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Review

Interpretation of Developmental Signaling at Chromatin: The Polycomb Perspective

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The Polycomb group (PcG) system represses the transcription of important developmental regulators and perpetuates this repression across multiple cell divisions. Inputs from outside the cell can influence PcG function by recruiting additional chromatin factors to PcG-regulated loci or by downregulating the PcG genes themselves. These types of PcG system modulation allow context-dependent induction of genes during development, in cancer, and in response to changes in the environment. In this review, we outline instances where molecular players in this process have been recently identified, comparing and contrasting different ways in which derepression is achieved, and projecting directions for future research.

Introduction

As development unfolds in an embryo, body-patterning signals activate distinct cohorts of genes in various cells. This results in setting aside groups of cells that make different parts of the body (Gilbert, 2006). These patterning stimuli, generated by cell-cell interactions, reaction-diffusion kinetics of ligands, or prepatterns set in an oocyte, are transient, although the gene expression initiated by these stimuli has to last for much longer (Wolpert et al., 2002). How do cells translate the temporary information of pattern-generating signals to stable and persistent gene expression states? This question puzzled developmental biologists for a long time. The discovery of the Polycomb group (PcG) factors, required to restrict Hox genes to their normal realms of action in *Drosophila,* paved the way to unravel the process at the mechanistic level. The molecular logic by which PcG proteins heritably silence expression has become clear over the years owing to genetic, biochemical, and recent genomic analyses (Sauvageau and Sauvageau, 2010; Simon and Kingston, 2009; Surface et al., 2010). The repertoire of known processes controlled by PcG has in the meantime expanded substantially, and includes cell cycle regulation, X inactivation, cell differentiation, and genomic imprinting. Indeed, genome-wide studies in the last few years have further extended the scope of this system beyond the classical Hox paradigm—several components of signaling pathways, cell cycle control, and stress responses are silenced by PcG in appropriate cell types (Sauvageau and Sauvageau, 2010; Surface et al., 2010).

Research on the molecular basis of regulation by this system has centered around four key issues. (1) What mechanisms govern the selection of a specific set of genes in the genome that become targeted by PcG for silencing? (2) What is the molecular definition of ''silencing'' achieved by this system? (3) How does silencing persist through several rounds of cell division? (4) How is PcG-mediated repression counteracted in a tissue- and locus-specific manner during cell differentiation? The first three issues have received major attention in the last few years with the exciting discovery that noncoding RNAs may be playing a crucial role in these aspects (Bracken and Helin, 2009; Guenther and Young, 2010). Insight into derepression of PcG targets, however, is only beginning, and is the central theme of this review. We begin by describing the molecular players involved in the PcG and the antagonistically acting Trithorax group (TrxG) with a brief summary of recent work on recruitment of the silencing machinery and its maintenance. We then discuss specific instances where PcG system plays a critical role in cellular transitions. Finally we sketch molecular details of how developmental signaling counteracts silencing at specific loci, and how this system is perturbed under pathological conditions.

The Molecular Complexity of PcG-Controlled Silencing

One clue to the precise role of PcG proteins in maintaining the expression state of Hox genes was the finding that the chromo domain, located also in the founding member Polycomb (Pc), binds modified histones (Bannister et al., 2001). PcG proteins act in a number of complexes with a variety of proteins that are conserved across metazoa and plants (Figure 1; Grossniklaus and Paro, 2006). Two major complexes, PRC1 and PRC2, regulate histone modifications (Schuettengruber and Cavalli, 2009): trimethylation of histone H3 lysine 27 (H3K27) by PRC2 component E(z) and ubiquitination of lysine 119 of histone H2A (H2AK119) by PRC1 component dRing. Both these marks are indicative of PcG action and gene repression. On the other hand, trimethylation of histone H3 lysine 4 (H3K4) is a modification associated with gene expression and is laid down by TrxG proteins, which counteract silencing set by PcG proteins (Kingston and Tamkun, 2006). Developing *Drosophila* embryos of trxG mutants show normal Hox expression pattern initially, but lose it once the initiating gradients of patterning factors disappear. PcG phenotypes are partially rescued by trxG mutations, confirming the view that PcG/TrxG systems are involved in maintenance of tissue-specific Hox expression but not establishing it. One major consequence of PcG misregulation is inappropriate expression of developmental regulatory genes such as Hox genes. This results in homeotic transformation—a conversion of one body segment into another without overtly affecting the rest of the body. This is seen in PcG mutants in *Drosophila* (Denell, 1978; Sato

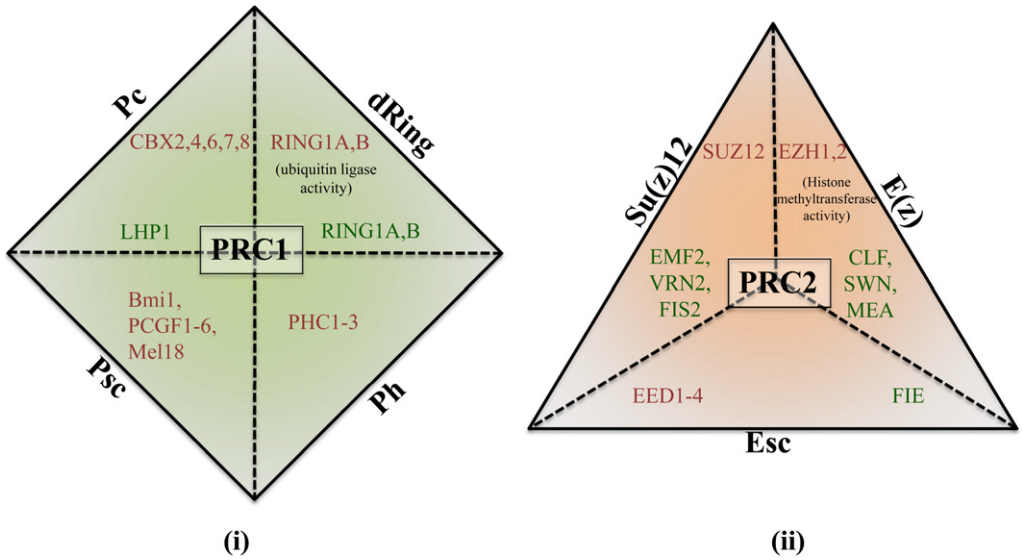

Figure 1. The Composition of Core Polycomb Repressive Complexes Primarily Based on Studies in *Drosophila*
Names at the edges of polygons are founding members from *Drosophila* polycomb repressive complex 1 (PRC1) (i) and PRC2 (ii). Names in red and green indicate mammalian and plant proteins, respectively, which are inferred from sequence or functional similarity. Note that plant PRC1 is not very well defined. The enzymatic activities associated with core components are indicated in brackets.

et al., 1983) and mammals (Surface et al., 2010; van der Lugt et al., 1994), emphasizing the conservation of function throughout metazoan development.

