Polycomb Group Proteins Set the Stage for Early Lineage Commitment

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Precise control of gene expression patterns is critical for the specification of cellular diversity during metazoan development. Polycomb group (PcG) proteins comprise a class of transcriptional modifiers that have dynamic and essential roles in regulating a number of key processes including lineage commitment. How this is accomplished during mammalian development is incompletely understood. Here, we discuss recent studies in embryonic stem cells (ESCs) that provide critical new insights into how PcG proteins may be targeted to genomic sites as well as the mechanisms by which these regulators influence gene expression and multilineage differentiation in mammals.

Introduction

First discovered in *Drosophila*, Polycomb group (PcG) proteins were found to be integral to the regulation of Hox genes and normal development (Duncan, 1982; Jürgens, 1985; Lewis, 1978). PcG proteins are now widely recognized in all metazoans for their roles in a broad range of biological processes including cell cycle control, genomic imprinting, X-inactivation, cell fate transitions, tissue homeostasis, and tumorigenesis (Bracken and Helin, 2009; Gieni and Hendzel, 2009; Sparmann and van Lohuizen, 2006). More recently, PcG proteins have garnered much attention as modulators of stem cell differentiation in mammals (Bracken and Helin, 2009; Pietersen and van Lohuizen, 2008; Schuettengruber and Cavalli, 2009). Thus, improved knowledge of how PcG proteins function has practical applications for understanding development in mammals and for designing new methods to control the differentiation of stem cells for patient-specific therapies.

PcG proteins comprise a class of transcriptional repressors that are found in multi-subunit complexes termed Polycomb repressive complexes (PRCs). Most PcG proteins are broadly associated with either PRC1 or PRC2. Their core components are conserved between *Drosophila* and mammals; however, the composition of these complexes can vary among different cell types and organisms (Kerppola, 2009; Levine et al., 2004; Schuettengruber et al., 2007). Although PRCs are functionally distinct, both PRC1 and PRC2 modify chromatin structure by covalent modification of histone proteins (Müller and Verrijzer, 2009; Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009). PRC2 (e.g., EZH2, SUZ12, and EED) catalyzes di- and trimethylation of lysine 27 on histone H3 (H3K27me2/3, a modification associated with transcriptional repression) (Cao et al., 2002; Cao and Zhang, 2004; Czermin et al., 2002; Kirmizis et al., 2004; Kuzmichev et al., 2002), while PRC1 (e.g., BMI1, RING1A, RING1B, CBX, and PHC) mono-ubiquitylates histone H2A on lysine 119 (H2AK119Ub1) (de Napoles et al., 2004; Wang et al., 2004a). Biochemical and genetic studies also support the idea that PcG-mediated repression requires both catalytic and noncatalytic activities. How the posttranslational histone modifications, termed chromatin marks, catalyzed by

these complexes contribute to gene regulation or how other associated factors may influence PRC activities remains to be fully elucidated.

Loss of PRC2 activity results in embryonic lethality in mice (Faust et al., 1995,1998; O'Carroll et al., 2001; Pasini et al., 2004), whereas inactivation of PRC1 generally results in less severe phenotypes that manifest later in development (Akasaka et al., 2001; Coré et al., 1997; del Mar Lorente et al., 2000; Endoh et al., 2008; Takihara et al., 1997; van der Lugt et al., 1996) (Table 1). These phenotypic differences, however, probably reflect the high degree of overlap in the function of PcG homologs and not their degree of importance during development (Kerppola, 2009; Leeb et al., 2010). Consistent with this, the PRC1 component RING1B, an E3 ubiquitin ligase, is essential for gastrulation during mouse development (Voncken et al., 2003). Thus, the precise mechanisms by which PcG proteins function in vivo have been difficult to dissect given their deleterious phenotypes.

Recent work demonstrates that the core features of how cells regulate cell fate decisions in mammals can be discovered in embryonic stem cells (ESCs) (Boyer et al., 2006a; Jaenisch and Young, 2008; Loh et al., 2008; Macarthur et al., 2009; Orkin et al., 2008). ESCs, which are derived from a transient population of pluripotent cells in the blastocyst, can self-renew while maintaining the capacity to differentiate in vivo and in vitro into any cell type in the body (Keller, 2005; Murry and Keller, 2008). Thus, ESCs are an important model system to elucidate the mechanisms that govern cell fate transitions during mammalian development. PcG proteins are required for the maintenance of multipotent and progenitor stem cell populations, in part, by regulating genes important for cell cycle control and cell proliferation such as *p16(INK4a)* and *p19ARF* (reviewed by Sauvageau and Sauvageau, 2010, in this issue of *Cell Stem Cell*). Interestingly, PcG proteins are not necessary for the maintenance of self-renewal in ESCs, perhaps reflecting their unique cell cycle properties including a shortened G1 and an extended S phase (White and Dalton, 2005) or their different developmental stage. Rather, PcG proteins are thought to prepare ESCs for lineage commitment by temporal control of

