

Transcriptional Silencing by *Polycomb*-Group Proteins

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SUMMARY

Polycomb-group (PcG) genes encode chromatin proteins involved in stable and heritable transcriptional silencing. PcG proteins participate in distinct multimeric complexes that deposit, or bind to, specific histone modifications (e.g., H3K27me3 and H2AK119ub1) to prevent gene activation and maintain repressed chromatin domains. PcG proteins are evolutionary conserved and play a role in processes ranging from vernalization and seed development in plants, over X-chromosome inactivation in mammals, to the maintenance of stem cell identity. PcG silencing is medically relevant as it is often observed in human disorders, including cancer, and tissue regeneration, which involve the reprogramming of PcG-controlled target genes.

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OVERVIEW

Organs of humans, animals, and plants are constructed from a large pool of distinct cell types, each performing a specialized physiological or structural function. With very few exceptions, all cell types contain the same genetic information encoded in their DNA. Thus, the distinctiveness of a given cell type is achieved through specific gene expression programs. As a consequence, cell lineages need to have these programs maintained during growth and cell division. This implies the existence of a memory system that ensures the faithful transmission of information (i.e., which gene is active or repressed) from mother to daughter cells. The existence of such a system is illustrated by the fact that cultured tissues of plants and animals usually maintain their differentiated characters even if grown in a foreign environment. By way of example, ivy plants regenerated after tissue culture produce the type of leaf corresponding to the phase of development from which the original tissue was taken (i.e., juvenile or adult leaf).

The major question to be addressed here and in Kingston and Tamkun (2014) concerns the molecular identity of factors contributing to the mechanism(s) of “cellular” or “transcriptional memory,” which maintains a determined state over many cell divisions. Genetic analyses in *Drosophila melanogaster* have identified regulators crucial in maintaining the morphology of individual body segments that are determined by the action of the *HOX* genes. In *Drosophila* males, the first thoracic segment has legs with sex combs. Legs on the second and third thoracic segment lack these structures. In the 1940s, *Drosophila* mutants were identified (*Polycomb* and *extra sex combs*) wherein males had sex combs on all legs. These morphological alterations reflect homeotic transformations of the second and third leg identities into the first leg

identity. Subsequent molecular studies showed that these mutations did not affect the products of the *HOX* genes themselves, but rather the way *HOX* gene activity was spatially controlled. Throughout the years, a large number of similar regulatory genes were identified, and were classified into two antagonistic groups: the *Polycomb* (PcG) and *Trithorax* (TrxG) group. Whereas the PcG proteins are required to maintain the silenced state of developmental regulators such as the *HOX* genes, the TrxG proteins are generally involved in maintaining the active state of gene expression. Thus PcG and TrxG proteins embody the molecular components of cellular memory.

Proteins of both groups form large multimeric protein complexes that act on their target genes by modulating chromatin structure. In this article, we will focus on the molecular nature and function of two of the major *Polycomb* repressive complexes, PRC1 and PRC2. The molecular nature of the TrxG complexes will be described in Kingston and Tamkun (2014). In *Drosophila*, it was shown that transcription factors recruit PcG complexes to a DNA sequence called a PcG response element (PRE). Once recruited, they establish a silent chromatin state that can be inherited over many cell divisions. Members of PRC2 are highly conserved between plants and animals, whereas PRC1 proteins are less well conserved. This implies conservation, but also diversity, in the basic building blocks of the cellular memory system. In addition to the function of PcG complexes in the maintenance of cell types, they may also play important roles in stem cell plasticity and regeneration. Also, their deregulation can lead to neoplastic transformation and cancer. Thus, PcG proteins play a crucial role in many fundamental processes of normal development and disease in multicellular eukaryotes.

1 INTRODUCTION

All multicellular organisms start from a single cell, the zygote, which during development gives rise to a multitude of distinct cell types with specialized functions. This poses the problem of how, once determined, cell types can be maintained over many cell divisions occurring during growth phases.

1.1 The Concept of Cellular Memory

An adult animal has 200 to 300 structurally distinct cell types, whereas a plant has between 30 and 40. The identity and function of a given cell type is determined by its characteristic gene expression profile. During development and adult homeostasis, it is crucial to remember and faithfully reproduce this state after each cell division. This is particularly critical during the replication of genetic material (S-phase) and the separation of chromosomes during mitosis (M-phase; see Almouzni and Cedar 2014). These are recurring events at each cell cycle that interrupt gene expression processes. Thus, how can differential gene expression patterns be inherited from one cell generation to the next, as illustrated in Figure 1?

We know from experiments performed in the 1960s and 1970s that plant and animal tissues remember a determined state even after prolonged passage in culture (Hadorn 1968; Hackett et al. 1987). Hadorn and colleagues

showed that imaginal disc cells found in *Drosophila* larvae have an intrinsic memory, allowing them to remember determined states that are fixed in early embryogenesis. Imaginal discs are clusters of epithelial cells set aside in the developing embryo as precursors for the formation of specific external structures and appendages during metamorphosis. For instance, of the two pairs of imaginal discs in the second thoracic segment, one forms a midleg and the other a wing (see Fig. 2 in Kingston and Tamkun 2014). Imaginal discs can be cultured by transplantation into the haemocoel of adult females, wherein they continue to proliferate, but do not differentiate. When transplanted back into a larva before metamorphosis, the disc will subsequently differentiate into the expected adult structures, even after successive passages in adult females. More recently, the PcG and TrxG proteins were shown to be required for the maintenance of the determined state of imaginal disc cells. Additionally, it was observed that in rare cases an imaginal disc could change its fate, a process called transdetermination. This process involves the down-regulation of PcG repression by the JNK signaling cascade in transdetermined cells (Lee et al. 2005). PcG mutants also have elevated frequencies of transdetermination, supporting a role for PcG proteins in maintaining imaginal disc cell fates (Katsuyama and Paro 2011). Thus, PcG proteins seem to play a crucial role in the maintenance and reprogramming of cellular fates during both normal development and regeneration.

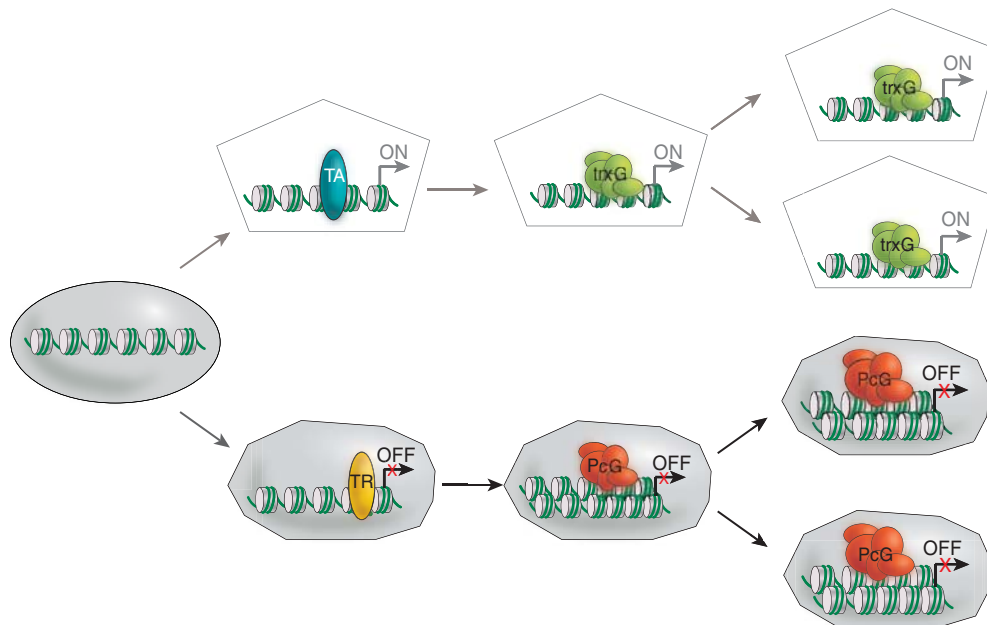


Figure 1. The concept of cellular memory. Schematic illustration of the involvement of PcG and TrxG complexes in the determination of active and repressed states of gene expression and, thereby, cellular differentiation, which is maintained over many cell divisions. TA, transcriptional activator; TR, transcriptional repressor.

1.2 The Genetic Identification of the *Polycomb* Group

In all metazoans, the anterior–posterior axis is specified through defined expression patterns of *HOX* genes (see Fig. 2 in Kingston and Tamkun 2014). During *Drosophila* embryogenesis, the activity of maternally (i.e., inherited through the oocyte) and zygotically produced transcription factors generates a specific combination of *HOX* expression patterns that define the morphology of each body segment. This segment-specific profile of *HOX* gene expression is maintained throughout the development of the fly, long after the early transcriptional regulators have disappeared. When the function of *HOX* genes was genetically characterized, many *trans*-acting regulators were isolated. Among the first, *Polycomb* (*Pc*) was identified and genetically analyzed by Pam and Ed Lewis (Lewis 1978). Heterozygous *Pc* mutant males have additional sex combs on the second and third legs. Homozygous mutants are embryonic lethal, showing a transformation of all cuticular segments toward the most posterior abdominal segment (Fig. 2C,D). These classical PcG phenotypes are caused by the ectopic expression of *HOX* genes. Thus, *Pc* and other genes with similar phenotypes were defined as repressors of *HOX* gene activity. Detailed analyses subsequently uncovered the fact that the PcG proteins are only required for the maintenance of *HOX*

repression, rather than the position-specific establishment of *HOX* activity. This latter task is performed by the transcription factors encoded by the early acting segmentation genes. Based on their repressing or activating influence on *HOX* expression, these newly identified *trans*-acting regulators were divided into two antagonistic classes, the PcG and TrxG, respectively (Kennison 1995).

The molecular isolation of *Drosophila* PcG genes made it possible to study the function of vertebrate orthologs in mice, which were subsequently also shown to be key regulators of *HOX* gene expression (van der Lugt et al. 1994; Core et al. 1997). In mammals, mutations in PcG genes typically lead to homeotic transformations of vertebrae (Fig. 2E,F). In addition, PcG genes play a crucial role in the control of cell proliferation, stem cell maintenance, and cancer (see Secs. 4.2 and 4.3).

In two other model organisms, namely, the worm *Caenorhabditis elegans* and the flowering plant *Arabidopsis thaliana*, the molecular characterization of mutants isolated in various genetic screens revealed the existence of other PcG protein orthologs in their genomes. In *C. elegans*, PcG members were identified in screens for maternal-effect sterile (*mes*) mutants and were shown to be involved in X-chromosome silencing in the hermaphrodite germline (Strome et al. 2014).

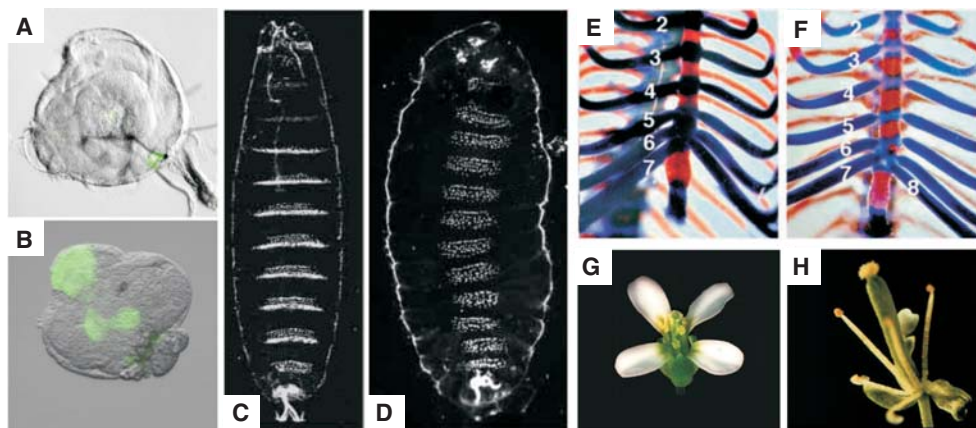


Figure 2. Homeotic transformations in PcG mutants of various species. (A–D) *Drosophila melanogaster*, (E,F) *Mus musculus*, (G,H) *Arabidopsis thaliana*. (A,B) Leg imaginal discs undergoing a transdetermination event as indicated by the expression of the wing-specific gene *vestigial* (marked by green fluorescent protein [GFP]). (C,D) Cuticles of a wild-type (C) and a *Su(z)12* mutant embryo (D). In the *Su(z)12* mutant embryo, all abdominal, thoracic, and several head segments (not all visible in this focal plane) are homeotically transformed into copies of the eighth abdominal segment because of misexpression of the *Abd-B* gene in every segment. (E,F) Axial skeleton of newborn wild-type (E) and *Ring1A*^{-/-} mice (F). Views of the thoracic regions of cleared skeletons show bone (red) and cartilage (blue). The mutant displays anterior transformation of the eighth thoracic vertebra as indicated by the presence of an eighth (1–8) vertebral rib, instead of seven (1–7) as in the wild type. (G,H) Wild-type (G) and *clf-2* mutant (H) flowers. The wild-type flower shows the normal arrangement of sepals, petals, stamens, and carpels. In the *clf-2* flower, petals are absent or reduced in number. (A,B, Courtesy of N. Lee and R. Paro; C,D, reprinted, with permission, from Birve et al. 2001, © Company of Biologists Ltd; E,F, reprinted, with permission, from Lorente et al. 2000, © Company of Biologists Ltd; G,H, courtesy of J. Goodrich.)