PcG Repression: Where and How?

The importance of selecting the right target genes to be silenced in a defined cell population cannot be overstated. Given that the core components of PRC1 and PRC2 do not bind specific DNA sequences themselves (Figure 1), they must rely on other factors that can recognize specific DNA sequences. *Drosophila* biologists narrowed the search by defining small stretches of DNA associated with target genes that are necessary and sufficient to perform the silencing and maintenance tasks of the PcG system. These sequences have been called Polycomb response elements (PREs). Sequence-specific DNA binding proteins like Dsp1, Zeste, GAF, and Grainyhead are thought to direct PcG proteins to PREs in *Drosophila* (Müller and Kassis, 2006; Ringrose and Paro, 2007; Schuettengruber and Cavalli, 2009; Simon et al., 1993). Recent studies identifying two segments of DNA controlling developmentally important genes have suggested that such PREs may also exist in mammalian cells (Sing et al., 2009; Woo et al., 2010). This was complemented by an identification of Jarid-2, a DNA-binding protein required for PRC2 anchoring (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). In addition, the last few years saw mounting evidence implicating RNA in the process of PcG recruitment, primarily in mammalian cells. Long (a few kbs) and short (50–200 bases) noncoding RNAs directly associate with components of PcG and are causally involved in their anchoring to target loci in *cis* or in *trans* (for reviews see Bracken and Helin, 2009; Guenther and Young, 2010). Long noncoding RNAs provide an opportunity to cotarget other complexes that may functionally complement PRCs in repression. The conundrum that active transcription (of noncoding RNAs) dictates repression is also observed in the case of heterochromatic gene silencing, possibly implying a common evolutionary origin (Grewal, 2010).

The fundamental question of precisely how the PcG system silences transcription is still not fully answered (Müller and Verrijzer, 2009). The emerging picture suggests that ubiquitination marks set by PRC1, in combination with the H3K27me3 marks of PRC2, prevent transcriptional elongation at the start sites of target genes (Stock et al., 2007). Additionally, studies from mammalian cells have demonstrated that PcG system is required for chromatin compaction (Eskeland et al., 2010) and DNA looping that may be linked to DNA methylation (Tiwari et al., 2008). The process of compaction may physically hinder the access of transcription factors, contributing to repression. Interestingly, compaction is lost upon expression during differentiation (Chambeyron and Bickmore, 2004). It remains to be seen how general the model is, because predictions made by the model do not hold true uniformly. For example, removal of RING1b that sets the ubiquitination mark does not lead to derepression of all targets (Eskeland et al., 2010). Moreover, many genomic targets are occupied by PRC2 alone and not by PRC1 as demonstrated by ChIP profiling in mammalian cells (Ku et al., 2008). Also, deubiquitination seems to be involved in repression, raising doubts about cause and consequence relationship in the model (Scheuermann et al., 2010).

The hallmark of the PcG silencing system is that it persists through cell division, resisting eviction by replication forks and mitotic condensation. What form of information passes through this cell cycle process is not clear, however. Recent studies show that PcG proteins have the ability to stay bound on replicating DNA, and the TrxG protein MLL stays on mitotic chromosomes, whereas most other proteins fall off during the condensation process (Blobel et al., 2009). Because noncoding RNAs have been shown to functionally associate with PcG/TrxG proteins, an inheritance model based on RNA as information

carrier is not implausible. Future research will certainly test this idea.

PcG/TrxG Memory System and Cell Fate Transitions

Mammalian Development, ESCs, and PcG

PcG proteins are vital for development and not surprisingly most loss-of-function PcG mutations result in early embryonic death. Knockout of some PcG members are viable and show strong homeotic transformations, similar to the ones described for *Drosophila* (reviewed in Surface et al., 2010). Major advances in our understanding of the cellular roles PcG plays during mammalian development have been possible due to investigations in embryonic stem cells (ESCs) that retain the core aspects of differentiation. Three key findings have been made on the significance of PcG in ESCs, as discussed in detail elsewhere (Surface et al., 2010). First, PcG proteins occupy and silence a large group of developmental and signaling genes such as the Hox clusters as well as genes coding for Wnt, Pax, and Fox families (Boyer et al., 2006). Many of these genes are selectively activated during in vitro differentiation of ESCs. Second, PcG-mediated repression is not important for ESC self-renewal, but it is vital for the ability to differentiate in response to extracellular cues (Chamberlain et al., 2008). This emphasizes the role of PcG in cell fate transitions rather than the establishment or maintenance of ESCs. Third and most importantly, PcG target genes in ESCs are occupied by both repressive H3K27me3 and activating H3K4me3 marks—so-called bivalent domains (Bernstein et al., 2006). The coexistence of these marks colocalizes with paused RNA polymerase II, waiting to enter elongation mode. PcG-mediated H2A ubiquitination largely causes this paused state of the polymerase (Stock et al., 2007), confirming the role of PcG in a repression poised for activation. Such bivalent domains are resolved during differentiation, at the end of which most genes bear either of the two marks, commensurate with their expression state. Additionally, DNA methylation may play an important role in this process (see below). The finding that such domains exist in a subset of genes within developing zebrafish embryos (Vastenhouw et al., 2010) has confirmed the validity of data from cultured ESCs.