Table 1. Loss of Function Phenotypes of Selected Polycomb Group Proteins

Subunit	Phenotype In Vitro (ESC)	Phenotype In Vivo	Key References
PRC2			
Suz12	De-repression of target genes; global loss of H3K27me3 and -me2, decrease in Ezh2 protein levels. Embryoid bodies lack proper structure.	Lethal at early postimplantation stage. Die ~E7.5–E8.5	Pasini et al., 2004, 2007; Jung et al., 2010
Eed	Target genes are de-repressed; genome-wide decrease in H3K27me1, H3K27me2, and H3K27me3. Decrease in Ezh2 protein levels. Eed null ESCs fail to differentiate properly in vitro, but can contribute to chimeras.	Disrupted axial patterning; Fail to properly gastrulate and to produce embryonic mesoderm. Die ~E8.5.	Faust et al., 1995; Montgomery et al., 2005; Chamberlain et al., 2008; Shumacher et al., 1996; Leeb et al., 2010
Ezh2	Fail to abolish H3K27me1 and H3K27me3 at some genes. Null ESCs fail to undergo mesoendoderm differentiation, but phenotype is less severe than Eed null, because of a partial redundancy with Ezh1, particularly at developmental genes.	Lethal at early postimplantation stage. Die ~E7.5–E8.5.	Shen et al., 2008; O'Carroll et al., 2001
Jarid2	Global H3K27 methylation unaffected; fail to properly differentiate.	Incompletely penetrant; neural, cardiac, liver, and hematopoietic defects. Die ~E11.5-15.5, depending on strain background.	Jung et al., 2005; Motoyama et al., 1997; Takeuchi et al., 1995; Shen et al., 2009;
Pcl2/Mtf2	Upregulated pluripotency regulators; fail to properly differentiate.	Mice are viable, but have growth defects. Incompletely penetrant; posterior homeotic transformation.	Walker et al., 2010; Wang et al., 2007
PRC1			
Ring1B	ESC lines are unstable and display a high propensity toward differentiation; de-repression of target genes; embryoid body formation is abnormal. Some ESC lines can differentiate into all three germ layers.	Developmental arrest in early gastrulation similar to PRC2 components. Die ~E10.5.	Voncken et al., 2003; van der Stoop et al., 2008; Leeb and Wutz, 2007; Leeb et al., 2010
Ring1A	Ring1A/Ring1B double knockout ESCs lose ESC identity and fail to self-renew after several passages.	Mice are viable. Anterior transformation and axial skeletal patterning abnormalities in both heterozygote and homozygote mutants.	del Mar Lorente et al., 2000; Endoh et al., 2008
Bmi1		Mice are viable, but with posterior homeotic transformation and severe immunodeficiency.	van der Lugt et al., 1996; Akasaka et al., 2001
M33 (Cbx family)		Mice are viable, but have growth defects; homeotic transformation; and increased sensitivity to retinoic acid during development.	Coré et al., 1997
Rae28 (Phc family)		Some null mice are viable. More severe phenotypes include perinatal lethality, posterior skeletal transformations, and neural crest defects.	Takihara et al., 1997

the expression of a key set of developmental genes (Pietersen and van Lohuizen, 2008), but we currently have an incomplete understanding of this process.

In this review, we highlight recent findings in ESCs that reveal important new insights into the regulation of early lineage commitment in mammals by PRC1 and PRC2. In particular, we focus our discussion on the mechanisms by which these complexes are targeted to genomic sites and how they function to modify chromatin to ensure that developmental gene expression patterns are faithfully executed during ESC differentiation.

PcG Proteins Silence a Key Set of Developmental Genes in ESCs

PcG proteins co-occupy and regulate the expression of a large cohort of developmental and signaling genes in ESCs, such as the Hox gene clusters as well as members of the *Dlx*, *Fox*, *Irx*, *Lhx*, *Pou*, *Pax*, *Sox*, *Tbx*, and *Wnt* gene families (Boyer et al., 2006b; Ku et al., 2008; Lee et al., 2006). This suggested that PcG proteins function in ESCs to prevent differentiation by repressing these genes. However, ESCs can self-renew and maintain the expression of key pluripotency genes in the
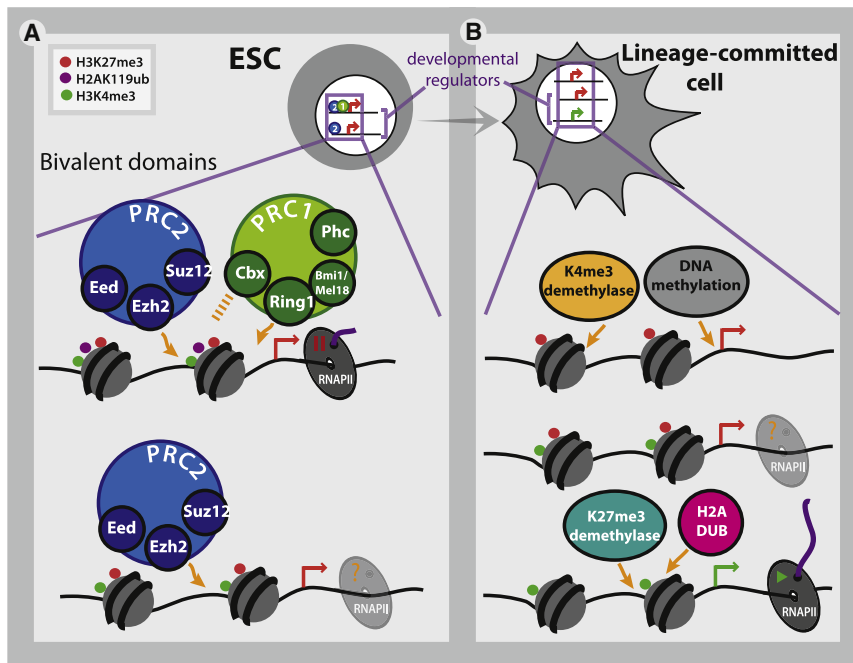
Figure 1. PcG Target Genes Have a Bivalent Chromatin Conformation in ESCs

(A) Bivalent domains are enriched with H3K4me3 and H3K27me3, modifications associated with TrxG and PcG activities, respectively. In mammals, PRC2 consists of SUZ12, EED, and the histone methyltransferase EZH2, which catalyzes the di/trimethylation of lysine 27 on histone 3 (H3K27me2/3). PRC1 subunits comprise CBX, PHC, BMI1/MEL18, and RING1A/B. RING1B monoubiquitylates lysine 119 on H2A (H2AK119ub). In ESCs, PRC1 and PRC2 colocalize at some promoters, whereas only PRC2 is targeted to others. Although genes are repressed in both cases, those with PRC1/H2AK119ub may harbor paused RNA polymerase II and experience transcription initiation but no productive elongation.

