

## Review

# Polycomb-mediated 3D chromatin interactions in gene regulation

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The Polycomb repressive system is a conserved, chromatin-based transcriptional regulator that is essential for the normal development of multicellular organisms. Polycomb-group proteins catalyze repressive histone modifications and organize 3D chromatin interactions. While the relationship between Polycomb-deposited histone modifications and gene repression is well-established, how Polycomb-mediated 3D chromatin interactions contribute to gene regulation remains poorly understood. In this review, we discuss current insights into the formation of these interactions and potential models for how they contribute to Polycomb-dependent gene regulation.

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## Introduction

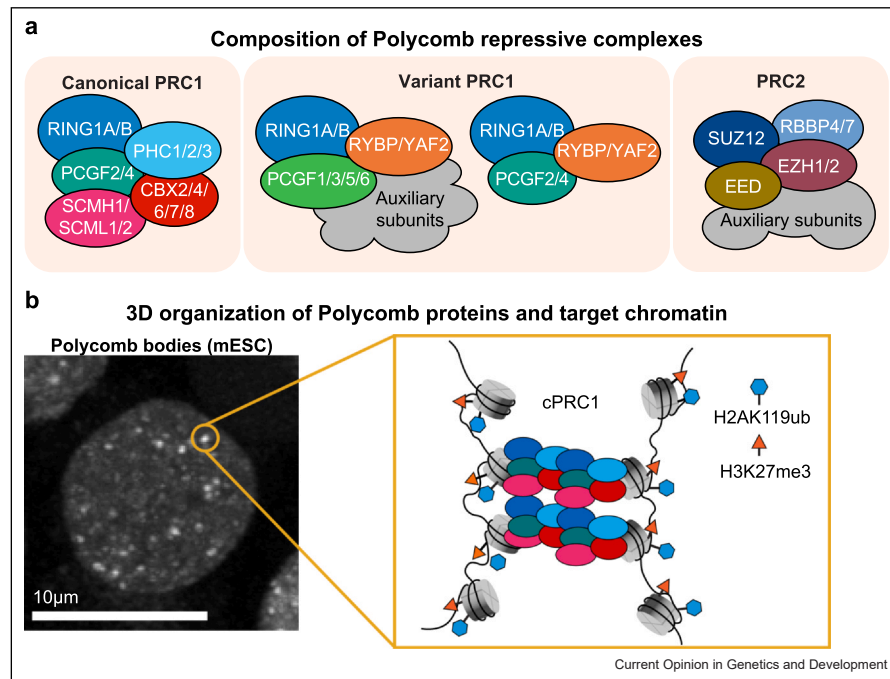
Polycomb group (PcG) proteins ensure proper development of multicellular organisms by establishing and maintaining repression of lineage-specific genes [1–3]. PcG proteins are important for maintaining pluripotency in stem cells and determining cell identity during differentiation, and mutations in these proteins are

associated with developmental abnormalities and cancer [4]. PcG proteins were first discovered in *Drosophila melanogaster* over 75 years ago through analyses of mutations that resulted in altered body segmentation due to Homeotic (*Hox*) gene dysregulation [2–4]. Subsequent work identified mammalian PcG orthologs [2], establishing them as a conserved system for transcriptional repression.

PcG proteins assemble into two major complexes: Polycomb Repressive Complex 1 (PRC1), which catalyzes monoubiquitylation of histone H2A (H2AK119ub) [1–3], and Polycomb Repressive Complex 2 (PRC2), which catalyzes trimethylation of histone H3 lysine 27 (H3K27me3) [1–3,5] (Figure 1a). In mammals, there are multiple paralogs for each subunit, which combinatorially form diverse complexes [1–3,6,7]. PRC1 complexes are broadly divided into canonical PRC1 (cPRC1) and variant PRC1 (vPRC1) depending on the inclusion of either CBX proteins or RYBP/YAF2, respectively [6]. cPRC1 forms around PCGF2/4, while vPRC1 can contain any PCGF protein. vPRC1 is more catalytically active than cPRC1 [6,8,9], whereas cPRC1 is believed to drive the formation of nuclear foci called Polycomb bodies and mediate higher-order chromatin interactions [10–12] (Figure 1b). cPRC1-mediated 3D chromatin structures include both long-range interactions between distal Polycomb chromatin domains and local chromatin compaction (Figure 2) [6,10–21]. Establishment and maintenance of these Polycomb chromatin domains, which exhibit PcG protein occupancy and the associated histone modifications, rely on the interplay between the PRC1 and PRC2 complexes: vPRC1 binds to target regions through direct DNA interaction and deposits H2AK119ub [1–3]. PRC2 binds to H2AK119ub through an auxiliary subunit [1–3], in turn depositing H3K27me3. cPRC1 subsequently binds H3K27me3 through its CBX subunit [5,22–24] or by recognizing PRC2 in an H3K27me3-independent manner, as emerging evidence suggests [25,26].