The role of PcG proteins in maintaining pluripotency of ESCs by repressing differentiation genes is well known. However, it is not clear how this repression is established in the first place. An understanding of processes that establish repression on specific genes in developing embryos may shed some light on how it may be counteracted appropriately. Two approaches have been taken to understand the setting up of PcG repression. One system makes use of the fact that fusion of a differentiated lymphocyte from human with a mouse ESC results in reprogramming of the lymphocyte genome (Pereira et al., 2008). It has been demonstrated recently that ESCs lacking EED or Suz12, core components of PRC2, are unable to reprogram lymphocytes after fusion (Pereira et al., 2010a). Curiously, the inability to reprogram is a dominant trait as shown by the fact that a concomitant fusion between wild-type and mutant ESCs also failed to reprogram lymphocytes. It is possible that derepressed PcG targets in mutant ESCs (such as coding or noncoding RNAs) may actively inhibit pluripotency. As a corollary, such RNAs made at one derepressed PcG target may override PcG activity at another target within the same cell. In other words, signaling-induced changes in PcG repression can have cascading effects, and genome-wide analyses should help us understand the mechanisms involved.

The other successful approach in finding out how repression is established early in development relates to recent analyses of activating and repressive marks in male gametes. In mammals, most histones bound to the genomic DNA are replaced with protamines during the course of sperm differentiation. However, it has been shown that some genes are occupied by nucleosomes with activating or repressive marks even in mature sperm from human (Hammoud et al., 2009). In particular, genes that will continue to be repressed, even after the onset of zygotic transcription in early embryos, are already marked with H3K27me3 in human sperm (Brykczynska et al., 2010). We do not fully understand how only a subset of PcG target sites avoids global nucleosomal eviction during spermatogenesis.

Although the role of PcG proteins in mammalian development in vivo has been difficult to discern because of early lethality, conditional knockout strategies have been used to decipher their importance in specific lineages. For example, Ezh2 deletion in mouse cortical progenitor cells before neurogenesis affects two subtle aspects of development. The cortical progenitor cells make more basal progenitor cells, instead of self-renewing (Pereira et al., 2010b). Additionally, neuro- and glio-genesis initiate prematurely. Together these events lead to a much smaller cortex.

Given the importance of PcG in repression of genes important in signaling and development, it is not surprising that PcG proteins play a vital role in initiation and pathogenesis of cancer, both in *Drosophila* and mammals. That PcG repression may be involved in carcinogenesis was first hinted at two decades ago, when PcG protein Bmi1 was found to enhance c-Myc's effects (van Lohuizen et al., 1991). Since then several PcG/TrxG proteins have been shown to be causally linked with cancers (Merdes and Paro, 2009). The mammalian Trithorax ortholog Mixed lineage leukemia (Mll) is known to cause cancers when translocated to generate fusion proteins with various partners (Marschalek, 2010). The combined role of PcG/TrxG proteins in regulating senescence, cell cycle checkpoint control, apoptosis, and DNA damage repair makes them an interesting model to study in the context of cancer. The exponentially growing links between PcG and cancer have been recently reviewed elsewhere (Mills, 2010; Sauvageau and Sauvageau, 2010).

PcG in Plants and Unicellular Eukaryotes: An Evolutionary Perspective

The importance of the PcG system in stabilization of cellular identities throughout evolution is exemplified by the fact that plants, which evolved multicellularity independently of animals, utilize this system in strikingly similar ways (Meyerowitz, 2002). Studies mostly conducted on *Arabidopsis* have confirmed the presence of PRC2-like complexes with H3K27 methylation activity as well as TrxG members with H3K4 methylation activity that antagonizes PcG function (reviewed in Köhler and Aichinger, 2010). Not only is there a molecular identity between plant and animal orthologs (Figure 1), they also perform similar developmental functions in *Arabidopsis*. A complete loss of PcG function in plant cells inhibits cell differentiation and activates an embryo-like state (Chanvivattana et al., 2004),