(B) Upon lineage commitment, many bivalent domains are resolved depending on the expression state of the gene. To stabilize the repressed state of a particular gene, DNA methyltransferases can methylate CpG sites to silence genes. The histone demethylases JMJD3 and UTX and possibly histone H2A deubiquitylases (H2A DUB) may allow for activation of PcG target genes by facilitating removal of the repressive H3K27me3 and H2AK119Ub1 marks during differentiation. Some genes remain in a poised, bivalent state until further lineage decisions are made.

absence of PcG proteins (Chamberlain et al., 2008; Pasini et al., 2007; Shen et al., 2008; van der Stoop et al., 2008). Rather, PRC2-deficient ESCs as well as those lacking RING1B fail to properly maintain the expression of lineage-specific genes (Boyer et al., 2006b; Chamberlain et al., 2008; Leeb and Wutz, 2007; Leeb et al., 2010; Pasini et al., 2007). These data led to the idea that PcG proteins are necessary for cell fate transitions and that PcG-mediated repression must be dynamic because many of the target genes in ESCs maintain the potential to become either activated or silenced during differentiation.

PRC2-Mediated H3K27me3 Is Enriched at Bivalent Chromatin Domains in ESCs

H3K27me3 is broadly associated with facultative heterochromatin and the repression of developmental programs in metazoans. An important open question then is how PcG-repressed genes in ESCs maintain the potential for gene activation. A remarkable finding in both mouse and human ESCs is the coenrichment of both activating and repressive chromatin modifications at PcG target genes (Azuara et al., 2006; Bernstein et al., 2006; Pan et al., 2007; Zhao et al., 2007). These "bivalent domains" consist of peaks of H3K4me3 enrichment that overlap with broader domains of H3K27me3 modifications (Bernstein et al., 2006; Mikkelsen et al., 2007) (Figure 1). This is consistent with the idea that control of developmental gene expression patterns is highly coordinated by the concerted activities of Trithorax group (TrxG) proteins and PcGs. Despite the overwhelming evidence that H3K4me3, a modification catalyzed by TrxG proteins, is associated with transcriptional initiation, bivalent genes display low expression levels. Bivalent domains are also found in other cell types albeit less frequently (Mikkelsen et al., 2007); however, in ESCs unlike in lineage-committed cells, most H3K27me3 is associated with H3K4me3. Importantly, recent studies in Zebrafish also found coenrichment of both

H3K4me3 and H3K27me3 at a subset of genes in early embryos (Vastenhouw et al., 2010) providing strong evidence that bivalent domains are not simply cell culture artifacts.

Bivalent domains are generally resolved during differentiation into either H3K27me3 or H3K4me3 regions depending on the expression state of the gene in a particular cell type. The binding of PcG proteins in ESCs may facilitate repression at a particular set of genes during differentiation by recruitment of more stable silencing mechanisms such as DNA methylation (Schuettengruber et al., 2007; Simon and Kingston, 2009). Indeed, promoters associated with H3K27me3 in ESCs are more likely to become DNA methylated during differentiation (Meissner et al., 2008; Mohn et al., 2008). The resolution of bivalent domains is also probably facilitated by a class of histone demethylases that selectively remove H3K4me3, consistent with their essential roles in development and differentiation (Cloos et al., 2008; Lan et al., 2008). Conversely, loss of PRCs or H3K27me3 may facilitate activation of genes necessary for lineage commitment. Two histone demethylases, JMJD3 and UTX, have recently been identified as H3K27me2/me3 demethylases (Agger et al., 2007; Hong et al., 2007; Lan et al., 2007; Lee et al., 2007; Xiang et al., 2007), making them likely candidates for counteracting Polycomb-mediated gene silencing during activation of lineage-specific genes. Jmjd3 and Utx are necessary for proper development and differentiation in a variety of systems including mammals and are targeted to developmental regulators such as *Hox* genes during ESC differentiation (Swigut and Wysocka, 2007). Moreover, inactivating mutations in *Utx* have been found in multiple tumor types (van Haaften et al., 2009), suggesting that disrupting the balance in H3K27 methylation patterns can lead to changes in cell state. Thus, demethylation of H3K27me3 may be one way to disrupt Polycomb-mediated gene repression, although there are probably other mechanisms that work in concert such as those mediated by

signaling pathways in response to developmental cues (Cole and Young, 2008).

