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Ruled by Ubiquitylation: A New Order for Polycomb Recruitment

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Polycomb complexes are found in most cells, but they must be targeted to specific genes in specific cell types in order to regulate pluripotency and differentiation. The recruitment of Polycomb complexes to specific targets has been widely thought to occur in two steps: first, one complex, PRC2, produces histone H3 lysine 27 (H3K27) trimethylation at a specific gene, and then the PRC1 complex is recruited by its ability to bind to H3K27me3. Now, three new articles turn this model upside-down by showing that binding of a variant PRC1 complex and subsequent H2A ubiquitylation of surrounding chromatin is sufficient to trigger the recruitment of PRC2 and H3K27 trimethylation. These studies also show that ubiquitylated H2A is directly sensed by PRC2 and that ablation of PRC1-mediated H2A ubiquitylation impairs genome-wide PRC2 binding and disrupts mouse development.

Biological problems often reach a cusp at which several laboratories with different approaches converge to provide unexpected solutions to longstanding questions. In the present case, the problem is how a major epigenetic mechanism, Polycomb silencing, is targeted to specific genes in the appropriate cells at the appropriate moment in lineage differentiation. Three new papers combine to upend widely held assumptions and open new ways of thinking about the way in which Polycomb complexes regulate genes in development and disease (Blackledge et al., 2014; Cooper et al., 2014; Kalb et al., 2014).

Polycomb group (PcG) proteins constitute a major epigenetic mechanism for controlling gene expression during the development of higher eukaryotes. To a large degree, this is done through repression of the key developmental genes, and the question of how PcG complexes are specifically recruited to these genes has been a major research problem. The recruitment mechanism is likely to be subtle, given that PcG components are present in the nuclei of most cells, but whether or not they are targeted to a given locus depends on its chromatin state. In general, transcriptionally active genes are not targeted, but genes that are not strongly transcribed are susceptible to PcG repression. Conversely, high levels of activators can override PcG silencing of a given gene, resulting in derepression. Some intermediate states are also possible, of which the bivalent domains found in pluripotent stem cells are the best known example. Once established, the repressed state of a gene tends to be transmitted through mitosis to progeny cells.

Polycomb Complexes

PcG proteins act as large multisubunit complexes of the two principal classes: PRC1 and PRC2. Each class comprises

several alternative variants (Figure 1). PRC1 complexes form around RING2 (or the closely related RING1) subunit to which one of the six alternative PcG RING finger (PCGF) proteins binds. The RING-PCGF heterodimers constitute a minimal core that can transfer a single ubiquitin group to lysine 119 of histone H2A (H2AK119). The identity of the PCGF subunits determines additional components and therefore some of the specific biochemical properties of the variant complexes (for a comprehensive discussion of the subject, see Schwartz and Pirrotta, 2013). In brief, the heterodimers between RING2/RING1 and MEL18 (also known as PCGF2) or BMI1 (also known as PCGF4) are incorporated in the so-called canonical PRC1 variants (also known as PRC1.2 and PRC1.4). The canonical variants have characteristic polyhomeotic (PHC) and chromobox-containing protein (CBX) subunits and can specifically recognize histone H3 trimethylated at lysine 27 (H3K27me3) via the chromodomain of the CBX subunit. All RING-PCGF heterodimers can also form noncanonical PRC1 complexes. These contain RING and YY1 binding protein (RYBP) instead of CBX and PHC. Noncanonical PRC1 complexes cannot recognize the H3K27me3 but have much higher H2A ubiquitylating activity because of the specific interaction of RYBP with H2AK119 (see below). The variant noncanonical complexes differ substantially in additional subunits (Schwartz and Pirrotta, 2013). Important for this discussion, the PCGF1-containing complexes (also known as PRC1.1) incorporate the KDM2B protein, a histone H3K36 demethylase whose CXXC domain confers the ability to bind unmethylated CpG-rich DNA (Farcas et al., 2012). The H3K36 demethylase activity, which removes what is considered to be a repressive histone mark, is apparently not necessary for Polycomb mechanisms (He et al., 2013).