Although the importance of vPRC1- and PRC2-mediated histone modifications in Polycomb gene repression is well-established [9,27–31], the role of cPRC1-mediated 3D chromatin interactions is less clear. 3D chromatin interactions driven by other mechanisms have been proposed as a key component of transcriptional

Figure 1



Composition and organization of Polycomb repressive complexes. **(a)** Schematic of canonical PRC1 (cPRC1), variant PRC1 (vPRC1), and PRC2 complexes. Only core protein names are shown for clarity. Grey ovals represent auxiliary subunits (excluding cPRC1, for which all subunits are named). cPRC1 contains RING1A or B, PCGF (Polycomb group RING finger) 2 or 4, PHC (Polyhomeotic) 1, 2, or 3, CBX (Chromobox) 2, 4, 6, 7, or 8, and SCM1 (Sex comb on midleg homolog 1) or SCML1/2 (Sex comb on midleg-like) 1 or 2. vPRC1 contains RING1A or B, PCGF1–6, RYBP (RING1 and YY1-binding protein) or its paralog, YAF2 (YY1-associated factor 2). PCGF2/4-containing vPRC1 does not have any identified accessory proteins. PRC2 core contains EZH (Enhancer of Zeste), EED (Embryonic ectoderm development), SUZ12 (Suppressor of Zeste 12), and RBBP (Retinoblastoma-binding protein) 4 or 7. **(b)** Organization of PcG proteins. PcG proteins form bright nuclear foci (Polycomb bodies) in mammalian cells (left), which are thought to be sites where cPRC1-mediated 3D chromatin interactions occur (right). Image: Endogenous RING1B was tagged and labeled with HaloTag using CRISPR/Cas9 in mouse embryonic stem cells as described in [34], and imaged on a Zeiss Airyscan LSM900 microscope (maximum intensity projection of one representative nucleus is shown).

regulation. Putative mechanisms include driving communication between regulatory elements and genes, establishing boundaries to prevent inappropriate information transfer, and limiting chromatin accessibility by compaction for gene repression [32,33]. Similarly, several roles have been suggested for Polycomb-mediated 3D chromatin interactions in contributing to Polycomb system function. Several recent reviews discuss PRC components and biochemical function in detail [1–3]; in this review, we focus on Polycomb-mediated 3D chromatin interactions, discussing recent work that challenges classical models of Polycomb repression and proposing additional models for how these interactions may contribute to Polycomb system function.

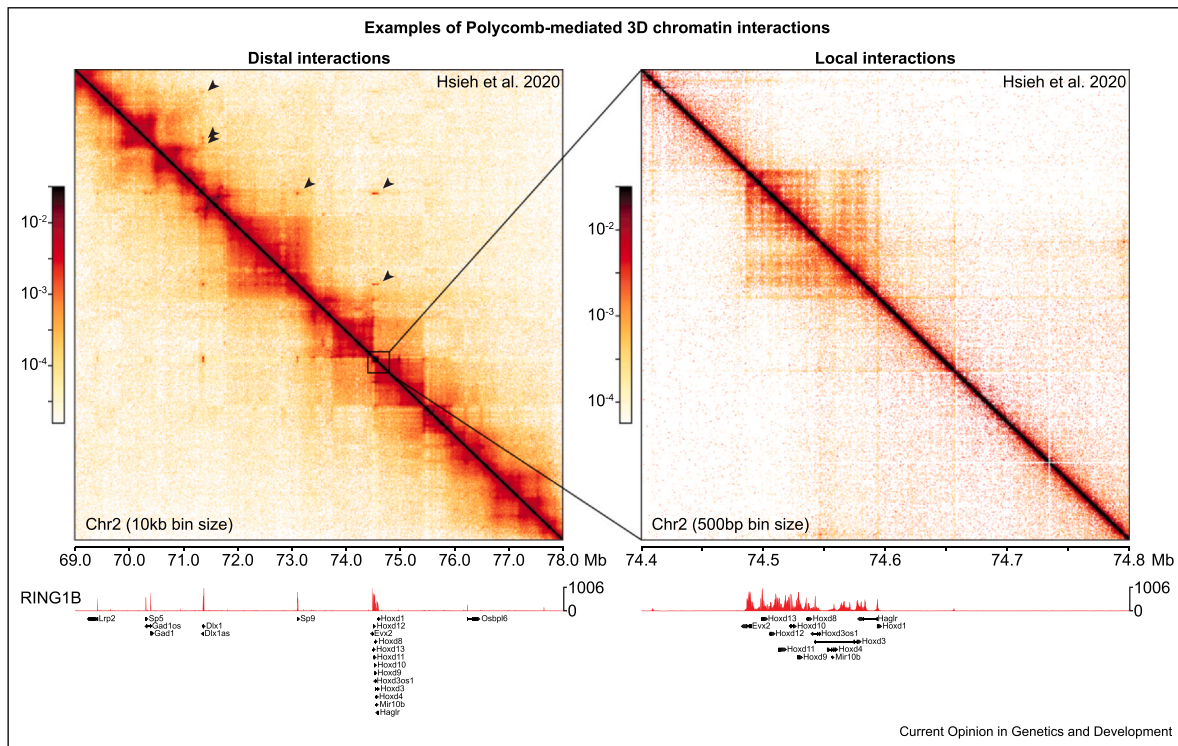
### Polycomb subunits that organize 3D chromatin interactions