It has been proposed that PRC2 establishes the silent state and that maintenance of gene repression occurs through recruitment of PRC1 via a direct interaction of the chromodomain of Polycomb (Pc)/CBX family members with H3K27me3 (Fischle et al., 2003; Min et al., 2003; Wang et al., 2004b). However, existing evidence does not support the exclusivity of this appealing model. Rather, the mechanisms utilized to target these complexes to specific sites are probably diverse and may result in distinct outcomes (discussed in Simon and Kingston, 2009). Consistent with this, recent genome-wide studies showed that although PRC1 and PRC2 colocalize at many important developmental genes in ESCs, these complexes could also occupy distinct genomic sites and act independently to repress genes (Boyer et al., 2006b; Ku et al., 2008; Leeb et al., 2010; Schoeftner et al., 2006). Moreover, these studies revealed two classes of bivalent domains: co-occupancy of both PRC1 and PRC2 or PRC2 alone (Ku et al., 2008) (Figure 1). Bivalent domains occupied by both complexes also harbor H2AK119Ub1 and are more likely to maintain H3K27me3 levels upon differentiation and are functionally distinct from the PRC2-only regions. Thus, it is important to understand how each of these complexes is recruited to target sites and how each influences chromatin dynamics and gene expression states during ESC differentiation.

PRC1-Mediated Histone Mono-ubiquitylation Contributes to Gene Repression

In ESCs most bivalent promoters and hence PcG target genes are thought to harbor a paused RNA polymerase II enzyme (RNAPII). These genes experience transcription initiation yet do not show evidence of elongation (Guenther et al., 2007), consistent with the earlier finding that PcG proteins do not prevent the binding of RNAPII to promoters in *Drosophila* (Dellino et al., 2004). Interestingly, PRC1-mediated H2AK119Ub1 may contribute to this paused state (Stock et al., 2007) (Figure 1). PRC1 appears to be responsible for the majority of H2AK119Ub1 in ESCs (Kallin et al., 2009). Deletion of *Ring1b*, an E3 ubiquitin ligase associated with PRC1, leads to loss of this modification, release of the paused polymerase, and gene de-repression (Stock et al., 2007). However, it should be noted that loss of Ring1b alters PRC1 integrity so it is possible that this observation is not a direct consequence of loss of H2AK119Ub1 in ESCs. Moreover, not all target genes become de-repressed upon loss of Ring1b activity (van der Stoop et al., 2008), indicating the existence of other mechanisms that maintain repression of these genes. Whether all bivalent genes harbor paused polymerases and whether this is tightly linked to histone ubiquitylation remain open questions.

These studies predict that specific histone deubiquitylases (DUBs) have roles in counteracting PcG-mediated repression during differentiation. In support of this, a distinct Polycomb repressive complex (PR-DUB), comprising additional factors such as Additional sex combs (Asx), has been shown to possess histone H2A deubiquitylase activity and to regulate Hox gene silencing in *Drosophila* (Scheuermann et al., 2010). Prior studies showed that Asx is necessary to maintain both homeotic gene activation and silencing (Fisher et al., 2006), indicating that addi-

tional studies are needed to fully understand its role in PcG-mediated gene regulation. Although Asx homologs exist in mammals (Fisher et al., 2006), a similar complex has yet to be identified. Nonetheless, there are several factors with known histone H2A DUB activity in vertebrates including Ubp-M (USP16), which is required for cell cycle progression in HeLa cells as well as *Hox* gene activation and posterior development in *Xenopus laevis* (Joo et al., 2007; Weake and Workman, 2008). Thus, the identification of H2A DUBs with specific roles in counteracting PcG silencing during ESC differentiation is expected to reveal another layer of regulation important for maintaining the balance between self-renewal and lineage commitment. Ultimately, identifying the downstream effectors of both PRC2-mediated H3K27me3 and PRC1-mediated H2AK119Ub1 as well as elucidating how these modifications crosstalk in ESCs will be necessary to fully understand how PcG proteins function in stem cell differentiation.

Chromatin Compaction and Higher-Order Chromatin Organization Do Not Require PRC-Enzymatic Activity

ESC differentiation is characterized by a dramatic reorganization of chromatin structure leading to a marked increase in heterochromatin formation and gene silencing (Mattout and Meshorer, 2010; Meshorer et al., 2006). Furthermore, the formation of extended PcG-mediated H3K27me3 domains, a mark of facultative heterochromatin, has also been observed during ESC differentiation (Hawkins et al., 2010). PcG proteins have long been thought to contribute to gene silencing by chromatin compaction; however, direct evidence in favor of this model was lacking. Indeed, recent work indicates the physical association of PRCs at genomic sites might contribute to gene repression by higher-order chromatin organization. For example, reconstituted PRC1 restricts chromatin-remodeling activity and can physically compact chromatin in vitro with unmodified nucleosome templates or those that lack histone tails (Francis et al., 2004; Francis et al., 2001). Although these studies argued that PRCs participate in the formation of higher-order chromatin states in the absence of histone modifications in vitro, whether this phenomenon could be observed in vivo remained an open question.