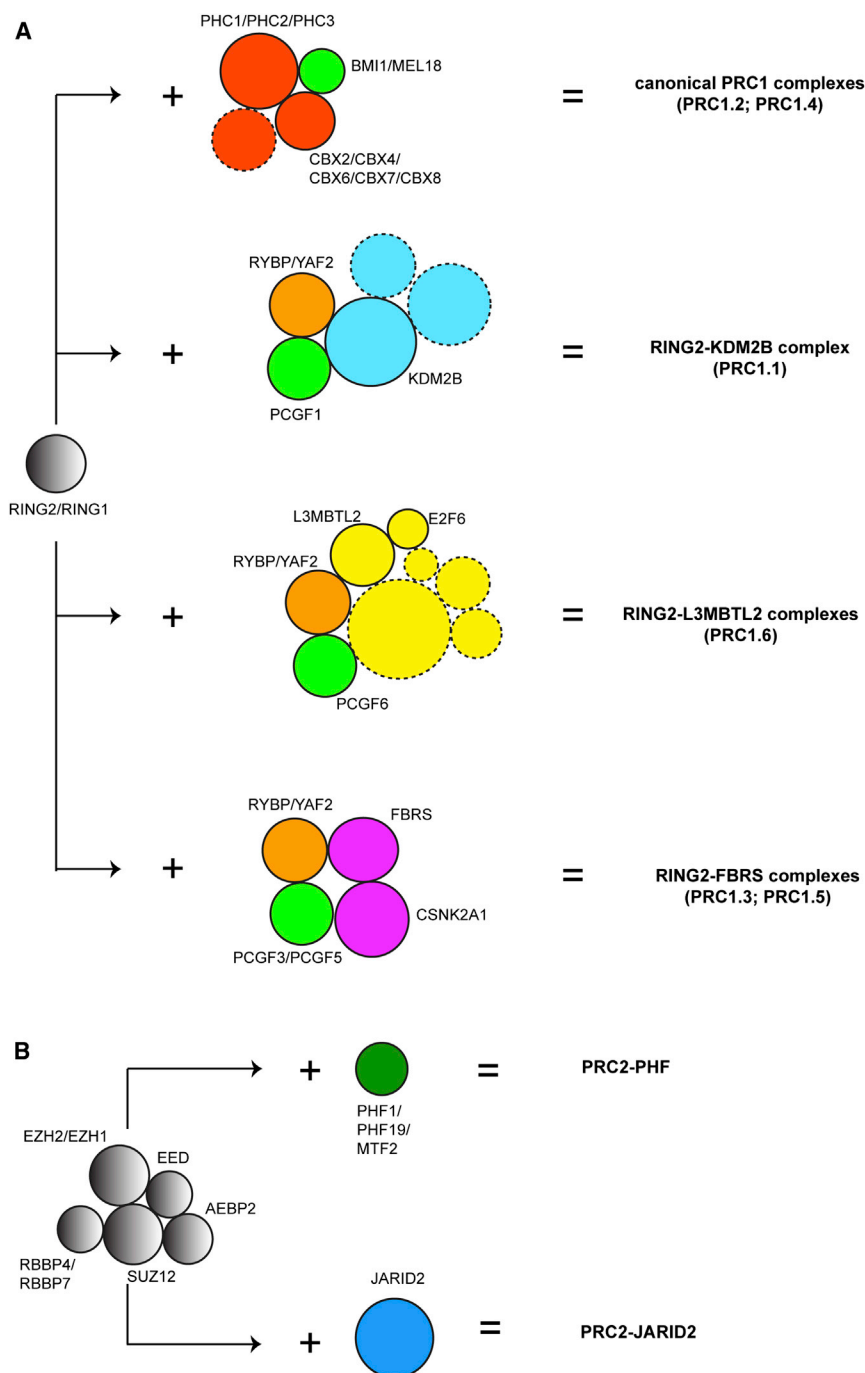


Figure 1. Varieties of PRC1 and PRC2 Complexes

(A) PRC1 complexes are built around the RING2 (or RING1) component. RING forms a heterodimer with one of six different PCGF proteins (green circles). Canonical PRC1 contains BMI1/PCGF2 or MEL18/PCGF4 as well as one of five CBX components. A variant PRC1 contains PCGF1 and KDM2B and the proteins RYBP or YAF2 (orange circles) instead of a CBX component. Variant PRC1 complexes containing RYBP or YAF2 are active H2AK119 ubiquityl transferases, whereas canonical PRC1 complexes have little activity.

