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EXTRA VIEW

Hierarchical recruitment of Polycomb complexes revisited

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ABSTRACT

Polycomb Group (PcG) proteins epigenetically repress key developmental genes and thereby control alternative cell fates. PcG proteins act as complexes that can modify histones and these histone modifications play a role in transmitting the "memory" of the repressed state as cells divide. Here we consider mainstream models that link histone modifications to hierarchical recruitment of PcG complexes and compare them to results of a direct test of interdependence between PcG complexes for recruitment to *Drosophila* genes. The direct test indicates that PcG complexes do not rely on histone modifications to recognize their target genes but use them to stabilize the interactions within large chromatin domains. It also shows that multiple strategies are used to coordinate the targeting of PcG complexes to different genes, which may make the repression of these genes more or less robust.

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Polycomb group proteins and complexes

Polycomb Group (PcG) proteins orchestrate development of multicellular animals by targeting genes pivotal to the onset of cell type-specific gene expression programs. Because of their remarkable propensity to repress genes if they have been repressed in previous cell cycles, PcG proteins epigenetically silence alternative gene-expression programs and ensure that cells maintain their identity throughout multiple generations.[1] First discovered in fruit flies (*Drosophila melanogaster*) as regulators of homeotic selector genes,[2,3] most PcG members were identified by genetic screens that used homeotic transformations as the readout. Orthologues of fly PcG proteins were later found in all metazoan animals and some of them were also found in plants and unicellular fungi.[4]

PcG proteins act as multi-subunit complexes. Two of these complexes, Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2), are evolutionary conserved and essential for PcG repression in all animals. However, some of the complexes, for example Pho Repressive Complex (PhoRC), are critical for PcG repression in flies but are not part of the process in mammals[5] and vice versa. *Drosophila* PRC1 consists of 5 core subunits: Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc) or its closely related homolog Suppressor of zeste 2 (Su(z)2), Sex combs on midleg (Scm), and RING1.[6-8] The heterodimer between RING1 and either of the 2 PCGF subunits Psc or Su(z)2 acts as E3 ligase that directs the transfer of one ubiquitin group to Lysine 118 of the histone H2A (H2AK118, the analog of H2AK119 in mammalian cells).[9,10] Mammalian PRC1 complexes have similar composition but are more diverse due to multiple closely related genes encoding each of the subunits. Thus, *Drosophila* RING1 is represented by 2 mammalian proteins RING1 and RING2. There are also 2 paralogs for Psc (BMI1 and MEL18, also known as PCGF4 and PCGF2), 3 for Ph (PHC1, PHC2, and PHC3) and 5 for Pc (CBX2, CBX4, CBX6, CBX7, CBX8).[11-13]

In addition to Psc and Su(z)2, *Drosophila* genome encodes one more PCGF protein called L(3)73Ah.[14] We and others have recently shown that the knockdown of L(3)73Ah leads to 70% reduction of the overall H2A118ub.[15,16] Together with observations that RING1 is responsible for all detectable H2A118ub,[9,15]

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this suggests that L(3)73Ah forms a heterodimer with RING1 and that L(3)73Ah-RING1 containing complex is a major *Drosophila* H2A118 mono-ubiquitylase. Although biochemical evidence for existence of L(3)73Ah-RING1 complex is currently lacking, complexes between RING2 and PCGF3 (mammalian ortholog of L(3)73Ah) have been reported.[12] Whether RING1/RING2-PCGF3 complexes are critical for bulk H2A119 ubiquitylation in mammalian cells is unknown.

