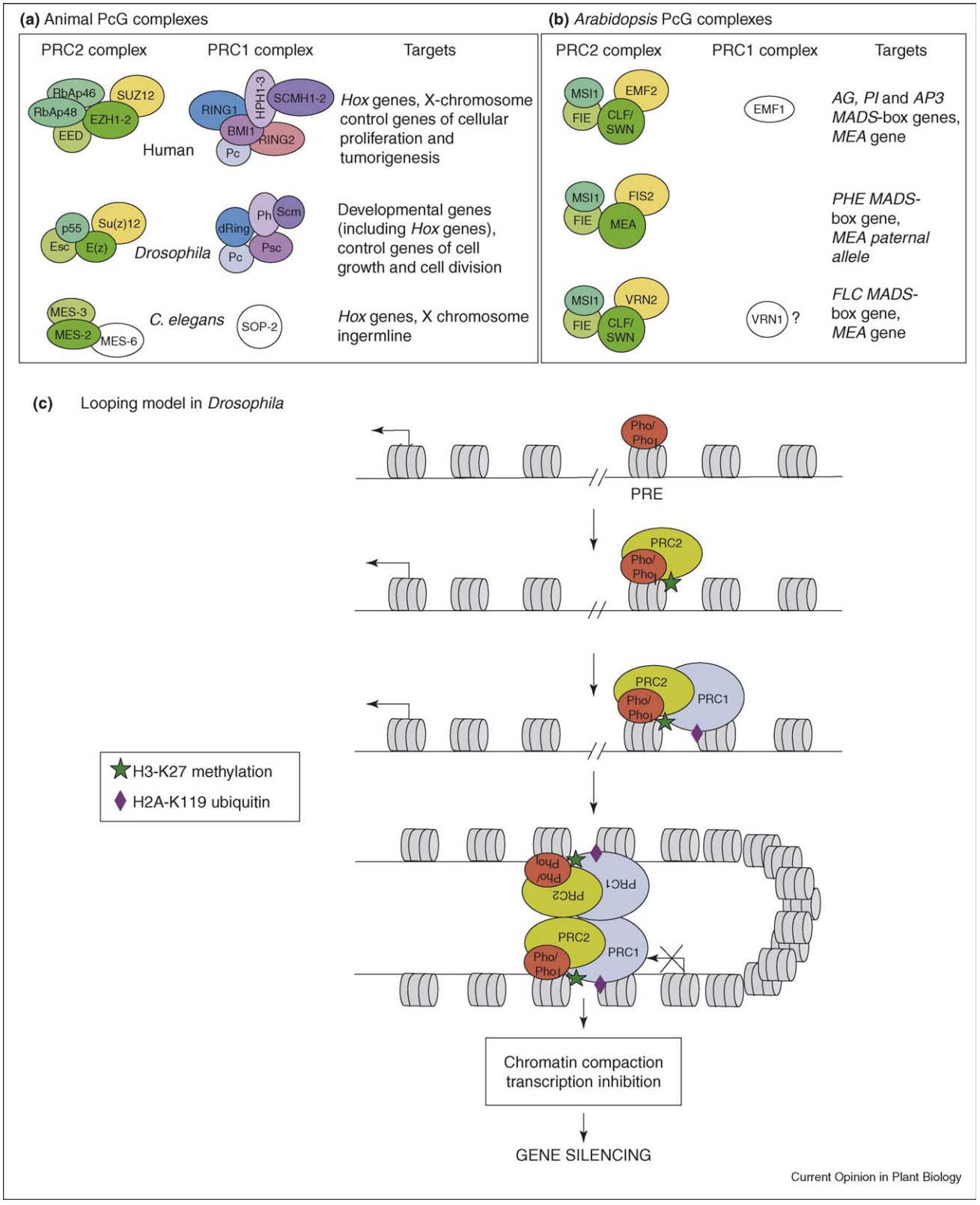
Drosophila	Vertebrates	Note[a]	*C. elegans*	Note[a]	*Arabidopsis*	Note[a]
PRC2 proteins						
E(z)	EZH1 EZH2	SET domain H3-K9 and H3-K27 HMTase	MES-2	SET domain H3-K27 HMTase	CLF MEA SWN	SET domain H3-K9 and H3K27 HMTase
Esc	EED	WD40 repeat protein Increases HMTase activity of E(z)	MES-6	WD40 repeat protein Increases HMTase activity of E(z)	FIE	WD40 repeat protein Required for HMTase activity of E(z)
Su(z)12	SUZ12	VEF domain[b] Interacts with E(z) Required for HMTase activity of E(z)			EMF2 FIS2 VRN2	VEF domain[b] Interacts with CLF Required for HMTase activity of E(z)
			MES-3	Novel protein Cofactor of MES-2 and MES-6		
p55/ NURF55	RbAp46 RbAp48	WD40 repeat protein Nucleosome-binding protein			MSI1	WD40 repeat protein Nucleosome-binding protein
PRC1 proteins						
Pc	HPC1 HPC2 HPC3	Chromodomain H3-K27 binding activity	SOP-2	SAM domain Protein–protein interaction RNA-binding activity	EMF1	Plant-specific protein
Ph	HPH1 HPH2 Rae28	SAM (sterile-α-motif) domain Protein–protein interaction FCS finger RNA-binding activity				
Scm	SCMH1 SCMH2	SAM domain Protein–protein interaction FCS finger domain RNA-binding activity				
Psc	BMI1 MEL-18	Ring-finger domain Inhibition of chromatin remodeling and transcription			VRN1?	B3-domain
dRing	Ring1A (RING1) Ring1B (RING2)	Ring-finger domain Ring-finger domain H2A-K119 ubiquitin E3 ligase activity				DNA-binding protein
DNA-binding recruiters[c]						
Pho	YY1	Sequence-specific DNA-binding protein				
Phol	YY1	Sequence-specific DNA-binding protein				
Psq		Sequence-specific DNA-binding protein				
Zeste		Sequence-specific DNA-binding protein				
DSP1		Sequence-specific DNA-binding protein				
GRH		Sequence-specific DNA-binding protein				