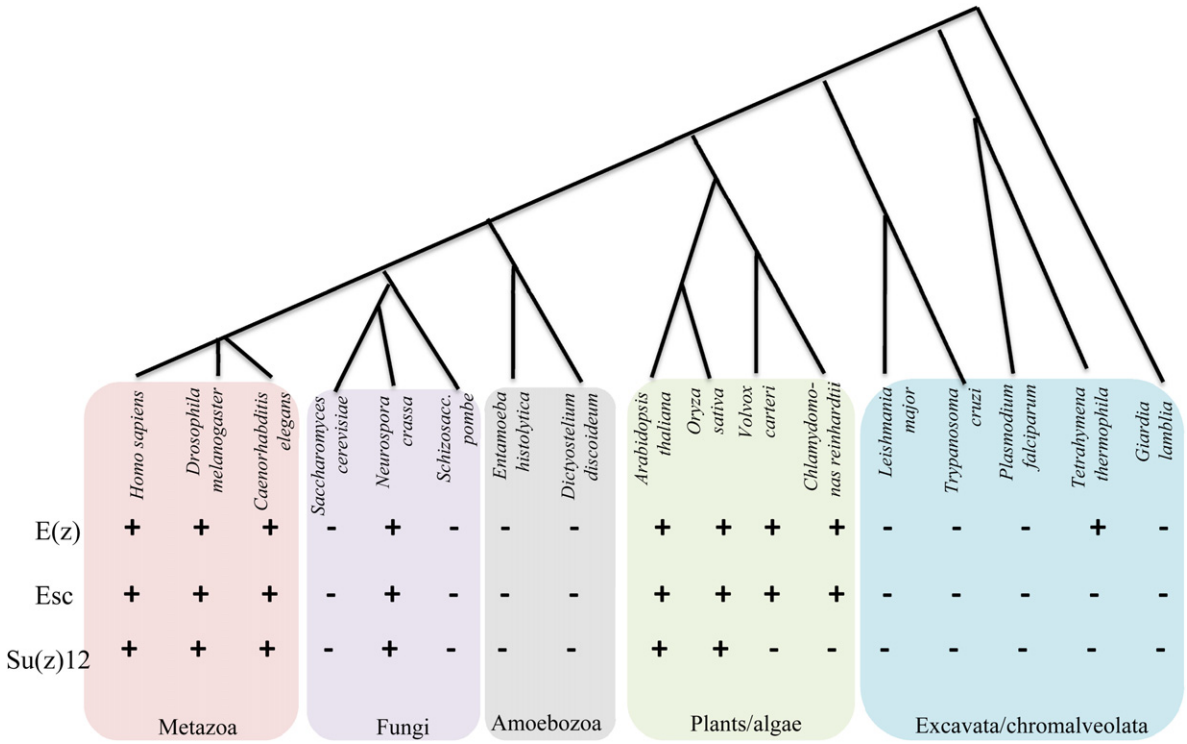

Figure 2. Phylogenetic Distribution of PRC2 Components

The existence of individual components has been inferred from the sequenced genomes (Shaver et al., 2010). The tree represents a possible proteome-based phylogeny (Song et al., 2005) and is not drawn to scale. Plus sign denotes present; minus sign denotes absent in the corresponding genome as judged by sequence similarity with known orthologs.

suggesting that PcG is required for the ability to form different tissues with diverse functions. PcG function is also required to maintain gametophytic identity of cells. Moreover, the system controls one of the most important developmental transitions plants make—from vegetative growth to reproductive flowers (Ito and Sun, 2009). By targeting key regulators in this transition, the PcG system inhibits flowering during early development and favors flowering after long periods of cold. The conservation of the system in its structural and functional entirety among metazoa and plants, despite its complete absence from yeasts, raises questions about the origin and diversification of this system (Whitcomb et al., 2007). Recent studies shed more light on this issue (Figure 2). Genomes of several single-celled eukaryotes code for the core subunits of PRC2 (Shaver et al., 2010). Moreover, an E(z) ortholog in *Chlamydomonas reinhardtii* is required for silencing retrotransposons and multicopy transgenic elements (Shaver et al., 2010). Thus, the PcG system may well have been present in the common ancestors of plants and animals in a rudimentary form, where it silenced genomic parasites such as transposons. As robust cell differentiation evolved along with multicellularity, the same system may have been deployed for additional silencing of developmentally regulated genes. Lineages leading to several extant unicellular organisms such as yeasts probably evolved more stable means of silencing retrotransposons, and thereafter lost the PcG system altogether. It will be fascinating to study other lineages that evolved multicellularity independently to that of plants and animals (Rokas, 2008) for a functional PcG system (Figure 2) and its role in cellular differentiation.

How Is Signaling Perceived at Chromatin to Override PcG Repression?

Research over the past decade has demonstrated the significance of the PcG system in repressing important developmental regulators and perpetuating this repressed state through cell divisions. However, cells within a developing embryo have to tackle an important problem in the face of PcG repression—how to counteract this persistent repression in response to appropriate differentiation signals? The issue becomes even more complicated by the fact that various signals induce different targets in a context-dependent way. Moreover, the kinetics and time frames of derepression are highly variable—there are developmental targets that are expressed only upon an extracellular signal, and there are cell cycle-regulated targets that are derepressed at regular intervals. Recent studies show that the PcG system also represses genes that are induced upon environmental stress (Beisel et al., 2007). These are induced in a very short time after stress exposure. On the other hand, different combinations of inputs from outside of the cell are interpreted by this system, allowing either rapid or highly regulated induction of targets. The last few years have seen a large number of studies addressing this question in diverse model systems. Two emerging themes underlying derepression are the ability to recruit activating chromatin factors to target loci and the ability to downregulate the PcG genes themselves (Figure 3).

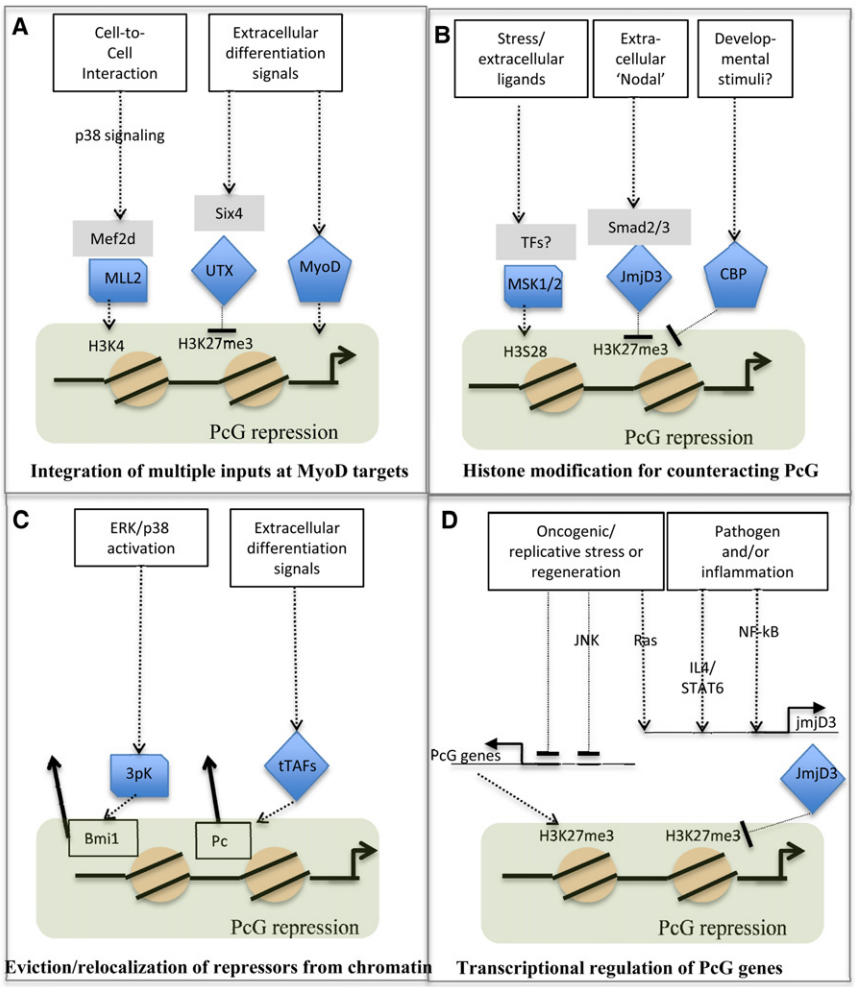

Figure 3. Cellular Mechanisms to Counteract PcG Repression in an Appropriate Context of Signaling

PcG target genes are represented within the light green box depicting associated proteins and nucleosomes with histone modifications. Initiating signals are indicated on top of each panel with normal and blunt-headed arrows showing positive and negative effects, respectively. The final effector proteins that directly bind chromatin are shown as blue polygons and intermediary proteins as gray rectangles. The various mechanisms have been classified and indicated as (A) to (D). Please refer to the text for further details and references.