In *Arabidopsis*, PcG genes were identified in several genetic screens investigating distinct developmental processes (Hsieh et al. 2003). The first PcG gene in plants, *CURLY LEAF* (*CLF*), was identified as a mutant with homeotic transformations of floral organs (Goodrich et al. 1997). Mutations in the *FERTILIZATION-INDEPENDENT SEED* (*FIS*) class of genes were found in screens for mutants showing maternal-effect seed abortion (Grossniklaus et al. 1998), or allowing aspects of seed development to occur in the absence of fertilization (Luo et al. 1999; Ohad et al. 1999). Finally, PcG genes were identified in screens for flowering time mutants, for example, mutants that flower directly after germination (Yoshida et al. 2001) or that disrupt the vernalization response (Gendall et al. 2001)—the process rendering plants competent to flower after prolonged exposure to cold (discussed in detail in Baulcombe and Dean 2014).

The variety of processes regulated by PcG proteins illustrates the importance of maintaining the repressed state of key developmental regulators in different organisms. On the one hand, there is an amazing conservation of some biological functions from plants to mammals (e.g., the regulation of key developmental regulators such as homeotic genes or the involvement in the tight regulation of cell proliferation). On the other hand, PcG complexes appear to be versatile and dynamic molecular modules that have been used to control a large and diverse variety of developmental and cellular processes.

2 ESTABLISHING SILENCING MARKS ON CHROMATIN

PcG proteins are grouped into two major classes based on biochemical characterizations: members of the *Polycomb* repressive complex 1 or 2 (PRC1 and PRC2; Table 1). The two complexes are required for different steps in the repression of gene expression. PRC2 has histone modifying activity, namely, methylating H3K27 at genes targeted for silencing. PRC1 components can recognize and bind to this modification and induce appropriate structural changes in chromatin. Additionally, PRC1 is able to monoubiquitylate H2AK118/119 at target loci. Both complexes are widely conserved across metazoa (Whitcomb et al. 2007) and the plant kingdom (Köhler and Hennig 2010).

2.1 Components and Evolutionary Conservation of PRC2

Several variants of PRC2 have been purified from *Drosophila* embryos, but all of these complexes contain four core proteins: the SET domain histone lysine methyltransferase Enhancer of zeste (*E(Z)*), the WD40 protein ESC, the histone binding protein p55, and Suppressor of zeste 12 (*SU(Z)12*; Table 1 and Fig. 3).

The *E(z)* gene encodes a 760-amino acid protein, containing a SET domain that exerts KMT (lysine methyltransferase) activity to histones. The SET domain is preceded by a CXC or Pre-SET domain (Tschiersch et al. 1994), which contains nine conserved cysteines that bind three zinc ions and is thought to stabilize the SET domain (see Fig. 1 in Cheng 2014). Such a structural role is supported by the fact that several temperature-sensitive *E(z)* alleles affect one of the conserved cysteines (Carrington and Jones 1996). In addition, *E(Z)* contains SANT domains implicated in histone binding, and a C5 domain required for the physical interaction with *SU(Z)12*. ESC is a short protein of 425 amino acids that contains five WD40 repeats, shown to form a β propeller structure. This serves as a platform for protein–protein interactions, hence, giving ESC a central role in PRC2 to physically interact with both *E(z)* and p55 in all model systems analyzed. The *SU(Z)12* protein is 900 amino acids long and characterized by a C_2H_2 -type zinc finger and a carboxy-terminal VEFS domain. The VEFS domain was identified as a conserved region between *SU(Z)12* and its three homologs in plants: *VERNALIZATION2* (*VRN2*), *EMBRYONIC FLOWER2* (*EMF2*), and *FIS2* (see Fig. 3). Several mutant *Su(z)12* alleles alter this domain, showing that it is required for the interaction with the C5 domain of *E(Z)* (Chanvivattana et al. 2004; Yamamoto et al. 2004).

The p55 protein was not identified as a PcG member by genetic approaches, possibly because it takes part in a multitude of other protein complexes associated with chromatin (Hennig et al. 2005). The p55 protein was, however, identified biochemically as part of PRC2. It is 430 amino acids long and contains six WD40 repeats, which physically interact with ESC or its orthologs in mammals and plants (Tie et al. 2001; Köhler et al. 2003a).

In addition to the core PRC2 proteins, some variants of the complex contain the RPD3 histone deacetylase (HDAC), or the *Polycomb*-like (PCL) protein. The interaction with RPD3 is noteworthy as histone deacetylation is correlated with a repressed state of gene expression (see Seto and Yoshida 2014). The different compositions of PRC2 variants likely reflect both the dynamic changes that occur during development or cater for tissue-specific regulation. PRC2 is highly conserved in invertebrates, vertebrates, and plants (Fig. 3). In *C. elegans*, only homologs of *E(Z)* and ESC are present: *MES-2* and *MES-6*. Together with a nonconserved protein, *MES-3*, they form a small complex of ~ 230 kDa required to repress the X-chromosome and somatically active genes in the hermaphrodite germline (see Strome et al. 2014). In mammals and plants, all four core proteins of PRC2 are present. As in *Drosophila*, the mammalian complex is ~ 600 kDa and is not only involved in regulating homeotic gene expression, but also in

Table 1. Core PcG proteins in model systems

	<i>Drosophila melanogaster</i>	<i>Mus musculus</i>	<i>Arabidopsis thaliana</i>	<i>Caenorhabditis elegans</i>
PcG DNA binding proteins				
PHO	Pleiohomeotic	YY1		
PHOL	Pleiohomeotic-like			
PSQ	Pipsqueak			
DSP1	Dorsal switch protein 1	HMGB2		
PRC2 core proteins				
ESC	Extra sex combs	EED	FIE	MES-6
E(Z)	Enhancer of zeste	EZH1/ENX2 EZH2/ENX1	CLF MEA SWN FIS2 VRN2 EMF2 MSI1 (MSI2/3/4/5)	MES-2
SU(Z)12	Suppressor of zeste 12	SU(Z)12		
p55	p55	RBAP48 RBAP46		
PRC1 core proteins				
PC	Polycomb	CBX2/M33 CBX4/MPC2 CBX6 CBX7 CBX8/MPC3 EDR1/MPH1/RAE28 EDR2/MPH2 (EDR3)		SOP-2
PH	Polyhomeotic	Zinc finger SAM/SPM domain		
PSC	Posterior sex combs	Zinc finger HTH domain	AtBMI1A AtBMI1B AtBMI1C	MIG-32
SCE/dRING	Sex combs extra/dRing	RING1/RING1A RNF2/RING1B	AtRING1A AtRING1B	SPAT-3

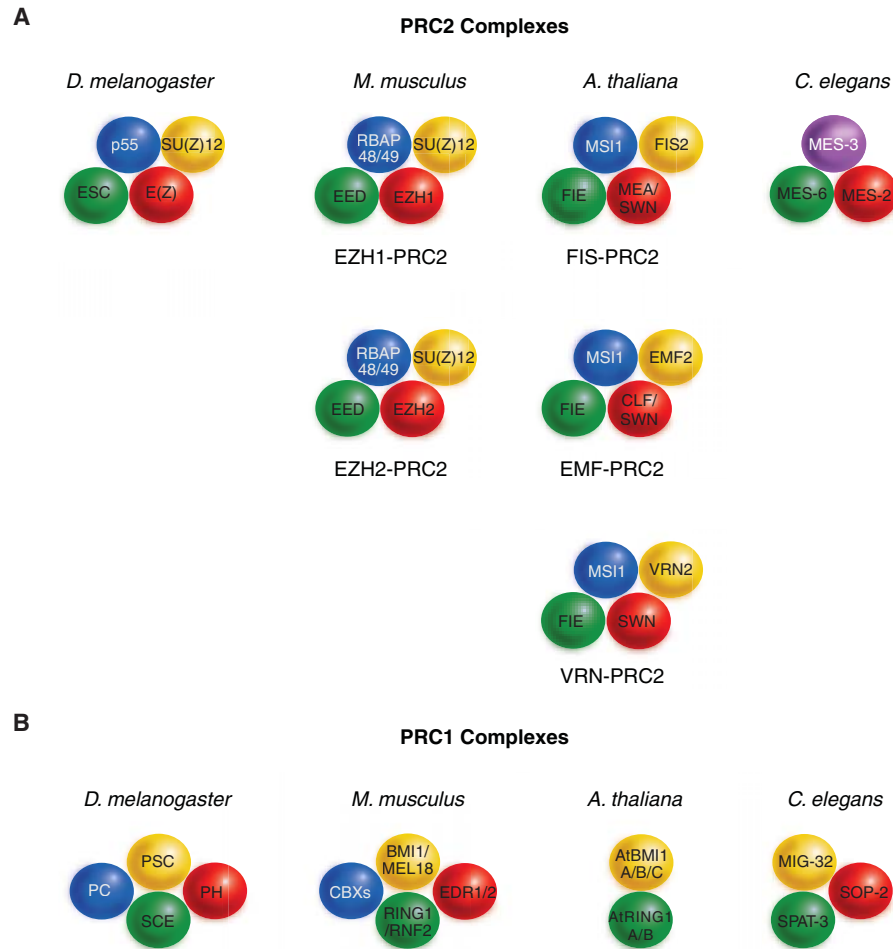


Figure 3. Conserved PRC2 core complexes. The conserved core proteins of PRC2 (A) and PRC1 (B) complexes in *Drosophila melanogaster*, *Mus musculus*, *Arabidopsis thaliana*, and *Caenorhabditis elegans* are shown. (A) In the mouse, PRC2 variants containing EZH1 or EZH2 have distinct functions, whereas in *Arabidopsis* the ancestral complex has diversified into at least three variants with discrete functions during development. In *C. elegans*, the PRC2 core complex contains only three proteins, with MES-3 not having homology with any other identified PRC2 protein. Apart from these core proteins, several other proteins, which are not shown here, interact with PRC2. For instance, mammalian complexes can contain the histone lysine demethylase JARID2, the Zn-finger protein AEBP2, and various homologs of the *Drosophila* PCL protein (PCL1/2/3). Proteins that share the plant homeodomain (PHD)-domain with PCL, but are otherwise not closely related, are also associated with the VRN-PRC2 complex in *Arabidopsis*. Homologous proteins are indicated by the same color. (B) The core proteins of PRC1 are less conserved than those of PRC2 across the four species. In mammals, all genes encoding the PRC1 core subunits have been expanded (see Table 1), such that a variety of complexes with different isoform composition can be formed. In addition to the core components, several additional proteins can be found in PRC1 that are, however, less well characterized and are not shown. In plants, only homologs of *Drosophila* PSC and SCE have been identified; these are encoded by small gene families. Homologous proteins are indicated by the same color. (Based on Reyes and Grossniklaus 2003, Chanvivattana et al. 2004, and Margueron and Reinberg 2011.)

the control of cell proliferation, X-chromosome inactivation, and imprinted gene expression (for more detail, see Sec. 4; Barlowe and Bartolomei 2014; Brockdorff and Turner 2014).

In plants, several genes encoding PRC2 components have undergone duplications such that now they are present as small gene families. In *Arabidopsis* there is only

one homolog of ESC, FERTILIZATION-INDEPENDENT ENDOSPERM (FIE), but three homologs of E(Z), three homologs of SU(Z)12, and five homologs of p55 (referred to as MSI1-5; Table 1). Varying combinations of these proteins form at least three distinct complexes that control specific developmental processes, namely, the FIS-PRC2, EMF-PRC2, and VRN-PRC2 complexes (Fig. 3).