(B) PRC2 complexes contain a core with the H3K27 methyltransferase activity stabilized by AEBP2. Associated factors such as JARID2 (blue circle) or PHF (green circle) modulate activity and binding specificities.

The Problem of Recruitment

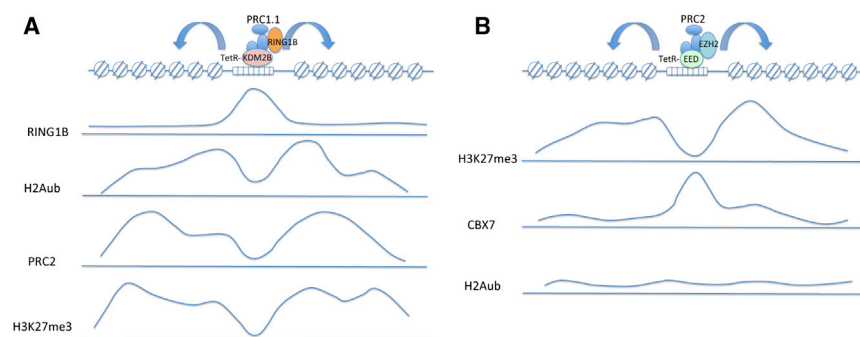
How the recruitment of PRC1 and PRC2 complexes is targeted and coordinated is not clear, given that none of the PRC1 or PRC2 subunits can bind DNA in a sequence-specific fashion. The conspicuous ability of canonical PRC1 complexes to recognize H3K27me3 led to the widely held idea that PRC2 is somehow recruited first and then trimethylates H3K27, and this, in turn, recruits PRC1. Although initially appealing, this hypothesis is at odds with observations that, in *Drosophila melanogaster*, the H3K27me3 produced by PRC2, forms a broad domain, whereas the binding of PRC1 is sharply localized at Polycomb response elements (PREs) and that, in mouse cells, not all H3K27me3 domains correspond to PRC1 binding sites. In any case, the binding of noncanonical PRC1 complexes would not be dependent on H3K27 methylation.

As mentioned above, in *Drosophila*, both PRC1 and PRC2 are recruited to specific target genes by PREs, ~1 kb DNA elements containing recognition sequences for DNA binding proteins. These DNA binding proteins are believed to combine their individually weak interactions with PcG proteins in order to yield

The known diversity of PRC2 complexes is less broad. All PRC2 variants contain a core of five proteins: EZH2 (or the closely related EZH1), EED, SUZ12, RBBP4 (or closely related RBBP7), and AEBP2. This core complex acts as a histone methyltransferase specific for H3K27. In addition to the core, PRC2 complexes incorporate either JARID2 or PHD finger protein (PHF) subunits. In mammals, insects, and worms, PRC2 complexes are the sole source of H3K27me2 and H3K27me3, and the latter is essential for PcG repression.

robust recruitment. The combinatorial nature of the PRE-mediated recruitment and the paradoxical ability of PREs to also recruit trithorax group proteins that help to counteract PcG repression provide the necessary plasticity to the *Drosophila* PcG-repressive system.

So far, the search for mammalian PRE-like elements has had limited success. Notable examples are a 1.5 kb DNA region from the mouse *MafB/Kreisler* locus that can recruit PRC1, but not PRC2 (Sing et al., 2009), and a 1.8 kb DNA element within



(B) In this experiment, PRC2 is directly recruited to the TetO array by fusing TetR to the EED component. The PRC2 bound at the TetO produces H3K27me3 on flanking nucleosomes. Surprisingly, canonical PRC1 containing CBX7 is recruited not over the methylated region but over the TetO array, where PRC2 is bound. Canonical PRC1 has poor ubiquityl transferase activity, and little H2AK119ub is produced.