Recent studies investigating the large-scale remodeling of chromatin at *Hox* loci in ESCs now provide in vivo support for the role of PcG proteins in this process. *Hox* gene clusters serve as a paradigm for studying the role of PcG proteins in chromatin organization and gene regulation because they are classical PcG targets and because they are temporally activated in a collinear fashion during development (Kmita and Duboule, 2003; Mallo et al., 2010). Furthermore, extensive domains of PcG proteins as well as of H3K27me3 have been observed across all *Hox* clusters in ESCs (Boyer et al., 2006b; Ku et al., 2008; Lee et al., 2006; Mikkelsen et al., 2007) and are lost upon induction of expression during differentiation in vivo (Soshnikova and Duboule, 2009). Prior observations noted a distinct nuclear reorganization of the *HoxB* cluster during ESC differentiation (Chambeyron and Bickmore, 2004; Morey et al., 2007). Using DNA FISH, this same group now provides striking evidence that PcG proteins can mediate chromatin compaction at the *HoxA* and *HoxD* clusters tested in ESCs (Eskeland et al., 2010). Both PRC1 and PRC2 were required to
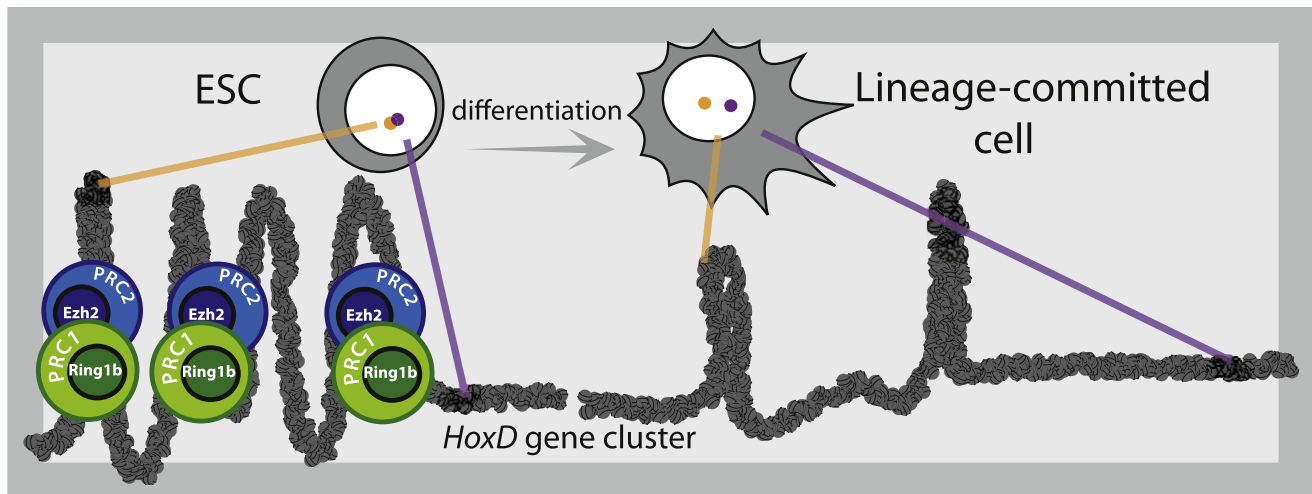
Figure 2. Polycomb Group Proteins Mediate Higher-Order Chromatin Structures
PcG proteins mediate chromatin compaction at both the *HoxB* and the *HoxD* gene clusters. In ESCs, the chromatin at these clusters appears compact, and de-compaction and gene activation are observed upon differentiation. Notably, although PRC1 is necessary for chromatin compaction, this function does not require the ubiquitin ligase activity of RING1B.

maintain compact chromatin (Figure 2). Remarkably, the E3 ubiquitin ligase activity of RING1B was dispensable for this organization. Moreover, an observable increase in the distance between DNA FISH probes temporally preceded *Hox* gene activation during ESC differentiation. Together, these data point to important noncatalytic roles for PRC1 in regulating higher-order chromatin organization and *Hox* gene expression during early development. Although the precise organization and function of this unique chromatin region is still under investigation, the role of H2AK119Ub1 is not clear in this context, given that this modification appears dispensable for chromatin compaction and gene repression.

EZH2, a core PRC2 component, has also been implicated in the formation of chromatin interactions. Recent studies mapped long-range chromatin interactions at the PcG target gene *Gata4* in embryonic carcinoma (EC) cells with a high-resolution chromatin conformation capture assay (Tiwari et al., 2008a; Tiwari et al., 2008b). These interactions were only partially lost upon knockdown of *Ezh2* and completely lost when EC cells were induced to differentiate. However, it was unclear whether the reorganization of chromatin structure during differentiation causes the observed changes in gene expression. Given the studies by Eskeland et al., it is possible that the changes in chromatin structure observed upon EZH2 depletion may be due to a concomitant loss of PRC1 at this gene. Nonetheless, these data suggest that PcG proteins may also repress gene expression in ESCs by mediating long-range chromatin interactions or DNA looping (Mateos-Langerak and Cavalli, 2008) and that this chromatin conformation may occlude access of activating factors to the DNA template. It remains to be determined whether the mechanisms that mediate chromatin compaction and long-range interactions are similar or functionally distinct.

Recruitment of Polycomb Complexes at Genomic Targets in ESCs
The precise localization of PRC1 and PRC2 within the genome is necessary to facilitate the specific changes in chromatin and

gene expression states that accompany lineage commitment. Because PcG proteins have no known DNA binding activity, they may require other factors for proper recruitment to target sites. Although considerable prior knowledge exists in *Drosophila*, how PcG proteins recognize specific regions within the billions of base pairs of DNA in mammals is still poorly understood. Below, we present recent work that suggests roles for DNA binding elements, transcription factors, accessory factors, and noncoding RNA in this process. These seemingly disparate pathways probably collaborate to regulate the targeting of Polycomb complexes and transcriptional fine-tuning of developmental programs in ESCs.

A Potential PRE Targets Polycomb Complexes to the HoxD Locus in ESCs
In *Drosophila*, Polycomb complexes bind to specific sites in the genome called Polycomb response elements (PREs) (Müller and Kassis, 2006; Ringrose and Paro, 2007; Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009). PREs contain clusters of DNA binding sites for several transcription factors, whose binding can mediate the recruitment of Polycomb complexes. Despite the wealth of available sequence data generated from high-throughput analyses of PcG binding sites in mammals, an analogous system has yet to emerge. Thus, evidence for transcription factor-mediated recruitment of PcG proteins is lacking in mammals.