The first of these complexes that was studied in detail is formed by members encoded by the FIS class genes, which play a crucial role in the control of cell proliferation in the seed (Grossniklaus et al. 2001). The FIS-PRC2 contains MEDEA (MEA), FIE, FIS2, and MSI1. Using chromatin immunoprecipitation (ChIP) against one of its components, the FIS-PRC2 was found to directly regulate *MEA* itself, as well as *PHERES1* (*PHE1*) and *FUSCA3* (*FUS3*), coding for transcription factors of the MADS-domain and B3-domain class, respectively (Köhler et al. 2003b; Baroux et al. 2006; Makarevich et al. 2006). Interestingly, the paternal allele of *PHE1* is expressed at much higher levels than the maternal allele. This regulation of gene expression by genomic imprinting is under the control of the FIS-PRC2, which specifically represses the maternal allele (Köhler et al. 2005). Thus, as will be outlined in Section 4.1, the FIS-PRC2 shares with its mammalian counterpart functions in regulating cell proliferation as well as imprinted gene expression.

The EMF complex contains CLF and EMF2 (Chanvattana et al. 2004). Mutations in either of them show weak homeotic transformations and an early flowering phenotype. The EMF-PRC2 is required to repress homeotic genes, whose combinatorial action determines the identity of floral organs (Goodrich et al. 1997). Thus, the EMF-PRC2 has a similar function in maintaining the repressed state of homeotic genes as PRC2 in *Drosophila* and vertebrates (Fig. 2). However, homeotic genes in plants do not encode homeodomain proteins, but rather transcription factors belonging to families containing the MADS-domain or the plant-specific AP2-domain. Strong *emf2* mutants, however, have more severe phenotypes, and produce flowers directly after germination, bypassing the vegetative phase of development (Yoshida et al. 2001). Thus, the EMF-PRC2 plays a role both early in development, in which it prevents immediate flowering, and later during floral organogenesis (Chanvattana et al. 2004). The EMF-PRC2 directly represses *FLOWERING LOCUS T* (*FT*) and *SHOOTMERISTEMLESS* (*STM*), which are both required for the transition to flowering, and the floral homeotic MADS-box gene *AGAMOUS* (*AG*), which together with the homeobox gene *STM* regulates the development floral organs (Fig. 4) (Schubert et al. 2006; Jiang et al. 2008). The FIS class proteins FIE and MSI1 have also been implicated in the control of homeotic gene expression (Figs. 3 and 4). Because mutations in both cause maternal-effect embryo lethality, this function was only revealed when partial loss-of-function alleles could be studied at later stages of development (Kinoshita et al. 2001; Hennig et al. 2003).

Finally, the VRN-PRC plays a key role in a well-known process called vernalization. This epigenetic regulation gov-

erns the timing of flowering in winter annuals, induced by extended periods of exposure to low temperatures, but the effect is only seen after many cell divisions (Fig. 4D; see Fig. 1 from Baulcombe and Dean 2014 for detail). A plant cell will remember that it was vernalized for many months, or even years, after the cold period. This cellular memory is even maintained through passages in cell culture, but not from one generation to the next (Sung and Amasino 2004). The *VRN* genes mediate the response to vernalization. *VRN2* encodes a SU(Z)12 homolog (Gendall et al. 2001), which interacts with the plant E(Z) homologs CLF and SWINGER (*SWN*) in yeast two-hybrid assays (Chanvattana et al. 2004). Floral induction is not only controlled by vernalization, but involves the perception of endogenous (developmental stage and age) as well as exogenous factors (day length, light conditions, temperature). Four pathways, two of which involve PcG factors, have been defined by genetic analyses (see Fig. 1B of Baulcombe and Dean 2014): (1) the autonomous pathway, which constitutively represses flowering presumably via PcG-mediated H3K27 methylation; (2) the vernalization pathway, which induces flowering in response to a prolonged exposure to cold temperature; (3) the photoperiod pathway, which accelerates flowering under long days; and (4) the gibberellin pathway, a phytohormone that promotes flowering. The flowering time gene *FLOWERING LOCUS C* (*FLC*), encoding a MADS-domain transcription factor, is a key integrator of the flowering response as a repressor of flowering. Although the initial repression of *FLC* during vernalization is independent of the VRN-PRC2, the maintenance of *FLC* repression requires *VRN2* activity. Both the vernalization and the autonomous pathways reduce *FLC* expression, integrating diverse signals (Gendall et al. 2001; De Lucia et al. 2008; Jiang et al. 2008). The VRN-PRC2 contains the core subunits *VRN2*, *SWN*, *FIE*, and *MSI1* (Fig. 3) and three associated PHD finger proteins (Wood et al., 2006; De Lucia et al. 2008). Interestingly, *VRN2* interacts with the *FLC* locus independently of cold, and *FLC* silencing is triggered by the association of the VRN2-PRC2 with *VRN5*, a PHD finger protein that is cold-induced and shares limited similarity to *PCL* (see Sec. 2.2 and Fig. 2 from Baulcombe and Dean 2014). In summary, the regulation of flowering time involves both the VRN-PRC2 and EMF-PRC2, which regulate *FLC* and *FT*, respectively (Fig. 4).

2.2 The Chromatin-Modifying Activity of PRC2

How does PRC2 mediate its repressive effect? In *Drosophila*, mammals, and plants, the hallmark histone modification H3K27me3 is produced by PRC2 (Cao et al. 2002; Czermin et al. 2002). This modification is generally thought to be



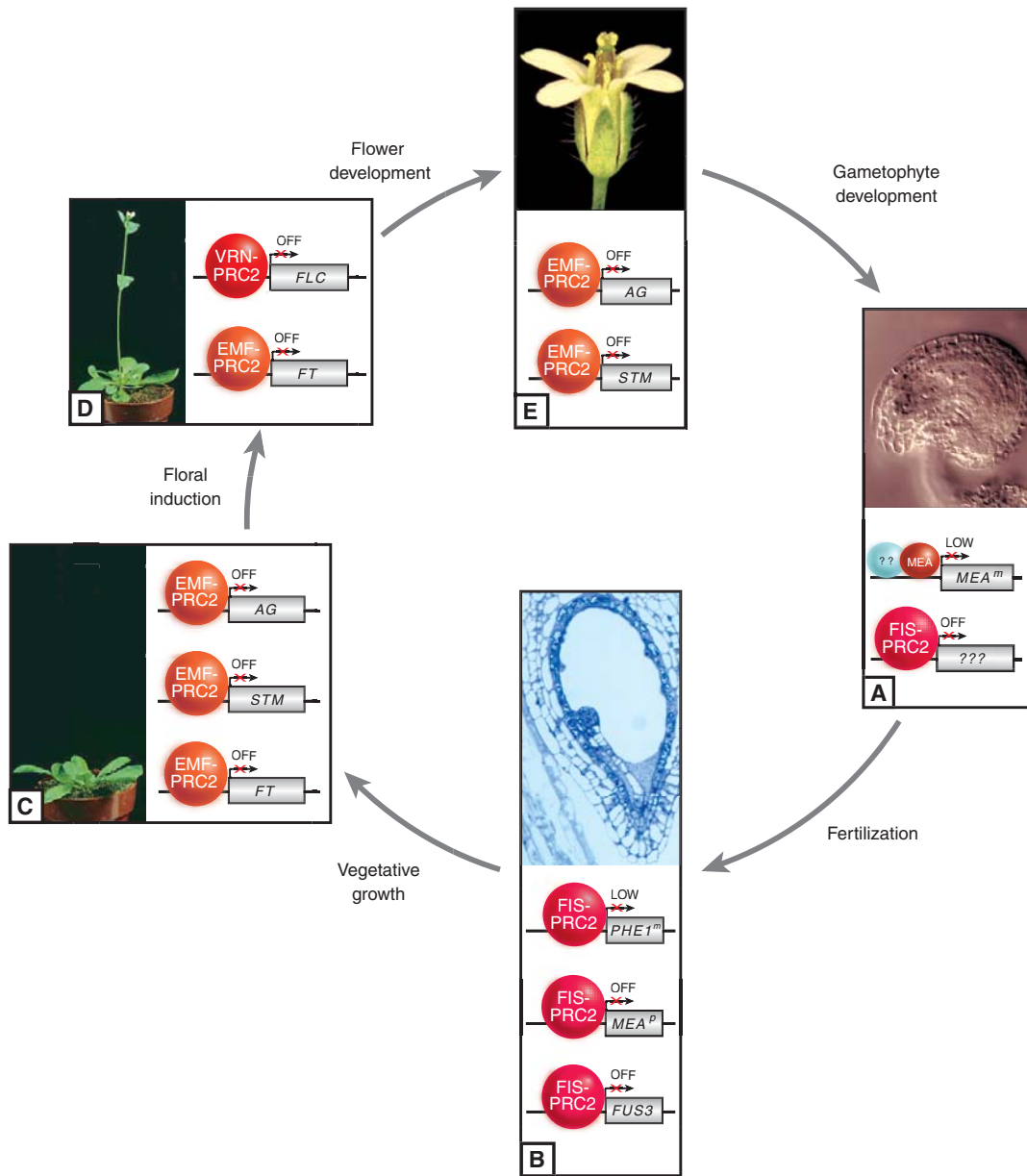


Figure 4. Involvement of distinct PRC2 complexes at various stages of plant development. During the plant life cycle, distinct variants of PRC2 (see Fig. 3) control developmental progression. (A) A cleared wild-type ovule harboring the female gametophyte in its center is represented. The FIS-PRC2 represses unknown target genes that control proliferation of the central cell; consequently, in all *fis* class mutants, this cell proliferates in the absence of fertilization. Around fertilization, *MEA* is also required to maintain expression of the maternal *MEA* allele (*MEA^m*) at a low level, but this activity is independent of other FIS-PRC2 components. (B) Section of a wild-type seed harboring embryo and endosperm, enclosed by the seed coat. After fertilization, the FIS-PRC2 is involved in the control of cell proliferation in embryo and endosperm. It maintains a low level of expression of the maternal *PHE1^m* allele and is involved in keeping the paternal *MEA^p* allele silent, although FIS-PRC2 only plays a minor part in its repression. Both parental alleles of *FUS3* are repressed by the FIS-PRC2. (C) Wild-type plant before flowering. The EMF-PRC2 prevents flowering by repressing *FT* and directly represses the floral genes *AG* and *STM*. (D) Wild-type plant after bolting—that is, floral induction induced by appropriate photoperiod and/or vernalization. The former relieves repression by EMF-PRC2 of *FT*, a promoter of flowering, whereas the latter leads to repression of the floral repressor *FLC*, thus inducing flowering. The maintenance of *FLC* repression depends on the VRN-PRC2. (E) Wild-type *Arabidopsis* flower. During flower organogenesis, the EMF complex regulates floral homeotic genes, such as *AG*, which determine the identity of floral organs, and *STM*, which is involved in floral organ development. (A, Courtesy of J.M. Moore and U. Grossniklaus; B, courtesy of J.-P. Vielle-Calzada and U. Grossniklaus; C,D, courtesy of D. Weigel; E, reprinted, with permission, from Page and Grossniklaus 2002, © Macmillan.)

crucial for PcG silencing because its distribution matches the binding sites of PcG components determined by genome-wide ChIP studies (Schuettengruber et al. 2009; Kharchenko et al. 2010). The PRC2 core complex contains E(Z), which catalytically adds up to three methyl groups at the target lysine residue K27 of H3 through its SET-domain (Fig. 5A). However, E(Z) alone seems to be inactive and needs to associate with the other PRC2 subunits, ESC and SU(Z)12, to provide the necessary catalytic activity (Cao and Zhang 2004; Pasini et al. 2004; Nekrasov et al. 2005).