Abbreviations: NURF55, Nucleosome remodeling factor 55; RbAp46, Retinoblastoma-binding protein 46.
[a] Characteristic domain(s) of the protein and potential or known protein function.
[b] VEF (VRN2, EMF2 and FIS2) family.
[c] PcG proteins and DNA-binding recruiters found in *Drosophila*, vertebrates, *C. elegans* and *Arabidopsis*. First column of notes apply to *Drosophila* PcG proteins and their vertebrate counterparts. The *C. elegans* and *Arabidopsis* PRC1 proteins are not homologous to the *Drosophila* and vertebrate proteins.

expression patterns and *in vitro* protein–protein interaction between members of the protein complex [8••]. No protein interaction evidence is available for the predicted VRN2–CLF/SWN–FIE–MSI1 complex [8••].

Plant PRC2s have histone methyltransferase activity. All four members of the complexes (i.e. EMF2/FIS2/VRN2–MEA/CLF/SWN–FIE–MSI1) are required for PRC2 function. This is based on the fact that H3-K27 methylation in euchromatin is considerably reduced in plants that are impaired in genes containing the SET domain, such us *CLF* and *SWN*, or in genes encoding other members of the complex, such as *FIE* [17••]. In addition, repression of *FLOWERING LOCUS C* (FLC) by VRN2 involves

Figure 1



PcG complexes and the silencing mechanism. **(a)** Subunit composition of PRC2 and PRC1 in animals. **(b)** *Arabidopsis* PcG complexes. **(c)** PcG silencing mechanism in *Drosophila*: the looping model [61••]. PcG proteins that are bound to distantly located PREs interact with PcG proteins bound at or close to the transcriptional start of the target genes, causing the chromatin to loop. The binding of DNA-binding recruiters to PREs initiates the recruitment of PRC2, which methylates H3–K27. This mark is recognized by the chromodomain protein Pc, which recruits PRC1 proteins. PRC1 represses transcription by a mechanism that involves H2A–119 ubiquitination, DNA compaction and inhibition of the transcription machinery.

methylation of H3–K9 and H3–K27 at specific regions of the ***FLC*** locus [18••,19].