Signaling-Activated Recruitment of Chromatin Factors

MyoD and Myogenesis

Myogenesis presents an instructive case for understanding how several inputs converge on chromatin to derepress Polycomb target genes. Myoblasts can be differentiated in multinucleated myotubes in vitro by a simple cue of serum withdrawal. This tractable model system provides a way to study the temporal order of events occurring at genome-wide Polycomb targets as cells differentiate and a means to manipulate the system to gain mechanistic insight into the process of derepression.

MyoD is a trans-activator that initiates the myogenic program in differentiating myoblasts and has been referred to as a master regulator of myogenesis. However, a recent genome-wide study revealed constitutive binding of MyoD to regulatory elements of a large number of genes in mouse myoblasts, only a small subset of which is activated upon muscle differentiation (Cao et al., 2010). MyoD binding is associated with local histone acetylation at most target loci. Many of these genes are repressed by the PcG system in myoblasts as indicated by the prevalence of H3K27me3 and EZH2 at these loci (Caretti et al., 2004). To overcome PcG repression as differentiation proceeds, at least three other events must happen besides increased binding of MyoD (Figure 3A). (1) The homeobox transcription factor (TF) Six4 along with the associated histone demethylase UTX is recruited to a subset of MyoD targets. This causes demethylation of repressive H3K27me3 only in promoter-proximal regions and not in gene bodies (Seenundun et al., 2010). (2) The MADS-box TF Mef2d binds to its target loci (Rampalli et al., 2007). Genome-wide studies indicated that a combination of Mef2 and MyoD binding is a better predictor of gene regulation during myogenesis than binding of either of them by itself (Cao et al., 2010). Binding of MyoD, Mef2d, and Six4 at target loci set the stage for gene activation. (3) The final signal comes from cell-to-cell contact, which results in p38 MAPK activation and consequent Mef2d phosphorylation. This signal reaches chromatin as phosphorylated Mef2d recruits TrxG histone methyltransferase Ash2L/MLL2 at its target loci (Rampalli et al., 2007). The newly recruited HMTases cause H3K4 methylation, initiating transcription. The process may be reinforced by phosphorylation of RNA pol II by pTEF-b, which is recruited by MyoD (Giacinti et al., 2006). UTX can then associate with phosphorylated polymerase engaged in elongation (Smith et al., 2008), erasing H3K27me3 marks throughout gene bodies. Later during differentiation, expression of both Ezh2 and YY1 is suppressed by specific microRNAs, further contributing to PcG target gene upregulation (Wang et al., 2008; Wong and Tellam, 2008).

This cascade of events results in full induction of PcG target genes only when all the conditions are met—binding of MyoD, Six4, Mef2d, and activated p38 pathway after extracellular cues (Aziz et al., 2010). It appears that the PcG-repressive system guards against precocious induction of genes during differentiation, while allowing for integration of multiple signals in a temporally ordered manner. The sequence of events described here has been shown to occur at only a few loci. With the advent of high-resolution genome-wide mapping techniques, a comprehensive picture relating signaling and transcription factor binding with gene activation during muscle differentiation will soon be possible.

Stress Signaling and Histone Phosphorylation

An elegant way to circumvent H3K27me3-repressive marks is to mask them from their readers rather than erase them. This can be achieved by a signaling-induced modification of a nearby residue, serine 28 (S28), for example. A recent study explored this idea with tantalizing conclusions—environmental stress and endogenous ligand-mediated signals activate kinases like MSK1/2 in human embryonic lung fibroblasts, which phosphorylate H3S28 even in the context of H3K27me3 on the same histone tail (Gehani et al., 2010). PcG proteins do not recognize the double modification thus formed, with an obvious consequence of loss of their binding and derepression of the target genes. The kinases are targeted to chromatin at specific promoters, possibly because of activation-specific interactions with certain transcription factors (Figure 3B). This is similar to the case of phosphorylation of H3S10 by Aurora kinase during mitosis and consequent loss of HP1 from chromatin (Hirota et al., 2005). The study of H3K27me3S28p also raises important technical questions undermining the general paradigm that PcG derepression is accompanied by the loss of H3K27me3: It is quite plausible that—like the H3K27me reader-proteins themselves—the H3K27me3-specific antibodies used for chromatin immunoprecipitations do not bind this mark in the context of an adjacent S28p, leading us to conclude erroneously that the repressive mark is lost (Gehani et al., 2010). However, the recruitment of specific demethylases to target loci may support an actual loss of H3K27me3 upon gene activation. It will be highly interesting to understand the specific uses cells use of these two ways of counteracting PcG—by removing the repressive mark or by masking it with S28p. Although the former may be more persistent in the context of development, the latter may be more meaningful in responding to transient stress.