Finally, *Drosophila* RING1-Psc heterodimer was said to be incorporated in a complex called dRAF, which differed from PRC1 in that it lacked Pc, Ph, and Scm but included Kdm2, RAF2, and Ulp1.[17] The same study reported that the dRAF complex produces the bulk of fly H2AK118ub and requires Kdm2 for its E3 ligase activity.[17] This contradicts our observation that cells completely deficient for Psc and Su(z)2 and, hence, lacking both PRC1 and dRAF, retain about 70% of the bulk H2AK118ub seen in wild-type cells.[15] The dRAF accounts are also hard to reconcile with drastic reduction of H2AK118ub in the L(3)73Ah deficient cells.[15,16] Interestingly, the Ring2 complex containing Kdm2B, one of the 2 mammalian paralogs of the fly Kdm2, was purified from mouse embryonic stem cells and appeared to contribute to PcG repression.[18,19] However, this complex is clearly different from dRAF. Instead of the Psc ortholog, it contains the protein called PCGF1, which does not exist in flies, as well as RYBP (or closely related YAF2) and BCOR subunits that were not found in dRAF. More work is needed to clarify the relation of the Kdm2 complexes to PcG repression in *Drosophila*. We should note, that human and mouse genomes encode 6 different PCGF proteins all of which form heterodimers with RING1 and RING2 and can mono-ubiquitylate H2AK119 *in vitro*. However, of the 6 PCGFs, only BMI1- and MEL18-containing dimers are incorporated in complexes that contain Pc and Ph orthologues (i.e. classical PRC1 complexes). All other PCGF-RING heterodimers form complexes that contain RYBP (or YAF2) and diverse sets of subunits that are specific for each PCGF.[12,20] Some of these RING complexes (at times referred to as non-canonical PRC1) were shown to activate transcription[21] and, probably, should not be considered as epigenetic repressors of the Polycomb group.

Drosophila PRC2 contains 5 core subunits: Enhancer of zeste (E(z)), Suppressor of zeste 12 (Su(z)12), Extra sex combs (Esc), Caf1, and Jing. PRC2 has histone methyltransferase (HMTase) activity and mono-, di- and tri-methylates Lysine 27 of histone H3 (H3K27).[22-25] E(z) is the enzymatic subunit of PRC2 but, similar to RING1 of PRC1, requires other subunits, namely Esc and Su(z)12, to form a catalytically active core.[22,23,26,27] EZH2 and EZH1, EED, SUZ12, and AEBP2 are mammalian orthologues for *Drosophila* E(z), Esc, Su(z)12, and Jing, respectively. *Drosophila* Caf1 has 2 mammalian homologs, RBBP4 and RBBP7.[20] In addition to these 5 core subunits, PRC2 comes with 2 mutually exclusive components. In flies, either Jarid2[28] or Pcl,[29] can be incorporated in PRC2 and this seems to confer some division of labor on the variant complexes.[20] Pcl-PRC2 was shown to stably associate with PcG target genes,[15,29] and is needed for extensive tri-methylation of H3K27 within the chromatin of repressed *HOX* genes.[29] Indeed H3K27me3 is a hallmark of the PcG-mediated repression state of the target genes.[30-32] However, the majority of methylated histone H3 is di-methylated at K27 and this, together with sparse tri-methylated H3K27, are scattered throughout transcriptionally inactive genome.[16,33] While the exact role of Jarid2-PRC2 is still unclear, it is tempting to speculate that, in *Drosophila,* it might have a role in global di-methylation of H3K27 by hit-and-run mechanism which does not involve stable binding of PRC2 to the chromatin.

Targeting Polycomb repression to specific genes

To epigenetically repress developmental programs, PRC1 and PRC2 need to be "targeted" to specific genes but also be able to "sense" whether a target gene is not transcriptionally active and, once the repression has first been established, "remember" it as cells continue to divide. Understanding molecular mechanisms that bring about these 3 essential properties of the system is a key challenge for the field.