Figure 2. The Recruitment Hierarchy

(A) The top diagram represents the reporter constructs and results of Blackledge et al. (2014). At the center, an array of TetO sequences binds TetR fused to KDM2B and thus recruits the associated variant PRC1 complex with its RING2 component. The lower diagrams from top to bottom illustrate chromatin immunoprecipitation experiments with antibodies against RING2, H2AK119ub, PRC2, and H3K27me3. The complex containing RING2 is found only over the recruiting TetO array. The H2AK119ub is found over the neighboring nucleosomes, and the PRC2 complex coincides with it. The H3K27me3 produced spreads even further away from the TetO array.

the human *HOXD* cluster that can recruit both PRC1 and PRC2 (Woo et al., 2010). In addition, several mouse promoter fragments containing binding sites for the RE1-silencing transcription factor or SNAIL proteins were reported to autonomously “recruit” PRC2 and possibly PRC1 (Arnold et al., 2013; Dietrich et al., 2012). The promoters of most PcG-regulated genes in mammals contain clusters of CpG dinucleotides, the CpG islands. Analysis in embryonic stem cells (ESCs) showed that, when CpG islands lack DNA methylation and are not associated with transcriptional activity, they bind PRC2 and contain H3K27me3 (Lynch et al., 2012; Mendenhall et al., 2010; Riising et al., 2014). The discovery of noncanonical PRC1 complexes containing KDM2B paved the way for understanding how such complexes might be recruited to CpG islands by the CXXC domain of KDM2B (Lagarou et al., 2008; Farcas et al., 2012; He et al., 2013; Wu et al., 2013). But how do we account for PRC2 recruitment?

A Link between PRC1 and PRC2

Two recent papers have studied how the full-fledged panoply of PcG complexes might be recruited to target genes or genomic regions. One paper (Blackledge et al., 2014) uses a specially constructed reporter region of 170 kb containing at its center a short array of Tet operator (TetO) binding sites for the bacterial TetR protein within a larger region devoid of other genes, enhancers, CpG islands, or elements that might recruit DNA methylation. Candidate components of PcG complexes can be targeted to the TetO array by fusion to the TetR DNA binding domain. The second paper (Cooper et al., 2014) exploits the fact that, in the absence of DNA methylation, CpG-containing regions in pericentric heterochromatin become targets for PcG complexes. In wild-type cells, these regions are DNA methylated, and proteins can be targeted to them by fusion with a 5meC binding methyl binding protein (MBP) domain, thus making the additional point that DNA methylation does not intrinsically prevent PRC2 complex binding or H3K27 methylation. Both approaches show that targeting the KDM2B protein recapitulates the recruitment of PRC2, H3K27me3, and CBX-containing PRC1 complexes. Therefore, the CpG binding KDM2B protein and its associated variant PRC1 complex can ultimately recruit both the PRC2 H3K27 methyltransferase complex and

the canonical CBX-containing PRC1. The key to this recruitment cascade is the RING1/RING2 protein, which forms the core of all PRC1-like complexes, and its ability to ubiquitylate histone H2A. Tethering a protein fragment that reconstructs the H2A ubiquitylating activity is sufficient to mimic the whole recruitment process. As a result of the RING1/RING2 activity, nucleosomes flanking the binding site become ubiquitylated. H2AK119ub now recruits PRC2, which in turn sets about trimethylating H3K27, which recruits canonical PRC1 complexes that include a chromodomain-containing CBX component.

This recruitment cascade implies that CpG islands must first bind variant PRC1 complexes that are ubiquitylation-competent in order to recruit PRC2, which recruits canonical PRC1 complexes, themselves poor ubiquityl transferases, but good at recognizing the H3K27me3 mark. Therefore, the initial step inverts the widely held notion that PRC2 is the initiator of the recruitment process, both clarifying and complicating the account as well as raising a number of interesting new questions.