Nodal Signaling in Mouse ESCs

A member of the TGF-β superfamily, Nodal, is involved in diverse developmental processes in vertebrates, including lineage specification and patterning of axes. Nodal activates signal transducers like Smad2/3, which along with co-Smad4 is recruited to chromatin to activate transcription. Not surprisingly, many Nodal target loci are PcG repressed and thus this model provides another opportunity to investigate the mechanism of signaling-induced derepression. A recent study highlighted a subtle role of PcG repression in mouse ESCs (Dahle et al., 2010). Both Nodal signaling and PcG in these cells target the genes encoding *Nodal* and *Brachyury*. In a situation similar to that of phosphorylated Mef2d and Ash2/MLL in differentiating myoblasts, Nodal-activated Smad2/3 binds Jmjd3 and recruits it to Nodal and Brachyury loci (Figure 3B). This reverses the repressive H3K27me3 mark set by PRC2 at these loci, allowing their activation. A critical point is that the interaction between Smad2/3 and Jmjd3 takes place only in the context of a functional Nodal signaling cascade in the cell (Dahle et al., 2010). More interestingly, Smad2/3 is required only to counteract PcG repression and not to activate gene expression as was thought earlier. In the absence of Suz12, and hence Polycomb repression, the target loci are active even without functional Nodal signaling. Thus in this system, PcG repression seems to function as a module that constrains gene activation to be Nodal responsive.

Dissociating PcG Proteins from Target Genes

During terminal differentiation of male germ cells to spermatocytes, PcG repression is overridden at many loci important for this developmental transition. *Drosophila* testis-specific TBP-associated factors (tTAFs), which are expressed during this differentiation process, are recruited to some of these PcG-repressed loci (Chen et al., 2005). Binding of tTAFs causes (directly or indirectly) a reduction in PRC1, promoting Trx-dependent accumulation of H3K4me3 and gene induction. Surprisingly, Pc is enriched in nucleoli of differentiating spermatocytes where there is little H3K27me3. Given that this repressive mark is required for anchoring Pc to chromatin, Pc localization to nucleoli may adopt a different mechanism for recruitment. This differentiation-specific enrichment of Pc is dependent on tTAFs (Figure 3C), indicating that Pc sequestration to nucleoli may be causally linked with derepression (Chen et al., 2005). This is reminiscent of EED relocalization to cell membranes in human T cells upon integrin receptor activation (Witte et al., 2004). The process is mimicked by the HIV-encoded Nef protein, which activates integrin receptors, causing relocalization of EED to the plasma membrane, depleting PcG complexes from HIV promoters and thus relieving repression. This results in an induction of tat-dependent HIV transcription (Witte et al., 2004). Both of these examples from different contexts highlight yet another mechanism of PcG derepression—by relocalizing repressive elements within the cell away from their target locus on chromatin. It has been difficult to prove that derepression is a direct result of relocalization, but advanced cell biological techniques combined with genomic analyses should be able to confirm this hypothesis. It must be noted that, similar to signaling-induced transcriptional downregulation of PcG genes (see below), relocalization also allows global rather than locus-specific derepression. Despite a global decrease in PcG binding to chromatin, only those genes with functional TFs bound can activate their expression.

The potential for signaling-induced posttranslational modifications of PcG proteins in the regulation of target genes has been demonstrated in several cases. ERK and p38 pathways converge on 3pK (MAPKAP kinase 3), which can directly phosphorylate Bmi1 in mammalian cells (Voncken et al., 2005). 3pK phosphorylation of Bmi1 dissociates it from its target loci (Figure 3C), permitting subsequent induction. EZH2, the catalytic component of PRC2, is known to be phosphorylated at two residues at least—Ser21 and Thr350. The former is modified by Akt (Cha et al., 2005), whereas the latter is phosphorylated by cyclin-dependent kinases 1 and 2 (Chen et al., 2010). Both of the modifications are important for chromatin recruitment of EZH2 and consequent silencing of the target loci. It is not clear, however, whether the kinases are themselves recruited to a subset of EZH2 target loci in order to activate them. In such a scenario it will be crucial to find out what targets kinases to chromatin in a context-dependent fashion.

Signaling-Induced Transcriptional Changes in PcG/trxG Genes

JNK Signaling in Drosophila Transdetermination

In the fascinating phenomenon of transdetermination within regenerating *Drosophila* imaginal discs, the role of JNK in PcG dynamics has been well illustrated. Embryonic imaginal discs are clusters of cells committed to differentiate in a particular

lineage. Upon fragmentation of such discs, cells proliferate to regenerate the disc. The PcG system is thought to maintain the identity of proliferating cells by heritably silencing specific genes. Rarely cells change their identity in a process called transdetermination, which is accompanied by switching on genes that were hitherto silenced by PcG. It has been shown that JNK signaling activated in fragmented discs downregulates transcription of PcG genes, thereby loosening the repression of target genes and allowing plasticity of cell fate (Lee et al., 2005). Thus signaling-induced depletion of PcG members may itself serve as a mechanism of derepression (Figure 3D). However, it must be noted that such derepression would act globally at all target loci, which are also bound by transcription factors, unless repressive marks at a subset of genes are actively retained.

Senescence Signaling and the CDK Inhibitor Locus

The *INK4b/ARF* locus is an important target of PcG-mediated repression. First, a large part of the phenotypic abnormalities in Bmi1 knockout mice are rescued by deleting the *INK4b/ARF* locus (Bruggeman et al., 2005). This suggests that developmental repression of this locus is one of the most significant functions of PcG proteins in mammalian models. Second, a noncoding RNA, *ANRIL*, encoded within the *INK4b/ARF* locus, recruits PRCs in *cis* (Yap et al., 2010). Finally, the locus codes for proteins that inhibit the cell cycle and is expressed during oncogene-induced or replicative senescence. Understandably, deletions of this locus have been associated with cancer predisposition and progression. While silenced in normal cells by the PcG system, the locus is induced upon culturing cells over several passages or by experimental activation of the Ras-Raf pathway. Earlier studies identified many TFs required to induce the locus in response to activating signals, but it is not completely clear how PcG repression is overcome. Recent studies have indicated at least two mechanisms by which this is achieved at the *INK4b/ARF* locus. In a striking parallel to the case of regeneration in *Drosophila,* where JNK activation causes transcriptional downregulation of Polycomb, senescence-inducing stress signals decrease the levels of *EZH2* transcript in human embryonic fibroblasts (Bracken et al., 2007). Concomitantly, Jmjd3 is transcriptionally upregulated in response to Ras signaling (Agger et al., 2009; Barradas et al., 2009). Both of these events lead to lower amounts of the repressive mark H3K27me3 at the locus (Figure 3D), allowing transcription factors to exert their influence on gene activity. The activation process may be aided in part by SWI/SNF recruitment and changes in DNA methylation (Paul et al., 2010), but this has not been directly shown.