Recently, two studies have each identified a cell-type-specific putative mammalian PRE (Sing et al., 2009; Woo et al., 2010). The first potential mammalian PRE identified was an element regulating the expression of a hindbrain segmentation gene, *MafB/Kreisler*, which can recruit both PRC1 and, less strongly, PRC2 to induce silencing of an ectopically introduced transgene in both flies and mice (Sing et al., 2009). Importantly, Sing et al. also provided genetic evidence for in vivo PRE function in the mammalian hindbrain, suggesting this PRE is not an artifact of an in vitro assay. PRCs occupy the entire *HoxD* locus in ESCs, but it was not known how PcG proteins were targeted to this
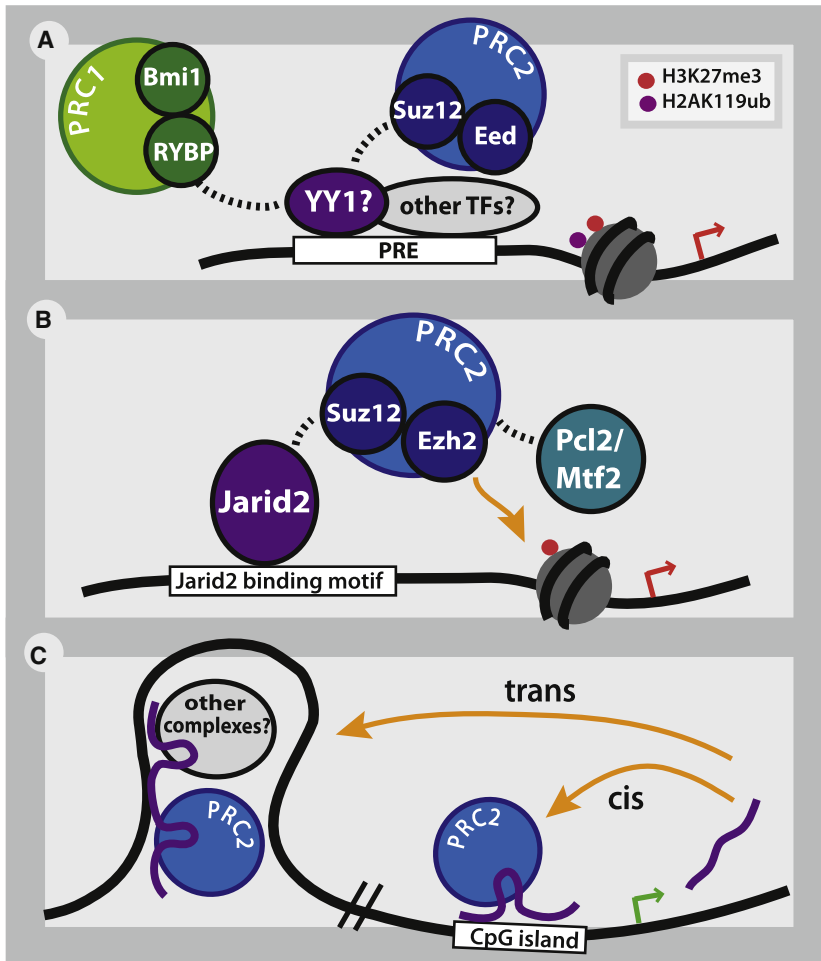
Figure 3. Potential Mechanisms of Polycomb Recruitment

(A) A potential Polycomb response element (PRE) may recruit PcG complexes to the *HoxD* locus in human ESCs. This element harbors several YY1 transcription factor binding sites and repression by this element of a reporter gene was modestly dependent on PRC2 and PRC1, intact YY1 binding sites and the YY1-binding protein RYBP.

(B) JARID2 copurifies with PRC2 components and occupies a similar set of target genes in ESCs. PCL2 also associates with PRC2 in ESCs in a biochemically separate complex. Although PCL2 may potentiate the histone methyltransferase activity of EZH2, the effect of JARID2 is less clear.

(C) PRC2 may interact with noncoding RNAs to regulate gene expression *in cis* or *in trans*, potentially by the noncoding RNAs acting as scaffolds that mediate interactions with chromatin modifying complexes, including PRC2.

region (Boyer et al., 2006b; Lee et al., 2006). A 1.8 kbp element between *HOXD11* and *HOXD12* (D11.12) was identified as a potential PRE with a remarkable approach that analyzed nucleosome position, nuclease sensitivity, and H3K27me3 modification profiles across this locus during hESC differentiation (Woo et al., 2010). The D11.12 element displayed characteristics similar to *Drosophila* PREs in that it was able to repress the expression of an ectopic reporter gene. Moreover, this activity was moderately dependent on BMI1, a core component of PRC1, and to a lesser extent SUZ12, a PRC2 component. RYBP, a known Yin yang 1 (YY1) binding protein that interacts with PRC1, also appears to modestly influence the targeting of PRC1 to the D11.12 region (Figure 3A).