Although the mechanistic basis of this enhancement remains elusive, the process is conserved in mammals. Several more proteins and subcomplexes related to PRC2 exist. For example, the ESC-like gene encodes a protein similar to ESC and is able to fully replace it in its absence (Wang et al. 2006; Kurzhals et al. 2008). Also, a PRC2 variant additionally containing PCL has been found to specifically enhance the last addition of a methyl group to generate H3K27me3 (Nekrasov et al. 2007). Without PCL, the bulk H3K27me3 is reduced in embryonic and larval tissues,

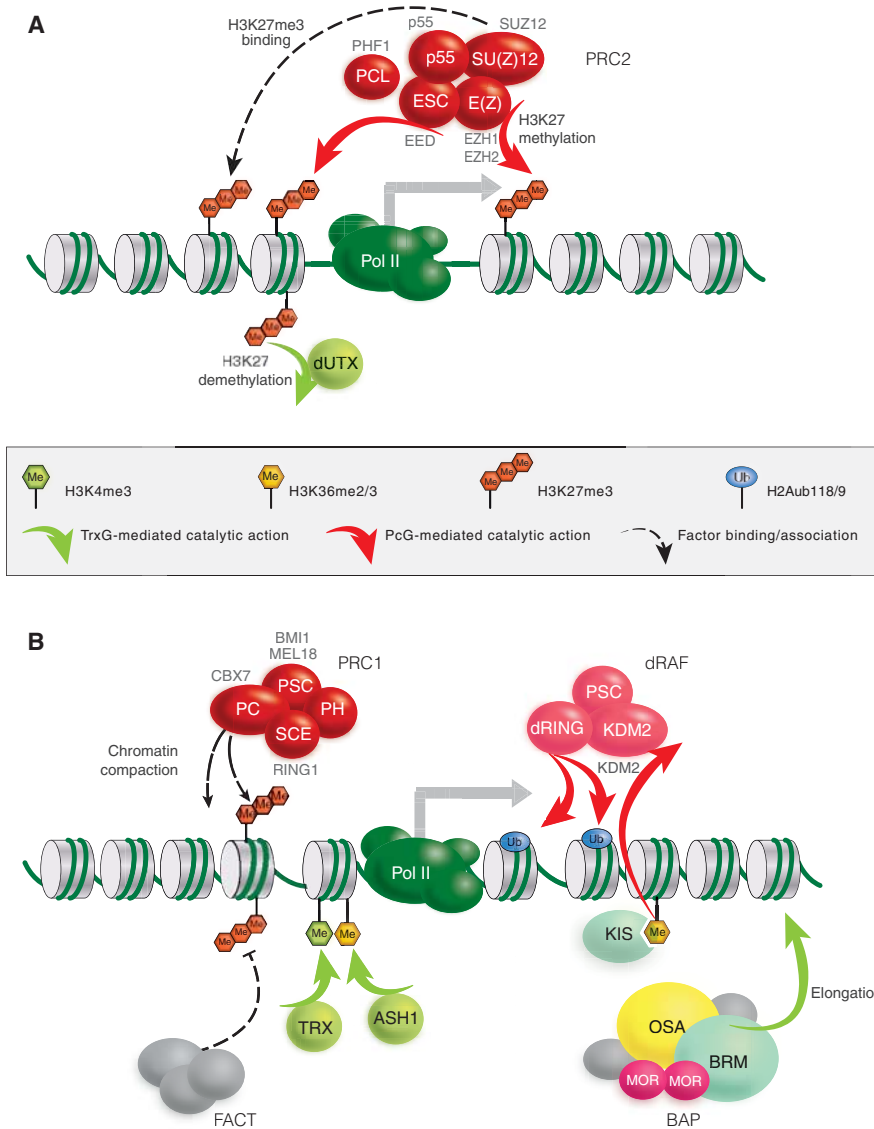


Figure 5. Schematic representation of the core PcG and TrxG protein complexes and their functions at promoters. *Drosophila* PcG proteins are depicted as red ovals with selected mammalian orthologs indicated in gray text. (A) Components and function of the PRC2 and counteracting activities of TrxG proteins (light green). (B) Components and functions of PRC1 and dRING-associated factor (dRAF) and the counteracting activities of the BAP SWI/SNF, facilitates chromatin transcription (FACT) remodeling complexes, and SET-domain histone KMTs TRX and ASH1. The TrxG protein Kismet-L is a member of the chromatin-helicase-DNA-binding (CHD) subfamily of chromatin-remodeling factors, stimulating elongation of Pol II. (Adapted from Enderle 2011.)

leading to the derepression of several target genes. Interestingly, a similar function and complex has also been described for the PHD finger protein PHF1 in mammals (Cao et al. 2008; Sarma et al. 2008).

The mammalian PRC2 has also been found to counteract H3K4 methylation, an active histone modification, by recruiting the H3K4me3 demethylase RETINOBLASTOMA BINDING PROTEIN 2 (RBP2) to target genes (Pasini et al. 2008) and control transcriptional elongation via JARID 2 (Landeira et al. 2010). The biological implications of these variations and additional activities, however, are not yet fully understood. Moreover, even the molecular function of H3K27me3 is still a matter of debate. There is no evidence yet that H3K27 methylation may directly alter nucleosomal structure to repress target genes. Rather, it seems to provide a binding platform for other PcG proteins; the PRC1 complex binds, albeit weakly, to H3K27me3 through the chromodomain of its PC subunit, and also mammalian PRC2 itself has been shown to bind H3K27me3 via the Embryonic ectoderm development protein (EED; Fischle et al. 2003; Margueron et al. 2009). Interestingly, this interaction seems to trigger the lysine methyltransferase activity of E(Z), providing a self-reinforcing positive-feedback loop (as illustrated in Fig. 13 of Allis et al. 2014), potentially contributing to the heredity of the PcG/TrxG system (Margueron et al. 2009). The counteracting force of the TrxG has also been described in mammals: UTX/KDM6A, the mammalian ortholog of *Drosophila* dUTX showing some genetic characteristics of a TrxG member (Smith et al. 2008), is able to demethylate H3K27me3 in vivo and in vitro (Agger et al. 2007; Lee et al. 2007). This reveals a direct antagonistic relationship between PcG and TrxG proteins in the modification of their target chromatin (Fig. 5A).

2.3 The Dynamic Function of PRC2 during Development

As pointed out in Sections 2.1 and 2.4, the PRC1 and PRC2 core complexes are associated with distinct factors that may play a role in recruiting PcG complexes to tissue-specific target loci or in modulating target gene activity. PcG complexes may even differ between target genes in the same cell, suggesting a highly dynamic behavior at different developmental stages. Studies performed in mammals and plants clearly show that PcG complexes have distinct memberships in specific tissues and their composition changes during cellular differentiation. Similar to the situation in plants described in Section 2.1, some of the genes encoding PRC2 subunits have been duplicated in mammals. For instance, PRC2 complexes containing either EZH1 or EZH2 are functionally distinct (Fig. 3). EZH1-containing PRC2

has weak KMT activity and is abundant in nondividing cells of adult organs, whereas EZH2 confers high KMT activity and is expressed strongly in proliferating cells (Margueron et al. 2008). Furthermore, different isoforms of EED, which are derived from the same messenger RNA but different translational start sites, are able to methylate H3K27 and H1K26 (Kuzmichev et al. 2005).

In *Drosophila*, PcG proteins maintain repressed states of homeotic genes, established during early embryogenesis, thereby fixing developmental decisions. Once the silent state of a PcG target has been fixed, it will often remain in that state for the remainder of an individual's lifespan. In plants, a similar situation may occur with the VRN-PRC2; once vernalized, the target gene(s) will be permanently inactivated and only reset in the next generation (see Baulcombe and Dean 2014 for more detail). Other plant PRC2 variants, however, seem to respond quickly to developmental or environmental stimuli. For instance, one function of the FIS-PRC2 is to repress cell proliferation in the absence of fertilization. Upon fertilization, however, cell proliferation is rapidly induced, presumably through the derepression of PcG target genes. This indicates that PcG repression is the default state, which has to be overcome by some unknown mechanism to allow developmental progression to occur. Indeed, the major function of the various plant PRC2 variants seems to be the regulation of developmental transitions, such as those occurring at fertilization, during seed development, and after germination when the plant progresses from the juvenile to the adult, and finally the reproductive stage (reviewed in Holec and Berger 2012).

2.4 Components of the PRC1 Complex

The PRC1 core complex purified from *Drosophila* embryos contains stoichiometric amounts of PC, Polyhomeotic (PH), Posterior sex combs (PSC), and Sex combs extra (SCE/dRing 1) (Shao et al. 1999). Its mammalian counterpart comprises the same core components, but the genes encoding them have been amplified (Table 1). As mentioned before, the PC subunit is able to bind specifically to H3K27me3 in vitro. This does not necessarily mean that H3K27me3 is the primary recruiter of PRC1 (Fig. 5B) because it is experimentally challenging to distinguish recruitment from subsequent stabilization of local chromatin binding. However, experiments directly increasing the H3K27me3 levels in human cells clearly enhance PRC1 binding in vivo, demonstrating the importance of the PC subunit as a chromatin anchor point for the PRC1 (Fig. 5B) (Lee et al. 2007). Additionally, flies with a mutated H3K27 fail to repress transcription of PcG target genes depicting a similar phenotype as *Polycomb* mutants (Pengelly et al. 2013). The reconstituted core components of the mamma-

lian PRC1 have been functionally tested on nucleosomal arrays and were found to inhibit chromatin remodeling by SWI/SNF and restrict access by RNA polymerase II (RNA Pol II) in vitro (Shao et al. 1999; King et al. 2002). Both the PRC1 subunits PSC and SU(Z)2 are crucial for chromatin accessibility, explaining the complete functional redundancy of PSC and SU(Z)2 (Lo et al. 2009). Another conserved hallmark function of PRC1 is its ability to monoubiquitinate H2A-K118/K119 (H2AK118/119ub1) by the E3 ubiquitin ligase SCE/dRing1 (Fig. 5B) (Wang et al. 2004b; Gutiérrez et al. 2012). This histone modification seems to be under a tight, dynamic control because PR-DUB, another PcG complex containing Calypso and Additional sex combs, actively removes this mark (Scheuermann et al. 2010). Moreover, there is cross talk with ubiquitination of histone H2B, extending the regulatory possibilities even further (reviewed in Weake and Workman 2008).

The function of H2AK118/119ub1 is not well understood, but somehow inhibits the recruitment of the FACT chromatin remodeling complex (Zhou et al. 2008). It was recently shown that the H2A ubiquitination activity of PRC1 is dispensable for target binding and its activity to compact chromatin at *HOX* loci, but is indispensable for efficient repression of target genes and thereby maintenance of embryonic stem (ES) cell identity (Endoh et al. 2012). The H2AK118/119ub1 mark in *Drosophila* is also set by the distinct and conserved dRAF complex (Fig. 5B) (Lagarou et al. 2008; Scheuermann et al. 2010). Interestingly, dRAF is able to demethylate H3K36 through its dKDM2 subunit, linking repression through H2A ubiquitination directly with the inhibition of transcriptional elongation by removing an activating mark.

The existence of a ubiquitously conserved PRC1 has been disputed because most of the core PRC1 subunits are not conserved in plants. However, although there is no clear PC homolog in plants, the *Arabidopsis* LIKE HETEROCHROMATIN PROTEIN1/TERMINAL FLOWER2 (LHP1/TFL2) protein serves as a functional counterpart to *Drosophila* PC. Like PC, LHP1/TFL2 binds H3K27me3 in vitro and is colocalized with this mark throughout the genome (Turck et al. 2007; Zhang et al. 2007). Also, *lhp1/tfl2* mutants show some of the phenotypes typical of PcG mutants. Furthermore, there are multiple homologs of PSC and SCE/dRing (Table 1), and double mutants for *Atbmi1a/1b* or *Atring1a/1b* result in phenotypes similar to those observed in mutants affecting PRC2. Indeed, the AtBMI1 homologous proteins have been shown to mediate H2A monoubiquitination in vivo (Bratzel et al. 2010). The *Arabidopsis* PSC/BMI 1 and SCE/dRING homologs interact with each other, the chromodomain protein LHP1, and EMF1, a plant-specific nucleoprotein. Thus, plants have a PRC1-like complex that contains some PRC1 homologs,

but also plant-specific factors. However, this PRC1-like activity only regulates a subset of the PRC2 targets as it was also reported for *Drosophila* (Gutiérrez et al. 2012). The PRC2 target gene *AG*, for instance, is not up-regulated in either the *Atbmi1a/1b* or the *Atring1a/1b* double mutant (Xu and Shen 2008; Bratzel et al. 2010).