PRC1s: making effective repression

The PRC1 core components are similar in *Drosophila* and humans (Figure 1a). *Drosophila* PRC1 contains Polycomb (Pc), Polyhomeotic (Ph), dRing1/Sex combs extra (Sce) [20,21], Posterior sex combs (Psc), Zeste, Sex comb on midleg (Scm) as well as several TATA-binding protein Associated Factors (TAFs) [22]. *In vitro* studies suggest several possible mechanisms by which PRC1 acts as an 'effector' of transcriptional repression. For instance, PRC1 inhibits both chromatin remodeling by the hSWI/SNF (human switch/sucrose non-fermenting) complex and transcription of a chromatin template *in vitro* [23]. In addition, the mammalian PRC1 complex possesses histone 2A (H2A)–K119 ubiquitin E3 ligase activity [24••,25••].

No PRC1 has been identified in *C. elegans*. Instead, SOP–2 (suppressor of pal-1 protein 2) appears to have assumed a PcG–like function in *C. elegans* (Figure 1a). SOP–2 is an RNA-binding protein and this property appears to be crucial to its role in silencing [26••]. Other animal PcG proteins (e.g. Ph, Scm and Rae28 [26••]) bind RNA, but to date there is no information about the identity and function of any RNA involved in PcG silencing *in vivo*.

Although homologs of PRC1 core components have not been found in plants, candidates for plant PRC1 equivalents might be identified by investigating mutants that have phenotypes similar to those caused by impaired PRC2 proteins (Figure 1b). EMF1, which encodes a plant-specific nuclear protein [27], has a mutant phenotype that is similar to, or stronger than, that of the plant Su(z)12 homolog EMF2. Recent results show that EMF1 has *in vitro* activities comparable to those of PRC1 proteins (M Calonje *et al.*, unpublished), indicating that plants have recruited different proteins to carry out the PRC1 task. *borgia* mutants have a fertilization-independent seed development phenotype. Cloning and characterization of *BORGIA* should determine whether it is a component of PRC1 or perhaps serves other functions [28•]. VRN1 is a B3–domain protein that has non-sequence-specific DNA-binding activity [29]. Both H3–K27 and H3–K9 methyl marks are required to maintain the vernalization response. Neither *vrn1* mutants nor mutants that are impaired in the Su(z)12 homolog VRN2 are able to maintain *FLC* repression. Neither mutant has H3–K9 methylation but *vrn1* has H3–K27 methylation. This is consistent with the involvement of VNR1 in methylating H3–K9 after the VRN2-complex methylates H3–K27, thus VRN1 might function as a PRC1 equivalent in interpreting the H3–K27 methyl mark.

A variety of targets

Animal PcG complexes target the much-studied *Homeobox* (*Hox*) genes and many others. *Drosophila* PcG proteins regulate genes at every stage of development and participate in chromosome organization and cell division processes [30–33]. Vertebrate PcG proteins repress the *Hox* genes [34] and are involved in inactivating the X chromosome [35], cellular proliferation and tumorigenesis [36]. In *C. elegans*, PcG proteins repress *Hox* genes during larval development [37] and are predicted to repress transcription from the X chromosome in the germline [7].

The *MADS*–box genes *PHERES1* (*PHE1*), *AGAMOUS* (*AG*), *APETALLA3* (*AP3*), *PISTILLATA* (*PI*), and *FLC* were the first PcG-target genes identified in plants (Figure 1b). Repression of endosperm proliferation during gametophyte and endosperm development is accomplished by FIS2–MEA–FIE–MSI1-mediated *PHE1* repression [38]. *PHE1* is expressed during early endosperm development but its role is not understood. The EMF2–CLF–FIE–MSI1 complex represses the flower *MADS*–box genes *AG*, *AP3*, and *PI* during vegetative development [39,40]. Loss of *EMF2* function leads to precocious transition from vegetative to inflorescence meristem. Epigenetic control of the vernalization response is maintained by VRN2-complex-mediated *FLC* repression [19]. Cold treatment induces a downregulation of *FLC* expression that is stably maintained after transfer to normal temperature and accelerates flowering.