Further analysis revealed several YY1 consensus-binding sites within the *HOXD* PRE, as well as in the mouse hindbrain *MafB/Kreisler* PRE. YY1 is the vertebrate homolog of PHO, a DNA binding factor that can recruit PcG proteins to *Drosophila* PREs (Brown et al., 1998; Wang et al., 2004b). Similar to PRC2 mutants, loss of *Yy1* in mice leads to embryonic lethality around the time of implantation (Donohoe et al., 1999). Although YY1 occupancy was coincident with PRC enrichment at the HOXD PRE region in ChIP assays, loss of YY1 binding sites had only a moderate effect on reporter repression. This suggests that YY1 is not deterministic for PRC recruitment to potential PREs

in mammals. Although promising, future studies will also need to address the consequences of deleting D11.12 on in vivo function and on the regulation of *Hox* gene expression at the endogenous locus during ESC differentiation.

Indeed, other studies argue against making simple conclusions about a targeting role for YY1. Although PHO is a component of PRC2 in *Drosophila*, evidence is lacking for a similar association between YY1 and PRC2 in mESCs (Li et al., 2010). For example, the binding sites for YY1 and PcG proteins do not directly overlap in murine embryonal cells (Squazzo et al., 2006). In fact, the vast majority of PRC2 binding sites in ESCs overlap with highly conserved CpG islands that are enriched for a variety of transcription factor binding sites (Ku et al., 2008; Tanay et al., 2007). Moreover, the collective enrichment of a set of transcription factor binding sites (e.g., MYC, E2F1, ZF5, TCFCP2L1, and CTCF) could also predict PcG occupancy with some success (Liu et al., 2010). Thus, it remains to be determined whether transcription factors play a predominant role in PRC recruitment during mammalian development. It is likely that PRC recruitment to specific genomic sites involves a considerably more complex set of interactions including cell-type-specific cofactors as well as noncoding RNA (see below). DNA sequence or structural features as well as the neighboring chromatin may also be relevant.

JARID2 and PCL2/MTF2 Associate with PRC2 in ESCs

Five groups have identified JARID2 as a previously unreported component of PRC2 in ESCs (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). JARID2 is the founding member of the Jumonji C (JMJC) domain protein family of histone demethylases that remove methyl groups from lysine residues. Thus, the association of JARID2 with PRC2 is predicted to be important for the balance between gene expression states. However, JARID2 lacks key residues for cofactor binding and so it is catalytically inactive (Cloos

et al., 2008). Notably, JARID2 levels are abundant in undifferentiated cells and decrease during differentiation (Mikkelsen et al., 2007; Walker et al., 2007; Zhou et al., 2007), suggesting that JARID2-PRC2 may be unique to ESCs. In further support, key pluripotency factors including OCT4, SOX2, and NANOG occupy the JARID2 promoter (Boyer et al., 2005; Kim et al., 2008), connecting its expression to the ESC transcriptional regulatory network. These data indicate that the JARID2-PRC2 complex represents a significant fraction of PRC2 in ESCs.

What is the function of JARID2 in PRC2? JARID2 can directly bind DNA through its C terminus (Li et al., 2010), suggesting that it directly recruits PRC2 to genomic sites (Figure 3B). Consistent with this, JARID2 co-occupied the same regions of the genome as PRC2. Moreover, PRC2 binding was diminished upon depletion of Jarid2 (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009) and JARID2 localization was dependent on PRC2 (Peng et al., 2009; Shen et al., 2009). PRC1 was also diminished upon JARID2 depletion (Landeira et al., 2010; Pasini et al., 2010), but this may be a consequence of loss of PRC2 at these regions. In addition to its recruitment role, JARID2 also regulates the histone methyltransferase activity of PRC2. Apparently, JARID2 can potentiate (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010) or attenuate (Peng et al., 2009; Shen et al., 2009) the catalytic activity of the complex. Regardless of the differences in the in vitro assay employed in each study, the reports generally agree that depletion of JARID2 reduces PRC2 recruitment to the target genes, albeit more substantially than H3K27me3 levels at those targets. These observations are also consistent with JARID2-independent mechanisms that maintain basal levels of PRC2 at genomic targets.

Whereas Jarid2-deficient ESCs displayed defects in their ability to differentiate in later stages of neural progenitor and embryoid body formation (Li et al., 2010; Pasini et al., 2010; Shen et al., 2009), Jarid2 null embryos proceeded further in development (Takeuchi et al., 1995) as compared to PRC2 mutants (see Table 1). Although this is consistent with the idea that JARID2 is not necessary to carry out all of PRC2 function, substantial evidence does support a role for JARID2-PRC2 in regulating gene expression in ESCs. Thus, the precise roles of JARID2 in ESCs remain to be elucidated.

Several recent studies, including three discussed above, reported the identification of a PCL2/MTF2 (Polycomb-like 2/ metal response element-binding transcription factor 2)-containing PRC2 complex in mESCs (Landeira et al., 2010; Li et al., 2010; Shen et al., 2009; Walker et al., 2010). PCL2 is one of three homologs of Drosophila Polycomb-like (dPcl), suggesting that it functions as a bona fide PcG protein. PCL2-PRC2 occupied a subset of PcG target genes in ESCs in a similar pattern as PRC2 (Li et al., 2010; Walker et al., 2010) and appeared to promote H3K27 trimethylation (Walker et al., 2010) (Figure 3B). On the basis of the gene expression effects observed upon PCL2 depletion in ESCs, the authors speculate that PCL2-PRC2 may function to regulate self-renewal to enable an appropriate response to differentiation cues. Notably, OCT4 and NANOG occupy the Pcl2 promoter in mESCs (Loh et al., 2006), and Pcl2 levels decrease upon differentiation (Walker et al., 2010; Walker et al., 2007). Thus, similar to Jarid2, Pcl2 gene expression may also be connected to the pluripotency

transcriptional regulatory network. Although this complex may also copurify with JARID2, current evidence supports the existence of biochemically distinct PRC2-like complexes (Landeira et al., 2010; Li et al., 2010; Shen et al., 2009). Interestingly, Pcl2 mutant mice display a phenotype more consistent with PRC1 knockouts, including posterior transformation of axial skeletons (Wang et al., 2007). It is possible that Pcl genes have critical roles for PRC2 function, but that this has yet to be appreciated because of the redundancy among these genes.