In *Drosophila*, PcG target genes are equipped with discrete DNA elements, called PREs (Polycomb Response Elements), to which PRC1 and PRC2 are stably bound when target genes are repressed.[34-37] Neither PRC1 nor PRC2 contain sequence specific DNA binding subunits, therefore it appears that at least one of the PRC complexes is anchored to PREs via sequence specific adaptor proteins. Indeed, over the years, several candidate DNA binding proteins were identified.[38] However, no candidate protein is, by itself, sufficient to reconstitute a PRE or recruit PRC1 or PRC2. This suggests that PREs are flexible

collections of binding sites for multiple DNA binding adaptor proteins, which combine their individually weak interactions to stably anchor PRC complexes.

PRC1 can specifically recognize H3K27me3 produced by PRC2 while PRC2 can specifically interact with H2AK118ub produced by PRC1.[39,40] Since during replication histones are randomly partitioned between sister chromosomes, they can, potentially, serve as media to transmit the "memory" of transcriptional state. Although more experimental work is needed to clarify whether after the passage of a replication fork the "old" histones are re-incorporated in the proximity of their original positions, recent genetic evidence support the role of H3K27 methylation in transmission of the memory of PcG repression.[41,42] If H3K27me3 or H2AK118ub are used to remember the repressed state, their presence within chromatin should, in some way, be advantageous for re-establishment of PcG repression after DNA replication and/or mitosis. In line with this, earlier reports have proposed that the main role of H3K27me3 and/or H2AK118ub is to coordinate the targeting of PRC complexes. One popular hierarchical model postulated that PRC2 is recruited to *Drosophila* PREs by sequence specific adaptor proteins, once at PREs it extensively tri-methylates H3K27me3, which recruits PRC1.[43] In a more recent extension of this model, one of the RYBP-RING complex was suggested to be recruited first and ubiquitylate H2A, which, in turn, was proposed to recruit PRC2, leading to H3K27 methylation and ultimately PRC1 recruitment.[44] Although attractive in their simplicity, both models are contradicted by some of the experimental data. First, it has been shown that, in *Drosophila*, PREs are the principal binding sites for both PRC1 and PRC2 while H3K27 tri-methylation is spread around PREs as broad domain.[31,35] Moreover PREs are depleted of nucleosomes and hence are low in histone modifications.[35,36] Second, in mammals, the catalytic activity of RING1/RING2 appears dispensable for PcG repression and early development.[45,46] Third, it has been shown that, flies with catalytically inactive RING1 display no misexpression of HOX genes or homeotic phenotypes, characteristic of PRC1 loss of function. Furthermore, replacement of endogenous *Drosophila* histone H2A with histones that cannot be ubiquitylated, does not disrupt the repression of *HOX* genes.[9] Finally, the H2A118ub deubiquitylase activity of Calypso subunit of the *Drosophila* PR-DUB complex is critical for repression of HOX genes,[47] and, in fact, the H2A118ub levels within some of the PcG-repressed HOX genes, are very low.[16]

Direct test of PRC1 and PRC2 recruitment hierarchy

Concerned with discrepancies in current hierarchical recruitment models we have set up direct test of interdependence of PRC1 and PRC2 for recruitment to *Drosophila* PREs.[15] For such test to be successful one has to devise a model system that provides enough material for biochemical experiments and allows to assay the consequences of the complete loss of individual PRC complexes without concomitant activation of target genes by developmental signaling. The latter is critical to separate direct relations between PRC1 and PRC2 from possible indirect effects caused by transcription and action of trithorax Group (trxG) proteins.[48-52] To this end, we derived cultured cell lines from *Drosophila* embryos deficient for closely related *Psc* and *Su(z)2* genes that produce no Psc and Su(z)2 proteins as well as embryos homozygous for null mutation of *Su(z)12* gene. Although *Psc/ Su(z)2* and *Su(z)12* deficient flies die during embryogenesis when maternally supplied protein runs out, corresponding mutant cells are viable and proliferate in cell culture. This is fully consistent with the idea that the key role of PcG system is to support cell differentiation in the context of a multicellular organism.