In spite of the already dazzling variety in PcG functions found, there is still scope for a better functional understanding of the role that proteins, other than the core subunits, play. There is a large group of loosely associated subunits such as TBP-associated factors in PRC1. This interaction might indicate a role in inhibiting the assembly of the preinitiation complex of RNA Pol II (Dellino et al. 2004). Several other enzymatic functions also seem to contribute to PcG silencing; PRC1 members associate with HDAC1 (Huang et al. 2002), indicating that histone-deacetylation may play a role in PcG-silencing. Furthermore, the PcG gene *super sex combs (sxc)* encodes an enzyme that posttranslationally modifies PH and RNA Pol II with β -O-linked *N*-acetylglucosamine residues and is necessary for the repression of several *HOX* genes (Gambetta et al. 2009; Sinclair et al. 2009). Yet another interesting link is the possibility of a switch-like mechanism through the acetylation of H3K27 by the acetyltransferase cAMP response element binding protein-binding protein (CBP), antagonizing its methylation (Tie et al. 2009). Most interestingly, in mammalian stem cells, PcG-based promoters marked by H3K27me3 frequently become DNA methylated during differentiation, suggesting that *Polycomb* repression and de novo DNA methylation are linked (Mohn et al. 2008). A direct physical interaction of PRC2 components and the MET1 DNA methyltransferase was recently also found in plants, indicating an evolutionarily old interaction between these major epigenetic pathways (Schmidt et al. 2013).

2.5 The PcG Connection to Paused RNA Pol II Promoters

The mechanism by which PcG complexes interact with the promoter via binding to specific *cis*-regulatory elements (PREs, detailed in Sec. 3.1) to prevent transcription in *Drosophila* has become clearer in recent years. The anchoring of paused RNA Pol II complexes at promoters, preventing initiation, has been attributed to PRE-PRC1 interactions described by the use of reporter constructs (Dellino et al. 2004). In mouse ES cells, Ring1-mediated ubiquitination of H2A was found to restrain paused RNA Pol II at PcG target genes (Fig. 6A) (Stock et al. 2007). Genome-wide ChIP-Seq profiling of *Drosophila* tissue culture cells uncovered a strong overlap between PRC1-binding sites and promoters with paused RNA Pol II (Enderle

et al. 2011). Indeed, this approach also found that many promoters of noncoding RNAs (ncRNAs) are targeted by PRC1. Among those, the promoters of primary transcripts for many micro RNAs stand out, suggesting that this important class of RNA regulators is under the control of the PcG system as well. Yet, the finding that paused Pol II at promoters is a major hallmark of PcG target genes indi-

cates that there is a mechanistic link between PcG-mediated silencing and mechanisms of transcriptional elongation. In addition, PRC1 was shown to counteract remodeling of nucleosomes in vitro and to induce a compact chromatin structure. Thus, PRC1 potentially blocks the accessibility to DNA of transcription factors and other complexes required for transcription (Grau et al. 2011).

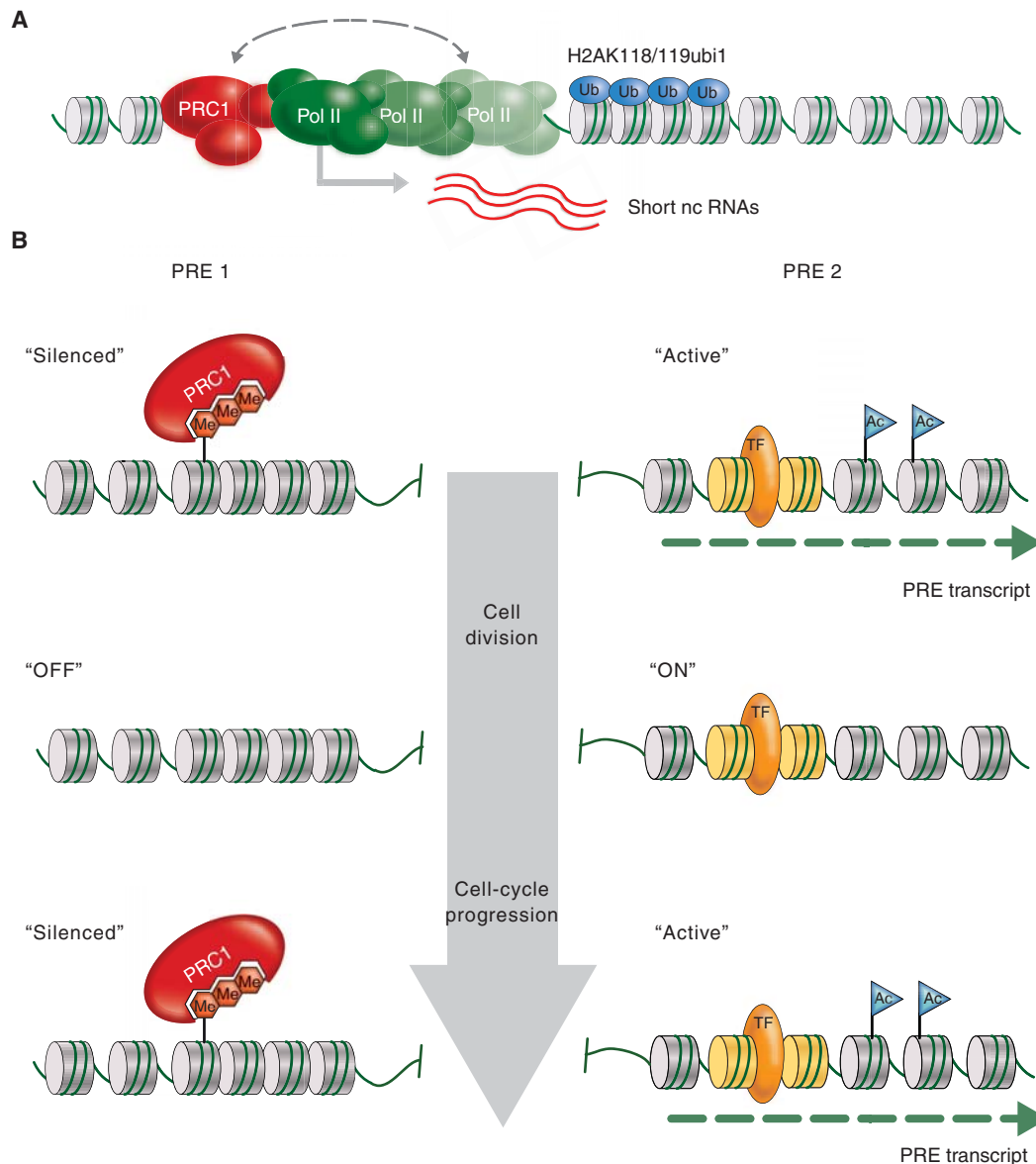


Figure 6. PRC1 at paused promoters and during cell division. (A) The PRC1 complex may repress target genes by stalling the elongation of RNA Pol II. This may be achieved by ubiquitination of histone H2A through the subunit SCE/dRING, compacting promoter proximal chromatin, or direct physical interaction with the transcriptional machinery (including the short RNAs produced by the paused RNA Pol II). (B) A possible model for how differential gene expression states can be inherited. The process of intergenic transcription places positive epigenetic marks (e.g., acetylated histone tails, histone variants) at PREs that control active genes (PRE 2). All other PREs are silenced by default (PRE 1). During DNA replication and mitosis, only the positive epigenetic signal needs to be transmitted to the daughter cells, ensuring that in the next interphase intergenic transcription is restarted at PRE 2 before default silencing is reestablished at all other PREs.

The stability of silencing complexes, as shown by anchoring via methylated histone tails, appears to be a major property of the long-term repressive function of PcG proteins. However, when analyzed *in vivo* at the cellular level, a remarkably dynamic behavior of the individual components is observed. PcG proteins cluster in PcG bodies, which vary in size and composition between cells (Bantignies and Cavalli 2011). Fluorescence recovery after photobleaching (FRAP) analyses of GFP-marked PC and PH proteins uncovered a very high rate of exchange between unbound proteins and their complexes at silenced target genes (Fonseca et al. 2012). These results suggest that long-term repression is primarily based on a chemical equilibrium between bound and unbound proteins rather than through high-affinity protection of DNA-binding sites. Additionally, a new method measuring nucleosomal turnover uncovered a rapid exchange over active gene bodies, epigenetic regulatory elements, and replication origins in *Drosophila* cells (Deal et al. 2010). Surprisingly, rapid nucleosome turnover is seen at many PcG- and TrxG-regulated elements. This finding questions whether PcG-associated histone marks can contribute to epigenetic stability. Indeed, it was recently shown that H3K4 methylation, a mark of active gene expression associated with the TrxG, is not essential; *Drosophila* cells completely lacking this histone mark show normal transcriptional activation in response to developmental signaling pathways (Hodl and Basler 2012).

2.6 Preventing Heritable Repression by Antisilencing

The binding of PRC1 complexes to PREs appears to be a default state, as many of the anchoring PcG components and DNA-binding proteins are expressed in all cells and transgenic constructs with PREs controlling reporter genes are globally silenced. The counteracting proteins of the TrxG do not, in fact, function as activators, but rather as antirepressors (Klymenko and Müller 2004; see Kingston and Tamkun 2014 and Fig. 7 therein). This antagonistic interplay of PcG and TrxG proteins seems to be conserved between animals and plants; for instance, several plant PRC2 targets such as the *AG* and *FLC* loci are similarly maintained in an active state through the activity of the homolog of *Drosophila* TRX, ATX1, which acts as a KMT specific to H3K4 (Alvarez-Venegas et al. 2003; Pien et al. 2008).

To maintain active transcription of a PRE-controlled gene, the silencing at that PRE, thus, has to be prevented in a tissue- and stage-specific manner. In *Drosophila*, for example, the early cascade of transcription factors encoded by the segmentation genes controls the activation of *HOX*

genes. Interestingly, these factors do not only induce transcription of the *HOX* genes, but also of intergenic ncRNAs that are transcribed through the associated PREs often found upstream or downstream. It was shown that transcription through PREs is required to prevent silencing and to maintain the active state of a reporter gene using transgenic constructs (Schmitt et al. 2005). The process of transcription most probably remodels PRE chromatin to generate an active state that is characterized, for instance, by a lack of repressive histone methylation and the presence of histone acetylation. Thus, although the DNA binding proteins will attract PRC1 to this particular activated PRE, the histone environment will not allow anchoring of PC via H3K27me₃, and no stable silencing would be established. Because silencing is induced by default in the PcG system, epigenetic inheritance of a differential gene expression pattern only requires the transmission of the active PRE state during DNA replication and mitosis (Fig. 6B). How this is achieved at the molecular level and which epigenetic mark(s) are responsible for maintaining an active PRE state is still an open question. It has been suggested that particular TrxG factors might act as “bookmarks” during epigenetic bottleneck stages, like DNA replication and mitosis, to mark a gene for continued expression (Blobel et al. 2009). Hence, finding the molecular constitution of chromatin components that self-template during DNA replication and carry over the signals for activity to the daughter cells may be key for advancing our understanding of epigenetic inheritance.

3 TARGETING PcG COMPLEXES TO SILENCED GENES

3.1 PcG Response Elements

A striking feature of the PRC1 and PRC2 core complexes is that they do not contain any obvious DNA sequence-binding activity, raising the question of how they might be targeted. Although PRC1 is bound to chromatin through its affinity for H3K27me₃ and a consistent hierarchical recruitment has been shown at the *bx1* genomic region in *Drosophila* (Wang et al. 2004a), H3K27me₃ alone is not sufficient to explain the targeting of the complex. First of all, PcG-binding sites are generally devoid of histones and a place of rapid nucleosome turnover (Mito et al. 2007; Deal et al. 2010). Furthermore, the generally broad distribution of H3K27me₃ does not fit the localized binding of PRC1 (Fig. 7B) (Schuettengruber et al. 2009; Enderle et al. 2011). Also, removal of H3K27me₃ does not lead to the immediate displacement of PRC1. Indeed, there are examples of PRC1 binding to sites without any apparent H3K27 methylation (Schoeftner et al. 2006; Tavares et al. 2012). Overall,

H3K27me3 may contribute to several different low-affinity steps working together in recruitment or, more interestingly, allowing PRC1 to reach and modify histones distant to its initial binding site in a local domain.