That PRC2 really recognizes H2Aub, and not the ubiquitylation of some other chromatin protein such as Ring1/Ring2 itself, is shown by the distribution of PRC2 recruited at the reporter target site (Figure 2A). The recruiting variant PRC1 in this experiment binds to the TetO array, but the PRC2 is not found associated to the array but to the flanking regions that contain the H2Aub. Therefore, PRC2 is recruited to a broader region than that which bound the original variant PRC1 complex. PRC2 would then methylate H3K27 over an even broader region. The canonical PRC1 complex is then said to be recruited by virtue of its CBX component binding to H3K27me3. However, strangely, when PRC2 is targeted to the TetO array (by fusing TetR to the EED component of PRC2), the distribution of CBX7 over the recruiting region does not fit the expectation that it would coincide with the H3K27me3 mark. Instead, it appears to be centered squarely over the TetO array, which is depleted of nucleosomes and of H3K27me3. As the authors remark, this resembles the situation seen in *Drosophila* where the binding of the canonical PRC1 complex coincides with that of PRC2 at the PRE rather than the distribution of H3K27me3 (Figure 2B), arguing against the idea that H3K27me3 directly recruits PRC1 (Schwartz et al., 2006). Does PRC1 recruitment involve specific interactions with the PRC2 complex in addition to H3K27me3?

PRC2 Reads H2AK119ub

How does PRC2 recognize H2AK119ub? PRC2 has three core components, in addition to E(z), that are essential for its methyltransferase activity on nucleosomes. These subunits also confer ability to recognize the presence of H3K4me3 and H3K36me2/H3K36me3, which inhibit H3K27 methylation activity, and H3K27me3 and nucleosome density, which stimulate this activity (Schmitges et al., 2011; Margueron et al., 2009; Yuan et al., 2011, 2012). Two other components, AEBP2 and JARID2, enhance stability, activity, and target specificity (Li et al., 2010; Son et al., 2013; Ciferri et al., 2012). Ability to recognize and bind to H2Aub would add an additional capability to a very versatile protein complex.

Work from Kalb et al. (2014) now sheds light on this key interaction. The authors used oligonucleosomes to affinity purify interacting proteins, which were then identified by mass spectroscopy. They found that, when the nucleosomes contained H2AK119ub, they bound PRC2 components and, in particular, PRC2 enriched in AEBP2 and JARID2. Using reconstituted PRC2 complexes, they found that addition of AEBP2 and JARID2 greatly increased the methyltransferase activity on H2AK119ub-containing substrates. The presence of AEBP2 was essential for this differential activity, which was further stimulated when JARID2 was also present. They concluded that PRC2 complexes that included these two components interacted specifically with H2AK119ub-containing nucleosomes. It is not clear whether the increased enzymatic activity is accounted for by the increased binding or whether H2AK119ub also enhances catalytic activity. In addition, the authors point out that the H2AK119ub-affinity purification from extracts prepared from ESCs revealed that KDM2B-containing PRC1 complexes were also selected. This would then result in a self-reinforcing loop, whereby KDM2B first brings a ubiquitylation-competent PRC1 complex to unmethylated CpG islands, but the ubiquitylating activity then stimulates further binding of PRC1 as well as of PRC2, whose methyltransferase activity then recruits chromodomain-containing PRC1 complexes. Although the basic features of this recruitment pathway are clearly conserved between *Drosophila* and mammals, some aspects must be modified to some extent, given that *Drosophila* lacks CpG islands and that the recruitment occurs instead at specific PREs, most likely via a series of weak interactions with DNA binding proteins. Then, interaction of PRC2 with H2AK119ub would further reinforce this process.

Broader Considerations

There is much that remains to be accounted for. In particular, the distribution of H2AK119ub in the genome of flies or vertebrates remains poorly characterized. Recent work from Riising et al. (2014) indicates that blocking RNA Pol II transcription in mouse ESCs is sufficient to induce ectopic recruitment of PRC2 to CpG islands. The H2AK119ub-dependent recruitment model described above would imply that arrest of transcription immediately results in the binding of the KDM2B-containing complex and H2A ubiquitylation of CpG islands. Other complexes have been claimed to produce H2AK119 monoubiquitylation (histone H2A can also be ubiquitylated at other lysines that are not relevant to the present discussion). For example, BRCA1 has been said to ubiquitylate H2AK119 in order to silence satellite

sequences (Zhu et al., 2011) or at DNA damage sites (Wu et al., 2009). A PRC1 complex containing BMI1 has also been reported to be involved in H2A ubiquitylation at DNA damage sites (Ismail et al., 2010). Does DNA damage also recruit PRC2? Or is PRC2 recruitment prevented by the further ubiquitylation and phosphorylation events that target H2A upon DNA damage?