In *Su(z)12* deficient cells no Su(z)12 protein is made, which leads to degradation of E(z) and possibly other PRC2 subunits. As a result, Su(z)12 minus cells completely lack H3K27me2 and H3K27me3.[15] Consistently, very little E(z) and no H3K27me3 or H3K27me2 can be detected at PcG target genes by Chromatin immunoprecipitation (ChIP). The loss of PRC2 and H3K27 methylation does not affect the overall levels of PRC1 and H2AK118ub.[15] Surprisingly, in Su(z)12 minus cells, despite complete absence of PRC2 and H3K27me3, PRC1 is still targeted to PREs. This finding argues that, at least in case of *Drosophila*, the models that place PRC2 and H3K27me3 at the base of recruitment hierarchy are not valid.

In *Psc* and *Su(z)2* deficient cells, corresponding proteins are not produced. This is accompanied by severe reduction in the level of Pc and 4-fold reduction in the level of RING1 proteins.[15] Since the amount of

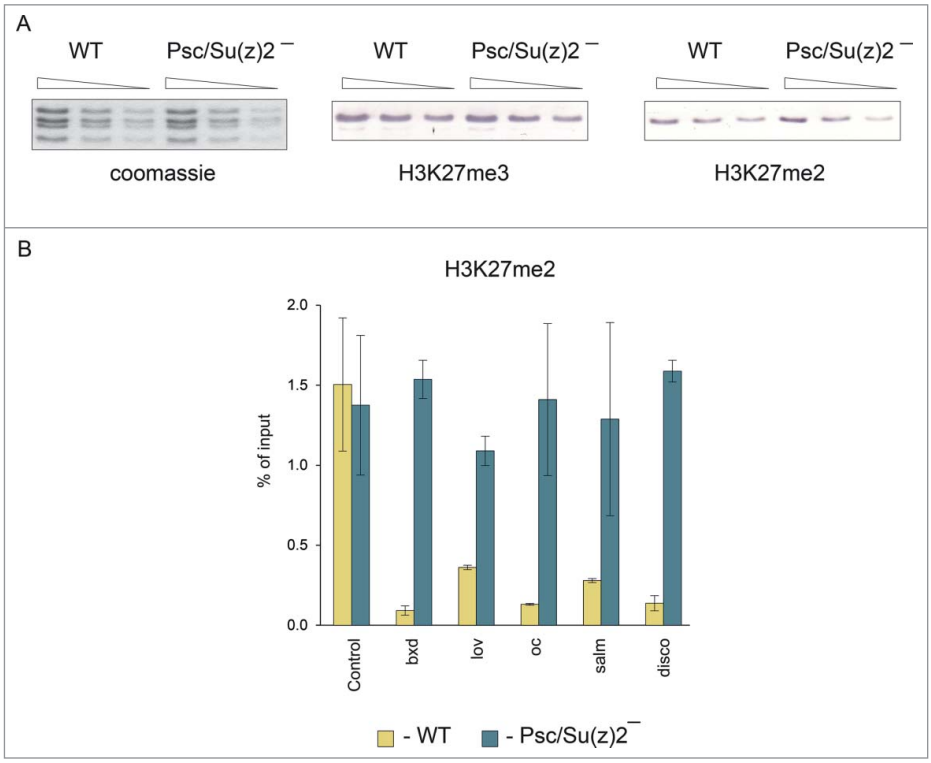

Figure 1. PRC1 is not required for untargeted methylation of H3K27 by PRC2. A. Two-fold dilutions of acid-extracted histones from control Ras3 and Psc/Su(z)2 deficient cells were analyzed by western-blot with antibodies against H3K27me2 and H3K27me3. The loss of Psc/Su(z)2 causes no significant reduction of the overall H3K27me2/me3, which suggests that much of the bulk H3K27me3 is produced by untargeted PRC2 activity and that the integrity and the activity of PRC2, *per se,* do not require PRC1. B. Di-methylation of H3K27 in the vicinity of PREs as measured by ChIP-qPCR. Histograms show the mean of 2 independent experiments with error bars indicating the scatter. Note that the loss of H3K27me3 in the PRC1 deficient cells allows di-methylation of H3K27 by untargeted PRC2. The high levels of H3K27me2 at "Control" amplicon from transcriptionally inactive intergenic region on Chromosome 3L are similar in wild type (WT) and Psc/Su(z)2 deficient cells. The data in (A) and (B) are from Kahn et al.[15]