The best characterized binding sites for PRC1 and PRC2 proteins were originally identified at the *bithorax* complex and subsequently termed PREs (Simon et al. 1993). PREs are thought to act as intergenic *cis*-regulatory elements, controlling gene expression by looping to the promoter regions of their target *HOX* genes. But mostly, PREs have been characterized by their ability to confer PcG silencing on reporter genes. PREs frequently contain binding sites for the zinc finger DNA-binding proteins Pleiohomeotic (PHO) and the related PHO-like (PHOL) protein, and these sites are essential for transgene and endogenous silencing functions (Fig. 7C). PHO forms a heterodimeric complex together with the Scm-related gene containing four mbt domains (SFMBT) protein, termed the Pho Repressive Complex (PhoRC) (Klymenko et al. 2006). The genome-wide distribution of PhoRC confirms its central role in the recruitment of PcG proteins: 45% of PHO-bind-

ing sites in larval and embryonic tissue are cooccupied by both PRC1 and PRC2. At the same time, the majority of PH-binding sites in embryos are enriched for PHO (Schuettengruber et al. 2009). Conversely, these data also show that PhoRC binding cannot be the only factor for targeting PRC1 because many loci bind PcG proteins without PhoRC being present. This is also reflected in PHO-binding sites being necessary, but not sufficient, for recruitment of PRC1 and PRC2. Several other proteins with DNA-binding abilities have been found as physical or genetic interactors of PcG proteins. Among them are Pipsqueak (PSQ) as subunit of the CHRASCH complex, Dorsal switch protein 1 (DSP1), Grainyhead (GRH), GAGA factor (GAF), and Sp1-like factor for pairing sensitive-silencing (SPSS), which is an Sp1/Klf protein family member (Fig. 7C). Despite this diversity, many PcG-binding sites do not contain any binding sites for the aforementioned transcription factors. Consistently, prediction algorithms based on their consensus binding sites are only able to predict a fraction of the many PRC1- and PRC2-binding sites found in a single cell type (Ringrose et al. 2003; Schwartz et al. 2006).

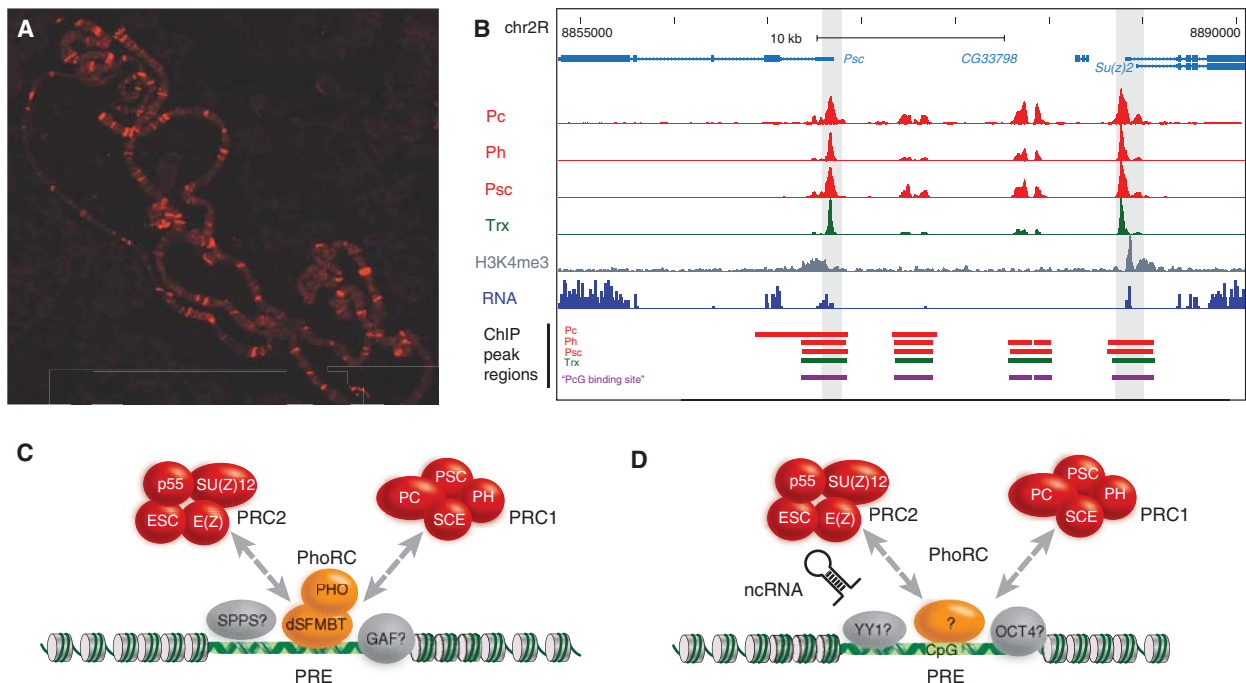


Figure 7. Chromosomal targeting of PRC1. (A) Immunostaining of *Drosophila* polytene chromosomes to visualize the distribution of the PC protein. (B) Genomic region encompassing the *Drosophila* PcG gene *Psc* and the *Su(z)2* gene. The genome browser section shows the result of a ChIP-Seq and RNA-Seq analysis of *Drosophila* S2 tissue culture cells. The distributions of PRC1 components (red) and the TRX protein (green) are shown (data from Enderle et al. 2011). (C) In *Drosophila*, the PhoRC is a key player in chromatin targeting of PRC1 and PRC2, but a number of other transcription factors also contribute to target gene specificity. (D) In mouse and human, several different anchoring factors have been proposed. These include the Pho ortholog Ying and Yang 1 (YY1), transcription factors like Jarid 2 and Oct4, long ncRNAs, and the CpG content of the target sequence. (Adapted from Enderle 2011.)

In contrast to *Drosophila*, PREs are ill-defined in plant and mammalian genomes, and only a few sequences have been described that, at least partially, fulfill the criteria for PRE function. According to Schwartz and Pirrotta (2008), the minimum criteria include (1) PREs attract H3K27me₃, (2) they should form a new binding site for PcG proteins when inserted at a new location within the genome, and (3) they confer PcG-based repression to a reporter gene. Although no PREs have fulfilled all three criteria in plants, some sequences at well-studied PcG targets likely function as PREs. For instance, a transgene including sequences overlapping the promoter and parts of the coding sequence of the *AG* locus, a target of the EMF-PRC2, renders the reporter gene responsive to CLF, leading to H3K27me₃ deposition (Schubert et al. 2006). More recently, the promoter of *LEAFY COTYLEDON2* (*LEC2*), which is regulated by EMF-PRC2, was shown to contain a repressive *LEC2* element (*RLE*), which is sufficient to trigger H3K27me₃ deposition and silencing of a reporter gene in seedlings (Berger et al. 2011). Although the *RLE* is close to a CT-rich *cis*-regulatory element with similarities to the GAGA-box of *Drosophila* PREs, a possible function of the BASIC PENTACYSTEINE (BCP) proteins binding these CT-rich sequences in recruiting PRC2 has not yet been shown.

DNA sequences from the *FLC* and *MEA* loci can similarly confer PcG-dependent silencing to reporter genes, although in the case of *MEA* the FIS-PRC2 plays only a minor part in the repression of the paternal allele (Sheldon et al. 2002; Wöhrmann et al. 2012). For *AG*, *FLC*, or *MEA*, no DNA-binding factors are currently known that recruit PRC2 variants to these loci. Thus, other factors, such as a specific chromatin structure or long ncRNAs (lncRNAs), may be involved in PRC2 recruitment. Recent studies have, however, shown that DNA-binding proteins do play a role in PRC2 recruitment at the *WUSCHEL* (*WUS*) locus, whose repression is crucial for the appropriate termination of floral meristems. *WUS* is a target of the EMF-PRC2, and H3K27me₃ levels at the *WUS* locus are reduced similarly in *ag*, *clf*, and *swn* mutants, which act in the same genetic pathway. As H3K27me₃ levels at *WUS* increase rapidly after the experimental induction of *AG*, the MADS-domain protein *AG* likely plays a role in recruiting PcG proteins to this locus.

In the mouse and human genome, PcG proteins predominantly occupy regions around gene promoters (Boyer et al. 2006; Lee et al. 2006; Ku et al. 2008). Two intergenic PREs, however, were identified in mammalian genomes (Sing et al. 2009; Woo et al. 2010). An intergenic region of 1.8 kb, termed D11.12, was bound by PcG proteins in the human *HOX-D* complex. The other intergenic PRE, a 3-kb “PRE-kr” at the *MafB/Kreisler* locus in mouse, also recruit-

ed PcG proteins and was able to regulate its expression pattern. PcG protein recruitment is necessary for their potential to exert gene silencing. Importantly, the D11.12 element is able to maintain repression of a luciferase transgene throughout cell differentiation, providing the first example of a mammalian PRE sequence. Interestingly, both elements contain binding sites for the mammalian homolog of the *Drosophila* PhoRC, which contains SFMBT and YY1 (Fig. 7D). Still, binding of YY1 only accounts for a fraction of the genome-wide PRC2 sites in ES cells (Squazzo et al. 2006). Although there is substantial overlap with the pluripotency factors NANOG, OCT4, and SOX2, these three proteins have not been copurified with PcG complexes yet (Boyer et al. 2006; Lee et al. 2006). Also, the recent identification of the mammalian GAF homolog may provide new insights into PcG recruitment by transcription factors in the future (Matharu et al. 2010).

A surprising aspect was found in ES cells, in which nearly all PRC2 binding sites are found at CpG islands or other highly GC-enriched sequences (Ku et al. 2008). Indeed, GC-rich DNA from bacterial genomes is able to initiate recruitment of PRC2 (Mendenhall et al. 2010). This is especially interesting because Mixed lineage leukemia (MLL), the mammalian TRX homolog, also has a preference for CpG dinucleotides, revealing a shared discriminant for targeting. Other interesting recruitment factors are specific lncRNAs, which are discussed in Section 3.2.

3.2 PcG Proteins Bind to Noncoding RNAs

Several ncRNAs have been proposed to recruit PcG proteins in mammals. The most prominent example may be *HOTAIR*, a 2.2-kb ncRNA from the human *HOX-C* cluster, acting in *trans* to mediate gene repression (described in Rinn 2014; also Rinn et al. 2007). *HOTAIR* is one of many transcripts identified in the intergenic regions of human *HOX* clusters and its depletion leads to the loss of H3K27me₃ in a large genomic region of *HOX-D*. Indeed, *HOTAIR* interacts with PRC2 components in vitro, suggesting that it may also recruit KMT activity to the *HOX-D* complex (Rinn et al. 2007). Another interaction between PRC2 components and an lncRNA is observed at the paternally imprinted *Kcnq1* locus in mouse (Fig. 8) (Wu and Bernstein 2008). Similar to *HOTAIR*, the 91-kb-long primary transcript of *Kcnq1 overlapping transcript 1* (*Kcnq1ot1*), coimmunoprecipitates with EZH2 and SUZ12, and additionally with the H3K9-specific KMT G9a (Kanduri et al. 2006; Pandey et al. 2008). The paternally transcribed ncRNA may facilitate silencing by recruitment of methyltransferases in *cis*, leading to the inactivation and compaction of genes at the locus (Terranova et al. 2008).