The recent and exciting discovery implicating PcG proteins in silencing *MEA* [41••,42••] indicates that plant PcG complexes target genes other than the *MADS*–box genes.

PREs and DNA-binding recruiters: how do PcGs find the target?

The target genes of *Drosophila* PcG and TrxG proteins contain specific DNA elements that enable the protein complexes to maintain the proper state of gene activity. These PcG response elements (PRE) span about 1 Kb of DNA and often have dual functions, mediating both PcG and TrxG regulation. Other than several short recurring motifs, some of which are recognized by DNA-binding proteins, there is little sequence homology among PREs. DNA-binding proteins, such as GAGA factor (GAF), Pipsqueak (Psq) [43–47], Zeste [48], Pleiohomeotic (Pho) and Pho-like (Phol) ([49,50]; Table 1), appear to be 'recruiters' that attach PcG or TrxG proteins to the PRE [32,33]. Pho and Phol were shown to interact directly with E(z) and/or Esc [51••], suggesting that PRC2 might be recruited to PREs through protein–protein interaction with Pho and/or Phol. The finding that other DNA–binding proteins, such as Dorsal Switch Protein 1 (DSP1) [52•] and Grainyhead (GRH) [53•], cooperate with Pho/Phol in recruitment suggests that *Drosophila* PcG protein targeting requires a combination of DNA-binding recruiters.

Despite the identification of several target genes for mammalian PcG and TrxG, mammalian PREs have

not been defined. The lack of mammalian homologs for Zeste, GAF and Psq has made finding vertebrate PREs difficult. Although the vertebrate homolog of Pho, Ying Yang1 (YY1), can functionally compensate for the loss of Pho in *pho* mutant flies [54,55•], how vertebrate complexes are recruited to specific genes is not known.

What targets plant PcG complexes to specific genes? Are DNA elements and DNA-binding recruiters involved? Several kinds of evidence suggest that DNA elements are likely to be involved in recruiting plant PcG complexes. Repression of *PHE1*, an intronless gene, requires the promoter region, whereas *FLC* and *AG* repression requires the promoter and intragenic regions located at the first and second intron, respectively ([8••,19,38,56–58]; M Calonje *et al.*, unpublished). Further defining DNA sequences that are required for PcG recruitment should elucidate plant PREs. VERNALIZATION INSENSITIVE3 (VIN3), a plant-homeodomain-containing protein, is the only known candidate for recruiting the PcG proteins to *FLC* (Table 1). None of the vernalization-mediated modifications in *FLC* chromatin are observed in *vin3* mutants. However, VIN3, unlike Pho/Phol, is transiently expressed, suggesting that it probably plays a role in the initiation rather than maintenance of PcG silencing [59••]. Furthermore, it is not known whether VIN3 has DNA-binding activity or interacts with DNA-binding factors.

Emerging models of silencing

Recent studies on PcG-mediated silencing in *Drosophila* point to a chromatin looping model (Figure 1c). In this model, PcG proteins that are bound to distantly located PREs can interact and mediate loop formation, bringing the PcG proteins into proximity with the transcriptional start of *Drosophila Hox* genes [60,61••]. The model envisions a hierarchical sequence of events, in which DNA-binding proteins, such as Pho/Phol, recognize and bind to specific sequences in the PRE, recruiting PRC2 to these sites. The PRC2 methylates H3–K27, recruiting PRC1 though Pc (a chromodomain-containing protein) by the affinity of the chromodomain for the H3–K27 methyl mark. Once recruited, PRC1 would repress gene activity by DNA compaction and inhibition of the transcription machinery [62•,63•,64]. The H2A–K119 ubiquitin ligase activity of PRC1 probably also interferes with transcription through a mechanism that has not yet been defined [24••,25••]. Ringrose and Paro [33] have shown, however, that Pc–binding sites and the histone methylation marks on polytene chromosomes do not overlap completely. This suggests that histone methylation could be a downstream event after Pc is recruited. They propose that H3–K27 methylation might fine-tune the strength of PcG binding at different loci through a dynamic equilibrium.