corresponding transcripts is not altered, this suggests that the majority of RING1 is in the complex with Psc or Su(z)2 and that, in their absence, PRC1 gets degraded. In contrast, the loss of Psc or Su(z)2 does not affect the overall levels of PRC2.[15] Surprisingly, ChIP assay indicates that, in the absence of PRC1, PRC2 binding to roughly 2 thirds of all PREs is significantly reduced (hereafter referred to as PRC2:PRC1-dependent PREs).[15] This argues that most PREs have to bind PRC1 first to efficiently anchor PRC2. However, the fact that one third of PREs retain PRC2 binding in PRC1 deficient cells (hereafter referred to as PRC2:PRC1-independent PREs) implies that there are multiple ways to coordinate the PRC1 and PRC2 recruitment (Fig. 2). Genome-wide survey of PRC2 loss in PRC1 deficient cells indicates that PRC2:PRC1-dependent and PRC2:PRC1-independent pathways to recruit PRC2 to PREs are not mutually exclusive.[15] It appears that at different PREs the 2 pathways can combine their inputs and contribute to different degrees (Fig. 2).

PRC2 methylates H3K27 by 2 distinct mechanisms. The bulk of the PRC2 activity is dedicated to pervasive di-methylation and sporadic tri-methylation of H3K27 throughout the entire transcriptionally inactive genome.[16] This process is untargeted and does not require stable binding of PRC2. It can be viewed as a hit-and-run action of PRC2 complexes free-floating in the nucleoplasm. The other PRC2 activity relates to extensive tri-methylation of H3K27 around PREs. Instructive, the loss of PRC1 has no significant effect on the level of bulk H3K27me2 and causes only small reduction of the overall H3K27me3 (Fig. 1A). This argues that global hit-and-run methylation of H3K27 by PRC2 is independent of PRC1 and that significant amount of tri-methylated H3K27 is scattered throughout transcriptionally inactive genome.