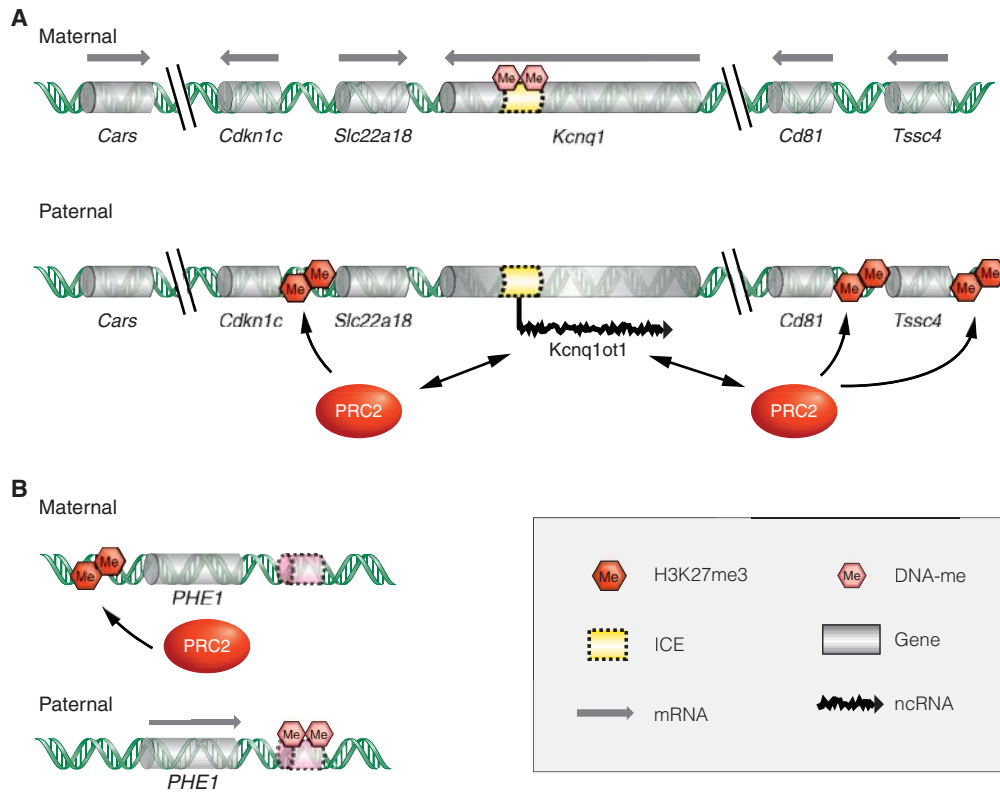


Figure 8. Interplay of PcG-mediated repression and DNA methylation regulates genomic imprinting in plants and mammals. (A) Regulation of genomic imprinting at the *Kcnq1* domain on distal chromosome 7. The imprinting control element (ICE) is maternally methylated and prevents the transcription of the lncRNA *Kcnq1ot1* from the maternal chromosome. The paternally expressed *Kcnq1ot1* associates with chromatin and recruits chromatin modifying complexes, such as PRC2, to mediate and maintain transcriptional silencing of several paternal, protein-coding alleles. (B) In *Arabidopsis* seeds, the paternally expressed *PHE1* gene is maternally repressed by the action of PRC2. A cis-regulatory element (shaded pink) downstream of the *PHE1* gene must be methylated for paternal expression, but demethylated for maternal repression.

Another cis-acting ncRNA that is a crucial component for inactivating one of the X chromosomes in female mammals is the 17-kb-long *X inactive-specific transcript* (*Xist*). It contains a 28-bp repeat element that interacts with EZH2 in vitro and in vivo (Zhao et al. 2010). This element folds into a double stem-loop structure and is necessary for X inactivation (covered in more detail in Brockdorff and Turner 2014). Recently, similar stem-loop structures have been identified in a class of short promoter-proximal ncRNAs, which are transcribed from H3K27me3-marked genes (Kanhare et al. 2010). The small ncRNAs interact with PRC2 in vitro through secondary structures, revealing PRC2-bound and folded ncRNAs as a repeating theme in mammalian cells. However, details of the recruiting mechanism and a common RNA motif have yet to be described. Also, RNA-interacting PcG proteins are not restricted to members of the PRC2 complex. A recent example is *ANRIL*, an ncRNA at the murine *Ink4b/Arf/Ink4a* locus. This transcript has been shown to specifically associate with

PRC1 members through the chromodomain of CBX7 (Yap et al. 2010). A local competition between *ANRIL* and H3K27me3 for the binding of CBX7 may therefore remove PRC1 from chromatin, leading to the derepression of the *Ink4b/Arf/Ink4a* locus.

An interaction between lncRNAs also seems to play a role in the regulation of the *Arabidopsis FLC* locus and, hence, vernalization (see Baulcombe and Dean 2014 for details). Expression of the sense ncRNA *COLDAIR* and the antisense ncRNA *COOLAIR* are induced by cold, and *COLDAIR* was shown to physically interact with CLF, indicating a possible role in VRN-PRC2 recruitment (Swiezewski et al. 2009). However, *FLC* transgenes without the *COLDAIR* promoter respond to cold, and also antisense *COOLAIR* is not required for vernalization-induced repression of *FLC*, such that the functional requirements for these ncRNAs are not clear (Sheldon et al. 2002; Helliwell et al. 2011). However, as the expression and processing of the *COOLAIR* ncRNA is affected by different genotypes and

environments, and these correlate with changes in chromatin marks (reviewed in Ietswaart et al. 2012), it is possible that ncRNAs also recruit PRC2 components to target loci as they do in mammals.

4 PcG REPRESSION IN DEVELOPMENT AND DISEASE

4.1 From Gene to Chromosome Repression

Mutations in members of the murine PRC1 complex show homeotic transformations of the axial skeleton. This can cause the appearance of additional vertebrae as a consequence of the derepression of *HOX* genes (Fig. 2E,F) (Core et al. 1997). In addition, the mutant mice display severe combined immunodeficiencies caused by a lack of proliferative responses of hematopoietic cells (Raaphorst 2005). The role of PcG proteins has been particularly well studied in blood cells, in line with the fact that most blood cell lineages are characterized by their well-described cell-type-specific transcription programs. Lineage commitment and restriction need to be faithfully maintained through cell division. In PcG knockout mice, B- and T-cell precursor populations are produced normally, indicating that the establishment of lineage-specific gene expression patterns does not depend on PcG proteins. These proteins do, however, contribute to the irreversibility of the lineage choice, rather than the decision to follow a particular developmental pathway.

PcG proteins play a major role in controlling proliferation as well as the control of *HOX* genes, whose expression patterns characterize different blood cell lineages. The *Bmi1* gene, an ortholog of *Drosophila Psc* belonging to the PRC1 group, was initially identified as an oncogene that, in collaboration with *myc*, induces murine lymphomagenesis (van Lohuizen et al. 1991). The *Bmi1* protein controls the cell-cycle regulators p16^{INK4a} and p19^{ARF} (Jacobs et al. 1999). Both *Bmi1* and the related protein *Mel-18* are negative regulators of the *Ink4c-Arf* locus required for normal lymphoid proliferation control. Misregulation of this important cell-cycle checkpoint affects apoptosis and senescence in mice.

Mammalian PcG proteins are also associated with X-chromosome inactivation as mentioned in Section 3.2 (see also Brockdorff and Turner 2014). The inactivation of one X chromosome in XX female cells is accompanied by a series of chromatin modifications that involve PcG proteins. In particular, components of the PRC2 complex, like the ESC homolog EED, or the E(Z) homolog ENX1 (Table 1), play a major role in the establishment of histone marks associated with transcriptional silencing. Transient association of PRC2 with the X chromosome, coated by *Xist* RNA, is accompanied by H3K27 methylation. In contrast, *eed* mutant

mouse embryos show no recruitment of the ENX1 KMT, and consequently no H3K27me3 is observed. However, the absence of these PRC2 components does not lead to a complete derepression of the entire inactive X chromosome; rather, some cells display sporadic reexpression of X-linked genes and an increase in epigenetic marks associated with an active state (H3K9ac and H3K4me3). This is likely because other, partially redundant, epigenetic mechanisms are in place to ensure the maintenance of one inactive X chromosome.

Recruitment of PRC2 to the inactive X chromosome is dependent on *Xist* RNA. As association of PRC2 to the inactive X is only transient, it appears that the complex is only required to set epigenetic marks (i.e., H3K27me3) for the maintenance of silencing. Currently, it is not known whether the PRC1 complex directly recognizes these marks. PRC1 is involved in the permanent silencing of the inactive X chromosome. The PRC2 component EED is required to recruit the PRC1 components MPH1 and MPH2, whereas RING1b, which can ubiquitinate H2A, is recruited independently of EED (Schoeftner et al. 2006). Thus, *Xist* RNA can recruit PRC1 components in both PRC2-dependent and -independent ways. In the absence of PRC2, *Xist*-dependent PRC1 recruitment is sufficient for PcG-based X-chromosome inactivation, which is further consolidated and maintained by DNA methylation.

PRC2 is involved in X-chromosome inactivation both in the embryo, in which an X chromosome is chosen at random for inactivation, and in extraembryonic tissues, wherein the paternally inherited X chromosome is systematically inactivated (imprinted X-chromosome inactivation). In addition, it was found that PRC2 is involved in the regulation of some autosomal imprinted genes. PRC2-mediated repression is, thus, a silencing mechanism that operates in addition to DNA methylation in the regulation of imprinted gene expression. By way of example, an analysis of 14 imprinted loci from six distinct imprinting clusters showed that four of these were biallelically expressed in *eed* mutant mice (Mager et al. 2003). At the *Kcnq1* imprinting cluster (Fig. 8), for instance, the predominantly maternally expressed genes *Cdk1*, *Cd81*, and *Tssc4* become biallelically expressed in *ezh2* mutant mice (Terranova et al. 2008). As similar results were also observed in mutants deficient for *Ring1b*, both PRC1 and PRC2 appear to be involved in regulating the monoallelic expression of some imprinted genes. Furthermore, it was shown that EZH2 is required for the association of the lncRNA *Kcnq1ot1* along the *Kcnq1* imprinting cluster (Terranova et al. 2008), confirming a link between PRC2 and ncRNAs in the regulation of imprinted genes (Fig. 8). Interestingly, all loci that lost imprinted expression were normally repressed when paternally inherited, whereas none of

the maternally repressed loci were affected. As there appears to be a cross talk between PcG-based repression and gene silencing by DNA methylation, it is possible that PRC2 complex plays a role in the regulation of these imprinted genes via DNA methylation (see Li and Zhang 2014).

An involvement of PRC2 in the regulation of imprinted gene expression has also been reported in *Arabidopsis*, in which the *PHE1* locus is expressed at much higher levels from the paternal allele (Köhler et al. 2005). In mutants affecting the *E(z)* homolog *MEA*, the maternal *PHE1* allele is specifically derepressed. *MEA* also regulates its own imprinted expression, noted by the strong repression of maternal *MEA* early in reproductive development in a *mea* mutant background. This effect, however, is independent of the other components of the FIS-PRC2 (Fig. 4) (Baroux et al. 2006). In contrast, later in development the FIS-PRC2 contributes to the stable repression of the paternal *MEA* allele (Baroux et al. 2006; Gehring et al. 2006; Jullien et al. 2006). In this latter case, the FIS-PRC2 is involved in the silencing of a paternally repressed imprinted allele similar to the situation in mammals. But *MEA* also has a role in keeping expression of the maternal *PHE1* and *MEA* alleles at low levels. Similar to the situation in mammals, regulation of imprinted expression at the *PHE1* and *MEA* loci involves both PRC2 and DNA methylation (Fig. 8). Although DNA methylation at the locus was suggested to regulate higher-order chromatin structure rather than directly distinguishing maternal and paternal alleles (Wöhrmann et al. 2012), both these epigenetic pathways seem to work together at the *PHE1* locus (Makarevich et al. 2008). The silencing of the maternal *PHE1* allele depends on a *cis*-regulatory region, which is differentially methylated. This regulatory element downstream of *PHE1* is methylated on the expressed paternal allele, but must not be methylated to mediate PRC2-dependent repression of the maternal allele (Fig. 8).

As PRC2 components are present in plants, invertebrates, and mammals, PRC2 represents an ancient molecular module suitable for gene repression that was already present in the unicellular ancestor of plants and animals, before the evolution of multicellularity. It was recently shown that DNA methylation at both the *MEA* and *PHE1* locus is affected in *mea* mutants, and PRC2 components directly interact with the DNA methyltransferase *MET1*, as found for the mammalian counterparts (Schmidt et al. 2012). Thus, although the interaction of these two major epigenetic pathways in the control of gene expression may have an ancient evolutionary origin, they were independently recruited for the regulation of imprinted genes in plants and mammals, the two lineages in which genomic imprinting evolved (Raissig et al. 2011).

4.2 Consequences of Aberrant Transcriptional Activation

The finding that *Bmi1* misregulation causes malignant lymphomas in mice raises the question of whether human BMI1 (a PRC1 component) itself contributes to the development of cancer in a similar fashion. There is now accumulating evidence that altered PcG gene expression is widespread in human malignant lymphomas (Shih et al. 2012). For instance, the level of BMI1 overexpression in B-cell lymphomas correlates with the degree of malignancy, suggesting that PRC1 components do play a role in the development of human cancers. However, the target genes of BMI1 in human cells appear to be different from those of mouse lymphocytes, as no obvious down-regulation of p16^{INK4a} could be correlated with the overexpression of the oncogenes.

PcG gene overexpression is not only observed in hematological malignancies, but is also found in solid tumors, including medulloblastomas, and tumors originating from liver, colon, breast, lung, penis, and prostate (Fig. 9). The high expression of a PRC2 marker, *EZH2*, is often found in early stages of highly proliferative lung carcinomas. This suggests that the well-known cascade of PRC2 initiation and PRC1 maintenance might also accompany the development of a tumor cell lineage (for a review, see Sauvageau and Sauvageau 2010).