Alternative models linking PcG proteins with non-coding RNAs have been proposed. The finding that the PcG proteins SOP–2, Ph, Rae28 and Scm can bind RNA supports this hypothesis [26••]. The best-documented process linking PcG proteins to non-coding RNAs is X inactivation in animals [35,65]. One of the earliest events in this process is the recruitment of PRC2, which methylates H3–K27 in a Xist RNA-dependent manner, although no direct binding to the non-coding RNA has been reported to date. Whether non-coding RNAs have a specific function in targeting PcG proteins or in regulating their function is not known.

Recent studies have provided the first mechanistic link between two essential epigenetic mechanisms: PcG protein activities and DNA methylation systems. Interestingly, the mammalian homolog of E(z), EZH2, interacts with DNA methyltransferases (DNMTs) and associates with DNMT activity *in vivo* [66••]. In some situations, however. histone methylation silenced gene expression without the involvement of DNA methylation [67]. In addition, another recent report shows that EED and EZH2, together with DNMT1, are essential to recruit the PRC1 protein BMI1 (B lymphoma Mo–MLV insertion region 1, a homolog of *Drosophila* Psc) to nuclear structures (called the PcG bodies) that are located on regions of pericentric heterochromatin [68••]. Homologs of DNMTs are found in *Drosophila* [69] but DNA methylation has not been reported at PcG-target genes.

How do plants interpret the PRC2-mediated marks? In *Arabidopsis*, methylation of H3–K27 and H3–K9 is required for the repression of *FLC* and *AG* ([9,18••,57]; D Schubert, J Goodrich: Abstract at *15th International Conference on Arabidopsis Research*, *2004*; http://www.arabidopsis.org/servlets/TairObject?type=publication&id=501714003), and H3–K27 methylation marks are present in the silenced *MEA* paternal allele in the endosperm and in the *MEA* genomic locus in vegetative tissues [41••,42••]. However, a Pc–like plant protein that can recognize H3–K27 methylation has not been identified. *Drosophila* Pc shares homology in the chromodomain with HETEROCHROMATIN 1 (HP1). In HP1, this domain recognizes and binds methyl H3–K9 and is involved mainly in maintaining constitutive heterochromatin. Some evidence links the plant homolog of *Drosophila* HP1, LIKE-HETEROCHROMATIN 1 (LHP1) or TERMINAL FLOWER2 (TFL2), with repression of *FLC*. This is consistent with the unstable *FLC* repression in *tfl2* mutants [70,71]. In addition, *tfl2* mutants flower early and mis–express flower homeotic genes, as do mutants that are impaired in EMF2 PcG complex genes [70,72]. LHP1/TFL2 does not bind H3-K27, however, and its mutant phenotype is much weaker than that of plants that are defective in the PcG genes. There must, therefore, be another factor that recognizes the H3–K27 methylation mark and makes repression effective, as animal PRC1s do. Most probably, plants have recruited other proteins to carry out this task. Finally, whether

histone ubiquitination or DNA methylation has a role in PcG-mediated gene repression is not known, as none of these marks has been reported at PcG target genes. It is expected that future research in this field will provide more evidence to solve this complex puzzle.

Conclusions

PcG proteins are versatile players in a wide range of cellular activities. The looping mechanism, which is emerging as the prevailing model of PcG-protein-mediated silencing of *Drosophila* homeotic genes, might also be employed by plants. However, evidence suggests that a combination of different proteins and mechanisms might be used in the different processes that are regulated by PcG complexes.

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