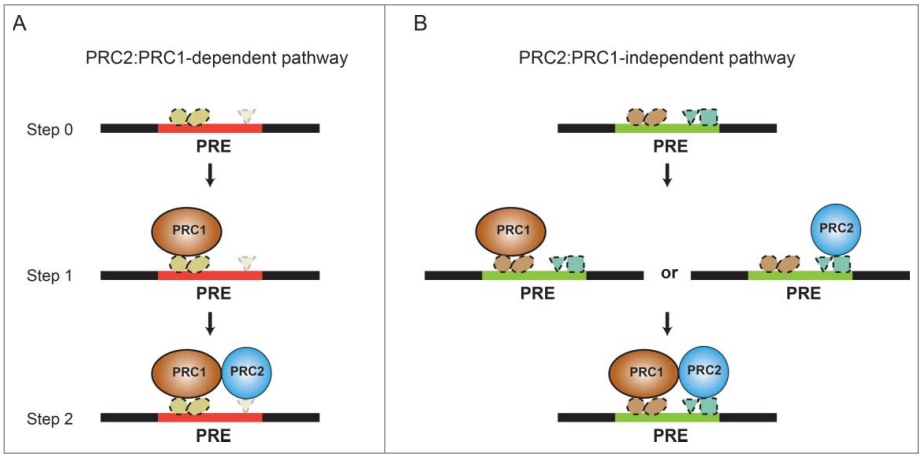

Figure 2. Revised model for hierarchical recruitment of Polycomb complexes. More than one pathway is used to recruit PRC2 to PREs. A. PRC2:PRC1-dependent pathway. At step 0, a PRE is bound by DNA-binding adaptor proteins (dashed circle and oval) that can cooperatively interact with PRC1 and anchor it. The PRE may also bind adaptor proteins that can interact with PRC2 (dashed triangle) but are not sufficient to anchor it. At step 1, PRC1 is recruited to the PRE. The recruitment of PRC2 at step 2 may require direct interaction with PRC1 and step 1 might be very transient. B. PRC2:PRC1-independent pathway. At step 0, DNA-binding adaptor proteins specific for both PRC1 (dashed circle and oval) and PRC2 (dashed triangle and square) bind the PRE. This is followed by independent recruitment of PRC1 and PRC2 via cooperative interactions with corresponding sets of adaptor proteins. Eventually, at step 2, the PRE is occupied by both PRC1 and PRC2. Step 1 might be transient and PRC1 and PRC2 may bind to the PRE stochastically or simultaneously. Genomic analysis indicates that the PRC2:PRC1-dependent and –independent pathways are not mutually exclusive. At many PREs both pathways contribute to PRC2 recruitment however their relative contributions vary between different sites.

In the absence of PRC1, chromatin around PRC2:PRC1-dependent PREs loses H3K27me3.[15] However, in contrast to PRC2 deficient cells, it gains H3K27me2 (Fig. 1B). This argues that PRC2 has to be anchored at PcG target genes to produce high density of H3K27me3 nucleosomes, which is consistent with *in vitro* preference of PRC2 for less methylated substrates (i.e., the H3K27me0 -> H3K27me1 -> H3K27me2 reaction is 6 times faster than H3K27me2 -> H3K27me3).[53]

Since PRC2:PRC1-dependent PREs are more common, the transgenic tests performed throughout the years were naturally confined to this group. From these tests we know that PRC2:PRC1-dependent PREs are able to autonomously recruit PRC1 and PRC2 complexes and repress reporter genes in stochastic clonally heritable fashion.[38] To determine if PRC2:PRC1-independent PREs are different in this respect, we generated a series of transgenic fly lines with reporter constructs containing both classes of PREs. These experiments suggest that PRC2:PRC1-dependent and PRC2:PRC1-independent PREs are equally efficient in recruitment of PcG complexes, forming a domain of H3K27me3 and repressing the *white* reporter gene.[15] What could be then a rationale for existence of PREs with different degrees of PRC2:PRC1-dependence if they provide the same level of repression? Although this could be a simple coincidence, the 2 modes of PRC2 recruitment may have evolved to fine-tune the robustness of PcG repression. While PcG proteins are ubiquitous and most of PcG target genes are repressed in any given cell, a subset of these genes has to be transcriptionally active in a cell-lineage specific manner.[30,54,55] This plasticity relies on the function of Trithorax Group (trxG) proteins which antagonize PcG repression. For a handful of PREs, all of which happen to be of PRC2:PRC1-dependent kind, we know that a transient activation of their target gene leads to "switching" of a PRE into a Trithorax-dependent elements that stimulates transcription.[50,56,57]In some cases, such switch is accompanied by displacement of PcG complexes from PREs.[30,58] It is, therefore, tempting to speculate that PRC2:PRC1-dependent and -independent PREs may have different susceptibility to the action of trxG proteins and, perhaps, that PRC2:PRC1-independent PREs cannot be "switched" to a transcription stimulating mode.