It is increasingly evident that the diversity of Polycomb complexes has functional consequences on gene expression as well as biological output. Moreover, PRC2 recruitment and the regulation of its enzymatic activity appear to be closely linked. Thus, PRC2 composition must be considered when investigating the effects of PRCs on gene regulation in pluripotent versus lineage-committed cells. These data also support the idea that multiple modes of recruitment are necessary to achieve the proper regulation of target genes (and PRCs) during cell fate transitions.

Noncoding RNA—Partners in Polycomb-Mediated Gene Regulation or Recruitment?

Recent work has highlighted possible roles for noncoding RNA (ncRNA) in the recruitment and regulation of Polycomb complexes (Koziol and Rinn, 2010; Morris, 2009; Ponting et al., 2009) (Figure 3C). In particular, long noncoding RNAs, defined by a length >200 nt and by the lack of protein coding capacity (Ponting et al., 2009), have recently garnered much of the spotlight. Interestingly, HOTAIR, a long noncoding RNA transcribed from the HOXC locus in human fibroblasts, interacts with SUZ12 and recruits PRC2 to the HOXD locus in trans (Rinn et al., 2007). Consistent with an important function, the misregulation of HOTAIR can promote cancer metastasis (Gupta et al., 2010). Other studies demonstrate that long noncoding RNAs that interact with PRC2, including Xist (Zhao et al., 2008) and Kcnq1ot1 (Pandey et al., 2008) function in cis to recruit PRCs to the inactive X chromosome or the Kcnq1 imprinted locus, respectively, in order to initiate changes in chromatin structure. Future studies will determine whether this phenomenon represents a more widespread mechanism for the recruitment of PRC2 to genomic sites in ESCs.

Nearly one-fifth of the ∼3300 large intergenic noncoding RNAs (lincRNAs), identified by a different group, associated with Polycomb complexes in various cell types including ESCs (Guttman et al., 2009; Khalil et al., 2009). Although many of these interactions require validation, the large number of interacting ncRNAs suggests that there are structural features shared among these lincRNAs. This idea is supported by a study showing that HOTAIR may act as a scaffold for interactions with other histone modifiers (Tsai et al., 2010). The role of ncRNAs in regulating PcG-mediated cell fate transitions is still emerging; however, it is interesting to speculate that many lincRNAs could be acting to mediate crosstalk between epigenetic regulators in a cell-type-specific fashion. Consequently, many long ncRNAs appear to be developmentally regulated, suggesting a function for this class of RNAs in mediating cell fate transitions (Dinger et al., 2008), but this intriguing possibility requires further study. Thus, defining the set of lincRNAs that interact with PcG complexes and their modes of action during

ESC differentiation will be a critical step toward understanding the role of long ncRNAs in mediating cell fate transitions.

In addition to long ncRNAs, new evidence suggests that CpG-rich sequences, which are highly enriched near Polycomb target genes (Ku et al., 2008; Mikkelsen et al., 2007), produce short transcripts ($\sim$50–200 nt) (Kanhere et al., 2010). Remarkably, these transcripts can interact with SUZ12 leading to recruitment of PRC2 and repression of the associated mRNA transcript in *cis*. Interestingly, this interaction depended on an intact double stem-loop structure within the RNA, suggesting that a conserved structure rather than a defined sequence mediates the function of this class of ncRNAs (Figure 3C). Consistent with a regulatory role, the authors found that an increase in mRNA production of PcG target genes corresponded with a decrease in the level of associated short RNAs. Although this study was largely performed in T cells, production of short RNAs near PcG target genes was also shown in ESCs, indicating that this could be a widespread mechanism. How the production of these short transcripts is regulated and how they may relate to CpG islands and DNA methylation status in ESCs still needs to be investigated, but this study highlights the complex interplay of mechanisms that may be instrumental for the proper localization and regulation of Polycomb in specific cell types.

Concluding Remarks

Current evidence indicates that PcG proteins set the stage in ESCs for the acquisition and maintenance of specific developmental gene expression programs during development. PRCs catalyze the posttranslational modification of histones to control developmental gene expression patterns. The physical presence of the PcG proteins themselves may also contribute to chromatin compaction, chromatin interactions, and gene silencing. Recent developments in the field indicate that PRCs work in conjunction with a range of other factors in a cell-type-specific manner. Such varied and diverse functions ascribed to PRCs are likely to be important for the transcriptional fine-tuning of the large number of target genes during lineage commitment.

Overwhelming evidence suggests that mis-regulation of PcG proteins, such as EZH2 and BMI1, is correlated with cancer progression. Given that transformed cells share many features with stem cells, functional analysis of PRC1 and PRC2 in ESCs may also contribute important new clues for understanding the progression from a normal to disease state and might also lead to the identification of new biomarkers or small molecule inhibitors. The potential discovery of mechanisms that govern the recruitment of PcG proteins to target sites in the genome may also facilitate efforts to direct the differentiation of stem cells in vitro and to control disease progression in vivo. Therefore, continued efforts to unravel the complexities of how PcG proteins function to control gene expression in pluripotent cells has practical significance for understanding development and for treating disease.

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