Why such a complicated recruitment? Which complex actually performs the transcriptional repression? We do not really know except that it seems to be linked to the ubiquityl transferase activity. H2AK119ub has been reported to interfere with the elongation of transcription by RNA Pol II (Zhou et al., 2008), but this does not exclude the possibility that other targets are ubiquitylated or that PRC1 complexes have additional repressive activities. Canonical PRC1 has been found to contribute little H2A ubiquitylation (Lagarou et al., 2008; Farcas et al., 2012; Wu et al., 2013), so why is it needed? One possibility is that it does, in fact, target some additional component important for transcription. An intriguing argument is that H2Aub generated by the initial noncanonical PRC1 complex is sufficient for transcriptional repression of nearby promoters, but the CBX-containing PRC1 is needed to mediate longer distance interactions through interaction with H3K27me3. Yet, recent evidence suggests that PRC2 is dispensable for repression in stem cells but necessary from the onset of their differentiation (Riising et al., 2014). Perhaps the best answer might be that the canonical PRC1, with its H3K27me3 recognition, might provide a more stable epigenetic maintenance function between cell cycles, which is particularly important for differentiating cells.

PcG recruitment by its nature cannot be hardwired but must remain flexible and dependent on the chromatin environment. The recruiting mechanism summarized here accounts for many of these features. KDM2B-dependent recruitment of PRC1 complexes is inhibited by transcriptional activity. Chromatin modifications associated with transcriptional activity modulate PRC2 enzymatic function (Margueron and Reinberg, 2011). In addition, transcriptional activity is associated with H3K27 acetylation both at enhancer sites and in the 5' region of active genes. Therefore, its presence would block H3K27 methylation. In contrast, the presence of pre-existent H3K27 methylation, as well as a high density of nucleosomes, promotes PRC2 methyltransferase activity (Margueron et al., 2009; Yuan et al., 2012). Therefore, the successful recruitment and maintenance of PcG repression would involve a complex interplay of these and probably other modulating circumstances. As a result, PcG mechanisms are better suited to maintaining a repressed state than turning off an active gene.

CpG methylation prevents the binding of KDM2B, and therefore the initiation of the entire recruitment cascade. This may avert the recruitment of PcG complexes to genes already silenced by the more long-term DNA methylation mechanism. It is possible that the KDM2B-based recruitment mechanism is particularly important in ESCs, where DNA methylation is relatively low and a large number of genes are kept in a bivalent state: neither silenced nor transcriptionally active but bearing both the H3K27me3 associated with repression and the H3K4me3 associated with transcriptional activation. In work that anticipated the approach of Blackledge et al. (2014), KDM2B was tethered at a reporter gene to demonstrate the

acquisition of H2AK119 ubiquitylation, but no recruitment of PRC2 and H3K27me3 was observed (Wu et al., 2013). The difference from the Blackledge et al. (2014) experiment is that Wu et al. (2013) used the differentiated HEK293T human cell line instead of mouse ESCs. It is very unlikely that such a fundamental recruiting difference is attributable to the difference between man and mouse but very possible that in differentiated cells PRC2 might become more selective. At later stages, when differentiating cell lineages are established, genomic regions that are not actively transcribed are likely to acquire DNA methylation, thus bypassing the PcG mode of transcriptional repression. Other recruitment mechanisms might become more important at specific sites or in specific cell lineages. A large number of noncoding RNAs have been said to bind PRC2 and, in some cases, PRC1 complexes and play a role in targeting them to specific genes (Khalil et al., 2009). PcG mechanisms are clearly highly versatile and nearly omnipresent. It would not be surprising if what was widely taken to be a well-understood mechanism revealed more new twists.

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