Histone modifications and PcG repression

Tri-methylation of H3K27 is an essential feature of PcG repression. Genes repressed by PcG mechanisms get embedded within broad domains of H3K27me3[30-32]

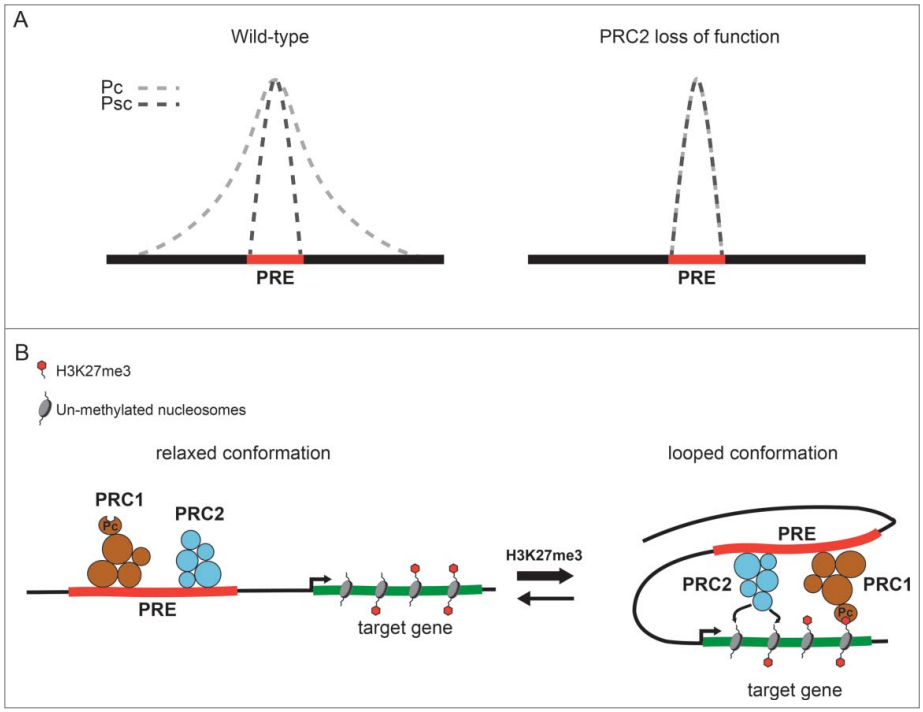

Figure 3. Tri-methyl H3K27 stabilizes PcG contacts with chromatin around PREs. A. When assayed by ChIP, Pc (light gray dashed line) but not the other PRC1 subunits, for example Psc (dark gray dashed line), shows immunoprecipitation profile that extends from PREs into the neighboring chromatin (left). Upon PRC2 loss, the Pc ChIP signals become confined to PRE cores (right). B. Polycomb group (PcG) complexes anchored at PREs can transiently loop out and interact with the chromatin of neighboring promoters, enhancers and transcription units where PcG complexes may interfere with transcription. Interactions between Pc and H3K27me3 seem to stabilize the looping, which shifts the equilibrium toward looped conformation. Assisting transcriptional repression, this may also help to restore high density of H3K27me3 after DNA replication.

and replacement of Lysine 27 of histone H3 to unmethylatable Alanine or Arginine (H3K27A or H3K27R) recapitulates the *HOX* gene misexpression and homeotic transformations, characteristic of PRC2 loss of function.[59,60] Moreover, recent studies provide genetic evidence that H3K27me3 is part of a short-term epigenetic memory whose replenishment requires PRC2 anchored to PRE.[41,42] Yet, our test of interdependence of PRC1 and PRC2 binding to PREs indicate that H3K27me3 is dispensable for PRC1 targeting. What is then the role of H3K27me3? Although it is likely to contribute to repression in multiple ways, one role of H3K27 methylation is to stabilize the interaction of PRE-anchored PcG complexes with surrounding chromatin. It has been noted for some time that PRC1 subunits give sharp peaks of ChIP signal over PREs. The exception from this rule is Polycomb (Pc) protein (Fig. 3A). While ChIP signals for Pc are also highest at PREs they spread as lower signal tails into surrounding chromatin.[31,35,36,61] The tails of Pc ChIP signals were proposed to reflect the direct interactions between H3K27me3-containing nucleosomes and Pc chromodomain[30,61] (Fig. 3B). Validating this model, we found that in PRC2 deficient cells, where tri-methylation of H3K27 at PcG target genes is ablated, Pc signal at core PREs is only slightly reduced but this signal drops to a background level at distance from PREs[15] (Fig. 3A). We speculate that prolonged interaction of the PRE-anchored PcG complexes with surrounding chromatin are integral part of "reading" the H3K27me3 memory mark. By making looping interactions more stable, H3K27me3 would deliver PcG complexes to enhancers, promoters and transcription units of target genes where the complexes can interfere with transcription. At the same time, stable loops between PREs and neighboring chromatin would help PRC2 to produce high level of H3K27me3 around PREs and quickly replenish the methylation after repressed genes undergone DNA replication (Fig. 3B).