Interestingly, PRC2 components also play a crucial role in the control of cell proliferation in *Arabidopsis*. Although aberrant growth does not lead to cancer and death in plants, a strict control of cell proliferation is essential for normal development. In mutants of the *fis* class, the two fertilization products of flowering plants, the embryo and endosperm, overproliferate and the resulting seeds abort (Grossniklaus et al. 2001; Hsieh et al. 2003). Effects on cell proliferation are also observed in double mutants of *clf* and *swn*, two of the three plant *E(z)* homologs. Such plants undergo normal seed development, but produce a mass of proliferating, undifferentiated tissue (callus) rather than a differentiated shoot after germination (Chanvivattana et al. 2004).

Although it is currently not known how exactly PRC2 controls cell proliferation in plants, it is likely to involve interactions with RBR, the plant homolog of the Retinoblastoma (Rb) protein (Ebel et al. 2004; Mosquna et al. 2004). Mutants of the *fis* class do not only show proliferation defects during seed development after fertilization, but the *FIS* genes are also required to prevent proliferation of the endosperm in the absence of fertilization. This latter aspect of the phenotype is shared with *rbr* mutants and can be explained by the fact that RBR regulates the expression of genes encoding PRC2 components and *MET1* (Johnston et al. 2008). Remarkably, the Rb pathway also regulates the

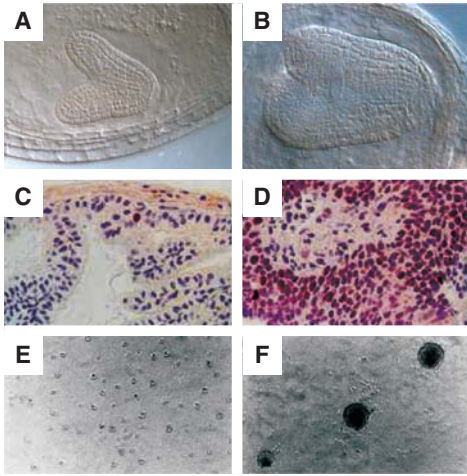


Figure 9. PRC2 regulates cell proliferation in mammals and plants. (A,B) Plant embryos derived from wild-type and *mea* mutant egg cells. *MEA* encodes a protein of the FIS-PRC2 and regulates cell proliferation. The *mea* embryo (B) is much larger than the corresponding wild-type embryo (A) at the same stage of development (late heart stage). Mutant embryos develop slower and have approximately twice the number of cell layers. (C,D) Normal and cancerous prostatic epithelium of mice. In the cancerous epithelium, *Ezh2* expression is highly increased (labeled with an anti-*Ezh2* antibody). Thus, both loss of E(Z) function in plants and overexpression of E(Z) function in mice can lead to defects in cell proliferation. (E,F) Control and RING1 overexpressing rat 1a fibroblast cells. Overexpression of RING1 leads to anchorage-independent growth in soft agar, typical of neoplastically transformed cells. (A,B, Courtesy of J.-P. Vielle-Calzada and U. Grossniklaus; C,D, reprinted, with permission, from Kuzmichev et al. 2005, © National Academy of Sciences; E,F, reprinted, with permission, from Satijn and Otte 1999, © American Society for Microbiology.)

mammalian *Ezh2* and *Eed* genes encoding PRC2 subunits (Bracken et al. 2003), illustrating conserved regulatory networks between plants and animals.

4.3 Maintaining Stem Cell Fate

PcG regulation plays a very early role during oogenesis in the mouse for the formation of totipotent cell identities in the progeny (Posfai et al. 2012). Genetic ablation of the PRC1 components RING1 and RNF2 results in loss of chromatin-bound PRC1 in oocytes, induction of massive transcriptional misregulation during oocyte growth, and a developmental arrest at the two-cell stage of embryogenesis. These results indicate that PRC1 functions during oogenesis to specify maternal contributions in the cytoplasm as well as on maternal chromosomes, both of which contribute to the developmental competence of preimplantation embryos. Indeed, cultured mouse embryonic stem cells were used very effectively to study the role of PcG proteins in many aspects of cell proliferation and differentiation.

The introduction of new technologies like ChIP-Seq allowed the correlation of PRC1/PRC2 components with many epigenetic marks, genetic regulatory elements of the murine genome, and the identification of functions related to ES cell pluripotency and plasticity during embryonic development (Boyer et al. 2006). Bivalent chromatin domains, characterized by the coexistence of the active H3K4me3 and repressive H3K27me3 histone marks, are resolved during differentiation (Mikkelsen et al. 2007). The cellular memory system takes a leading role in this process. Permanently repressed genes are tagged by the PcG system, together with DNA methylation, to establish stable silencing marks. Conversely, the TrxG system reiterates the H3K4me3 mark to keep the corresponding differentiation genes active.

Stem cells play an ever-increasing role in medicine. Their potential to provide progenitors for the healing of damaged tissue places them into a well treasured tool box of regenerative medicine. Not surprisingly, it is in the very well characterized blood cell lineage wherein we know most about the identity and location of stem cells. Hematopoietic stem cells (HSCs) maintain the pool of blood cells by self-renewing as well as producing daughter cells that differentiate into the lymphoid, myeloid, and erythroid lineages. The stem cell niche in the adult bone marrow provides the cells with specific external signals to maintain their fate. On the other hand, cell intrinsic cues for the maintenance of the “stemness” state seem to rely on the PcG system.

Mouse mutants affecting PRC1 genes (e.g., *bmi1*, *mel18*, *mph1/rae28*, and *m33*; see Table 1) suffer from various defects in the hematopoietic system, such as hyperplasia in spleen and thymus, reduction in B and T cells, and an impaired proliferative response of lymphoid precursors to cytokines. The requirements for *Bmi1* and *Mel18* in stem cell self-renewal during different stages of development suggest a changing pool of target genes between embryonic and adult stem cells.

The PcG system is also required for neural stem cells (NSCs) as indicated by the neuronal defects observed in *bmi1* mouse mutants (Bruggeman et al. 2005; Zencak et al. 2005). In particular, these mice are depleted of cerebral NSCs postnatally, indicating an *in vivo* requirement for *Bmi1* in NSC renewal. It appears that embryonic NSC maintenance is thus under a different PcG network control than adult NSC self-renewal, similar to the regulation of the hematopoietic system.

External signals like the *Sonic hedgehog* (*Shh*) signaling cascade modulate the *Bmi1* response in NSCs and ensure a proliferative/self-renewal capacity (Leung et al. 2004). The identification of these external cues controlling PcG repression came through the analysis of the development of cerebellar granule neuron progenitors (CGNPs). A postnatal

wave of proliferation is induced by the signaling factor Shh, secreted by the Purkinje cells. The *Shh* signal branches to control N-Myc and Bmi1 levels (Fig. 10). Thus, *Bmi1*-deficient CGNPs have a defective proliferative response upon Shh stimulation. The *Shh* signal is able to control proliferation of these stem cells ultimately by modulating both the downstream Rb pathway (via N-myc and Bmi1/p16^{INK4a}) and p53 pathway (via Bmi1/p19^{ARF}). This mechanism explains why hyperactivation of *Shh* signaling leads to the development of medulloblastomas. HSCs are regulated by a similar *Indian hedgehog*-controlled pathway. And, in NSCs, expression of the *Hoxd8*, *Hoxd9*, and *Hoxc9* loci is under the control of Bmi1. The appropriate *HOX* expression profile confers the necessary stem cell fate.

Indeed, as stem cells represent a defined and committed cellular state, it is not surprising that the PcG system maintains this particular fate in a mitotically heritable fashion. In the future, it will be interesting to identify the pool of targets of the PcG system in the different adult stem cell populations, and to learn how to influence the mainte-

nance system to allow for the controlled reprogramming of stem cell fates. At the moment, little is known about the role of PcG genes in stem cell maintenance in plants. However, the reprogramming of plant cells, which are totipotent and have the potential to form a complete new organism under appropriate conditions, involves PcG regulation. Indeed, plants lacking the *E(z)* homologs *CLF* and *SWN* produce a mass of undifferentiated cells after germination, suggesting that PcG genes are required to maintain a differentiated state (Chanvivattana et al. 2004). Interestingly, the same PcG genes are required for the in vitro reprogramming of differentiated leaf cells into totipotent callus cells, possibly because PRC2 is required to repress leaf differentiation genes during this reprogramming process (He et al. 2012). In floral meristems, PRC2 plays a role to repress *WUS* (see Sec. 3.1), which itself is required for stem cell maintenance (Liu et al. 2011). Thus, although the molecular mechanisms differ greatly between animals and plants, PRC2 has been recruited to regulate cell stem identity and cell differentiation in both lineages.

5 CONCLUSION AND OUTLOOK

It has been remarkable to follow the development of our understanding of PcG epigenetic regulation, starting with the initial genetic identification of a *Drosophila* mutant possessing additional sex combs on the second and third leg. This eventually led to the discovery of a new class of regulators found to be required for fundamental epigenetic processes such as vernalization in plants and silencing of the mammalian X chromosome. Control of genetic information is highly influenced by chromatin structure and composition of histones in their various modified forms. The proteins of the PcG are directly involved in generating epigenetic marks, for instance, H3K27me3 and H2AK118/119ub1, as a consequence of developmental decisions. The same group “reads” (i.e., shows high affinity to) these epigenetic marks through the action of the PRC1 proteins, and translates them into a stable, transcriptionally repressed state. In the model organism *Drosophila*, we have a relatively clear picture of how PcG complexes are anchored at PREs, for a defined group of target genes that are subject to long-term repression. However, to date very few PREs have been identified in other organisms. Although the basic functions of PcG proteins remain the same, it is not well understood how they are targeted to their site of action. Additionally, we need to get a better understanding of how an apparently dynamic group of proteins can impose a stable state of transcriptional repression through a chemical equilibrium.

The other major question in PcG research focuses on the heritability of the repressed state, the very essence of epigenetics. What is the identity of the molecular marks

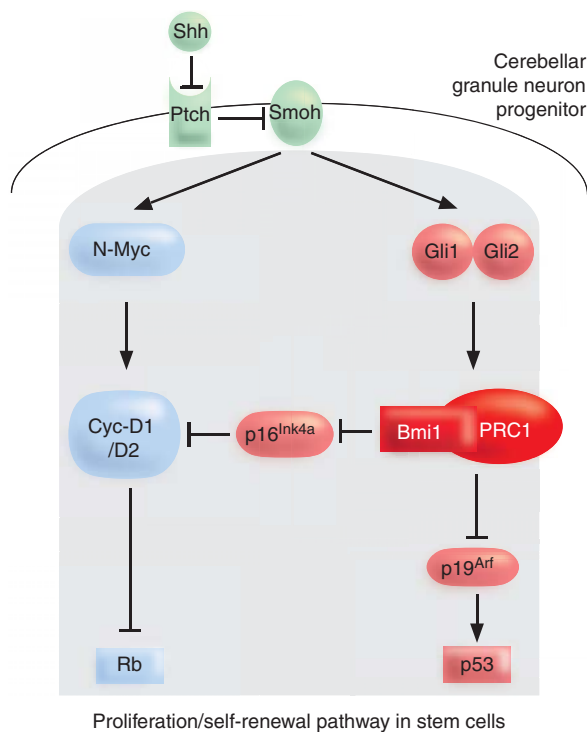


Figure 10. Sonic Hedgehog signaling maintains proliferation/self-renewal of cerebellar progenitor cells. The Shh signaling cascade regulates both the Rb pathway (which can be bound by the PRC2 RbAp48 protein) as well as the p53 pathway via Bmi1 control of the p16/p19 proliferation checkpoint. Inhibition of Smoothed (Smoh) by the Shh receptor Patched (Ptch) results in downstream signaling in the nucleus. One part of the signal induces N-Myc, Cyclin D1, and Cyclin D2, whereas the other part activates Bmi1 via the Gli effectors. (Adapted, with permission, from Valk-Lingbeek et al. 2004, © Elsevier.)

required to transmit a state of gene expression through DNA replication and mitosis? Do both active and repressed states need corresponding epigenetic marks, which are transmitted to daughter cells, or is only one sufficient, whereas the other represents the default state? The mechanism by which PcG proteins impose silencing on transcription during the interphase of the cell cycle has become increasingly clear. In the future, the focus of research will be on how the information regarding a state of gene expression endures the DNA replication process and is faithfully transmitted to the daughter cells following mitosis.

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