Transcriptional Regulation of Jmjd3

The histone demethylase JmjD3 is transcriptionally upregulated in response to various stimuli other than those mentioned above. In macrophages, it is induced by pathogens and inflammatory cytokines in an NF-κB-dependent manner (De Santa et al., 2007) or by the IL-4/STAT6 pathway (Ishii et al., 2009). In both cases, the increased cellular levels of JmjD3 correlate with its heightened occupancy at PcG target sites and concomitant loss of repressive marks (Figure 3D), leading to gene expression. During neurogenesis, the gene encoding Jmjd3 is repressed by SMRT-Corepressor complexes and requires retinoic-acid-receptor-dependent activation for its expression (Jepsen et al., 2007). This transition is critical for differentiation of neural stem cells into neurons. However, the molecular interplay between PcG repression and Jmjd3 in this case remains to be characterized. This H3K27-specific histone demethylase seems to be a focal point of regulation during PcG derepression in these diverse systems, highlighting a major area of research for the near future. Not only the transcriptional regulation of demethylases, but also their specific recruitment to target loci, has been extensively utilized by cells to counteract PcG silencing (Swigut and Wysocka, 2007).

DNA Methylation and PcG

Promoter-proximal DNA methylation in mammalian cells has been linked with gene repression, thus representing a parallel pathway possibly connected with the PcG system (Viré et al., 2006). The in vitro differentiation of ESCs to neurons via intermediate progenitor cells reveals a correlation between the two silencing systems. In mouse ESCs, most H3K27me3 domains are also marked with activating H3K4me3, representing a bivalent state. As differentiation proceeds, genes become enriched with either of the two opposing marks. It turns out that most genes, which are repressed by PcG in ESCs and are not activated during lineage commitment, are de novo DNA methylated during differentiation (Mikkelsen et al., 2007; Mohn et al., 2008). Thus, a poised state of bivalency dictated by a labile PcG system in pluripotent cells is more stable, once silenced by DNA methylation upon terminally differentiation. How this transition occurs is not yet understood, but this example highlights a context-dependent collaboration between PcG and DNA methylation.

Recently, the genome-wide role of nonpromoter DNA methylation in antagonizing PcG repression during differentiation has come to the fore (Wu et al., 2010). In postnatal neural stem cells (NSCs) in mouse, a de novo DNA methyltransferase Dnmt3a binds and methylates several nonpromoter regions besides promoter-proximal CpG islands. In *Dnmt3a* knockout NSCs, genes associated with nonpromoter DNA methylation were downregulated with concomitant increases in H3K27me3, Suz12, and Ezh2 occupancy at these sites. This was due to DNA methylation per se at these sites, and not just localization of Dnmt3a protein at nonpromoter regions. Many of these loci code for neurogenic genes essential for proper differentiation. Thus in postnatal NSCs, de novo methylation at nonpromoter regions by Dnmt3a is required to counteract PcG repression (Wu et al., 2010). This provides a fascinating potential mechanism whereby specific targeting of the de novo DNA methyltransferase, under the control of developmental stimuli, may contribute to derepression of PcG targets.

Possible Additional Mechanisms for Derepression

Studies over the last several years have identified various accessory proteins that are required for PcG silencing. When mutation or inhibition abrogates the activities of such proteins, there is a loss of repression. As a corollary, cellular signaling that modulates these activities may lead to context-dependent derepression at specific PcG target loci. This idea has not been tested thoroughly, but provides an additional opportunity to connect developmental signaling and PcG repression. For example, H3K27 acetylation by CBP has been thought to antagonize PcG silencing (Tie et al., 2009) during *Drosophila* development. CBP may be recruited or activated at target sites in a signaling-dependent way, thus counteracting PcG in only

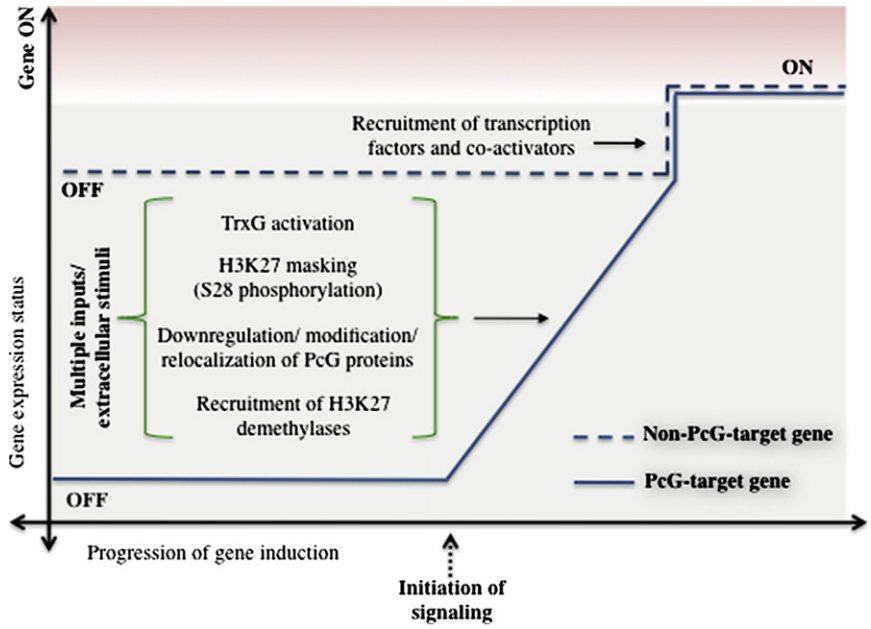

Figure 4. A Conceptual Framework to Understand Derepression of PcG Targets in Comparison with Induction of Genes that Are Not Silenced by PcG

The lines on the graph trace the events during signaling-induced expression of a gene. TF and coactivator binding is necessary and usually sufficient for activation of conventional genes. However, PcG target genes require a variety of processes depicted on the graph before they can be induced by TFs. This very property of derepression allows for an integration of multiple inputs from within and outside the cell, forbidding gene induction until an appropriate combination of inputs is achieved. Note that the precise sequence of various events indicated to occur during derepression of PcG target genes is not known.

a certain subset of cells and loci. Similarly, glycosyltransferase activity of a PcG protein Sxc is known to be required for efficient silencing by the complex in *Drosophila* (Gambetta et al., 2009). An allosteric modulation of such an activity by other proteins, or small molecules like second messengers, may be a way of overriding PcG silencing. Future research in this direction will certainly be fruitful.