While tri-methylation of H3K27 is, no doubt, a key component of PcG repression, the implication of H2AK118 ubiquitylation is less clear. Initial studies suggested that H2AK119ub (mammalian analog of fly

H2AK118ub) directly blocks transcription elongation.[62] Later, H2A119ub was reported to recruit PRC2 in mouse ES cells.[44,63] However, recent studies including our own indicate that, at least in *Drosophila*, H2AK118 ubiquitylation is unlikely to play a role as critical as that of H3K27 methylation. First, we found that in the absence of H2A118ub one third of the PREs is still able to recruit PRC2.[15] Second, we saw that repressed *Antennapedia* and *Bithorax* complex homeotic gene clusters display very low level of H2A118ub.[15,16] Third, flies severely impaired for their ability to ubiquitylate H2A118 were reported to have no homeotic transformations and display no misexpression of *engrailed* and *HOX* genes.[9] In line with these observations, it appears that the bulk of H2AK118ub is produced by RING1-L(3)73Ah complex,[15,16] and that many genomic regions enriched in H2AK118ub are not appreciably bound by PRC2 or PRC1 or tri-methylated at H3K27.[16] The latter argues that, in flies, H2AK118ub is not sufficient to recruit PRC2 and that most of the H2AK118ub is unrelated to PcG regulation. Perhaps the most surprisingly, the steady-state level corresponds to just 15% of H2AK118 ubiquitylation produced by RING1-L(3)73Ah complex as most of H2AK118ub is being continuously removed by deubiquitylase activity of PR-DUB complex.[15]

Outlook

The direct test of the interdependence between PcG complexes for recruitment to *Drosophila* PREs changes our view of how PcG repression is targeted. It refutes some of the popular hierarchical recruitment models and places histone modifications downstream of the targeting step. It also raises some exciting possibilities and important new questions. The core components of PRC1 and PRC2 are evolutionary conserved and so are many of the target genes. This makes it highly likely that at least some parts of the molecular mechanism that targets PcG repression to specific genes are common between *Drosophila* and vertebrates. As PREs that require PRC1 binding to recruit PRC2 are far more common, it is tempting to speculate that the use of compact DNA elements to recruit PRC1 may be that evolutionary ancient feature of the targeting. It would be very interesting to test this possibility experimentally.

Linked to this, is the question of how PRC1 promotes the binding of PRC2. One possibility is that PRC1 and PRC2 interact directly, although this interaction must be relatively weak as the 2 complexes appear as separate entities in biochemical purifications. This possibility is supported by observations that the Esc subunit of PRC2 co-immunoprecipitates with PRC1 from nuclear extracts made from early *Drosophila* embryos[64] and that Scm subunit of PRC1 can be recovered as an interactor of PRC2 after formaldehyde crosslinking.[65]More complex scenarios are also possible. For example, the binding of PRC1 to PREs may stabilize the binding of sequence specific adaptor proteins implicated in PRC2 recruitment, much the same way as PRC1 presence enhances the binding of PhoRC complex.[5]

As H3K27 methylation appears to function downstream of the targeting, it raises the question of whether it is directly involved in transcriptional repression and, more generally, how do PcG proteins repress transcription? Here it would be interesting to explore whether H3K27me3-stabilized looping contacts between PREs and the chromatin of the neighboring genes may directly inhibit their transcription.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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