Recent studies employing conditional knockout strategies in developing mouse brain substantiate earlier experiments implicating a TrxG member Mll1 in counteracting H3K27me3 (Lim et al., 2009). In the absence of this protein, postnatal NSCs differentiate only as glia but not neuronal lineages. This is thought to be caused by a failure to upregulate Dlx2, an important regulator of neurogenesis. During differentiation in wild-type cells, the *Dlx2* locus is bound by Mll1 in correlation with H3K4me3 deposition. In Mll1-depleted cells, however, the locus is predominantly marked by H3K27me3, suggesting a causal role of Mll1 in derepression (Lim et al., 2009). What brings about the changes in Mll1 levels at chromatin during differentiation is not clear, but this study emphasizes the role of this TrxG member not only in differentiation, but also in lineage choice.

Monoubiquitination of H2A by PRC1 component Ring is associated with gene repression (Wang et al., 2004). Counterintuitively, a deubiquitinating enzyme is also required for repression in *Drosophila* (Scheuermann et al., 2010). How these two opposing enzyme activities, colocalized on chromatin, manage persistent repression is not clear, and these enzymes may well be directed toward different substrates. However, it seems plausible that alternating cycles of addition and removal of ubiquitin on H2A are involved. Thus, another opportunity arises, to intervene in PcG repression, by signaling-induced alteration of one of the two enzymatic activities. This indeed may be the case—removing the deubiquitinating activity in *Drosophila* derepresses Hox genes in most embryonic tissues, but abrogates Hox expression in the nervous system (Scheuermann et al., 2010). The tissue-specific effect of shifting the equilibrium of histone ubiquitination suggests the deployment of the deubiquitinase to upregulate Hox expression after specific signaling.

Nucleosome turnover is an important factor affecting the stability of a repressive/activating mark. It has been thought that some histone modifications may persist across cell division and thus mediate inheritance of information about locus-specific gene activity (Hansen et al., 2008; Margueron et al., 2009). However, new studies that quantify turnover kinetics paint a different picture—nucleosomes are turned over faster than a cell cycle (Deal et al., 2010). Moreover, nucleosomes at TrxG-dominated target sites in *Drosophila* cells are turned over at a higher rate than those at PcG-dominated targets (Deal et al., 2010). Although this may simply be a result of a higher transcriptional activity at TrxG targets, it is possible that nucleosomal turnover/eviction mechanisms also initiate the process of derepression. Modulation of turnover rates by chromatin remodeling machinery may collaborate with decreased levels of PcG proteins to counteract repression. How this is fine-tuned at specific loci should be an interesting avenue of research.

Concluding Remarks

The emerging picture from recent research suggests that counteracting PcG repression is achieved by a combination of multiple inputs converging at chromatin (Figure 4). Besides the normal requirement of TFs and coactivator recruitment, PcG targets need the activity of specific demethylases and methyltransferases. Why have eukaryotes evolved a complex mechanism for a simple act of reversible gene repression? The system of PcG silencing offers at least three advantages in addition to the ability to heritably repress target genes. First, it imparts a state of robust repression in the milieu of biochemical noise and stochasticity. PcG targets important genes, which upon misexpression can cause uncontrolled proliferation and disease. Given that TF binding is not sufficient for their expression, PcG targets are not expressed in the face of stochastic TF binding that would otherwise lead to gene expression noise (Raser and O'Shea, 2004). Second, multiple inputs from outside the cell have to reach chromatin in the context of the appropriate intracellular signaling

to switch on PcG targets. This allows for an integration of a variety of stimuli before gene induction, akin to logical "AND" gate operating between different inputs. The PcG system thus interprets multiple signals before an important developmental regulator is turned on. It will be a long time before this system is utilized in synthetic genetic circuits that currently deploy only simple components (Zhan et al., 2010). Finally, the PcG system may facilitate cocoordinated expression of specific target loci in response to a particular combination of extracellular stimuli. During development, such an orchestrated response to transient signals must be critical for ensuing growth and differentiation. This concerted action of the PcG system may furthermore be important in minimizing phenotypic variability, a hallmark of canalized development.

The molecular basis of PcG derepression is only beginning to be clear. We still do not know all the molecular players in this process. The sequences of events that lead to target expression are known for a very small subset of loci. Also, most of our knowledge is derived from metazoan development, with only a faint picture of plant PcG and almost no understanding of the system in other eukaryotes that have a PcG system (Figure 2). Is there a unifying theme in counteracting PcG? Specific histone demethylases have been linked with many independent instances of PcG target derepression. Future research on the genome-wide dynamics of these proteins under different conditions will shed light on exactly how prevalent this mechanism is. Identifying cellular interaction networks for the demethylases will be immensely helpful in predicting hitherto unknown connections between signaling and the PcG system. Noncoding RNAs play an important role in recruitment of PcG members. Some controversial evidence exists for their involvement in PcG derepression (Petruk et al., 2006; Sanchez-Elsner et al., 2006; Schmitt et al., 2005). It is not clear whether transcription of noncoding RNAs is a cause or a consequence of a derepression and is a major area of future investigation.

Although most of the present research focuses on small subsets of PcG proteins in one or two cell types, the future will certainly see PcG research enter the exciting arena of systems biology. With the advent of high-throughput quantitative proteomics, the signaling-induced dynamics of PcG protein interactions can be captured in different cell types. Identification of such biochemical networks, combined with the explosion of data on genome-wide occupancy of chromatin proteins, will lead to a much-required mechanistic understanding. Such studies chaperoned by computational and theoretical biologists in conjunction with in vivo analyses will certainly provide a holistic picture of PcG regulation.

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