In mouse embryonic stem cells (mESCs), vPRC1 deletion results in a large number of differentially expressed genes, whereas removing cPRC1 has a minor impact on gene expression [9]. Despite this finding, cPRC1 is critical for proper development [4] (Table 1). Further,

PCGF2-containing cPRC1 is highly abundant in mESCs, suggesting functional importance [7,34]. Several studies suggest cPRC1 as the mediator of 3D chromatin interactions [10–12], with key evidence coming from a study using a synthetic tethering system: Tethering PCGF2 to an artificial locus in the genome is sufficient to recruit cPRC1 and form chromatin contacts with endogenous Polycomb loci, and inducing PCGF2 and PCGF4 deletion in mESCs results in significant loss of Polycomb chromatin interactions [19]. Because PRC2 recruits cPRC1 [5,22,23,25,26], these interactions are also dependent on PRC2, which is consistent with findings in *Eed*<sup>-/-</sup> mESCs showing a lack of Polycomb interactions [14,17,35]. Whether PRC2 can contribute to Polycomb interactions independently of cPRC1 is unclear and would require studies to assess PRC2 function in the absence of cPRC1.

How does cPRC1 mediate 3D chromatin interactions? Biochemical analyses show that PCGF, PHC, and CBX proteins that comprise cPRC1 have the capacity to oligomerize, polymerize, and compact chromatin or form

Figure 2



Polycomb-target loci form 3D chromatin interactions Polycomb-mediated 3D chromatin interactions are visible in chromosome conformation capture (Micro-C) data in mouse embryonic stem cells. Polycomb mediates distal (left) and local (right) interactions at sites with RING1B ChIP-seq signal. Distal interactions are marked with arrowheads. Colormaps indicate contact frequency. Micro-C data are from GSE130275 [18] and RING1B ChIP-seq tracks are from GSE119619 [9]. Only Polycomb-target gene annotations are shown for clarity.

condensates, respectively [36,37]. The biochemical characterizations and developmental relevance of these proteins are summarized in Table 1. PCGF2/4 has a ubiquitin-like domain, which has been shown to simultaneously allow homo-oligomerization and interaction with PHC2 [36], though whether this domain has any role in mediating chromatin contacts is unknown. PHC proteins are speculated to mediate chromatin contacts by polymerizing *via* their sterile alpha motif (SAM) domain [10,11,38–41]. PHC1-knockout mESCs exhibit decompacted *Hox* loci as measured by both genomics and super-resolution imaging [11], and *Hox* loci are farther apart in mouse embryonic fibroblasts expressing polymerization-deficient PHC2 mutant [10], consistent with PHC proteins playing a key role in forming 3D chromatin interactions *via* their SAM domains. Mixing nucleosome arrays *in vitro* with mammalian PRC1 components, including PHC2, results in large, branched condensates consistent with polymerization [41]. However, arrays mixed with PHC1 form round condensates, suggesting that polymerization may vary between the PHC paralogs [41]. As the PHC paralogs are differentially expressed depending on the cell type, the role of SAM domain polymerization in Polycomb function may also be cell-type specific. PHC1 is more highly

expressed than PHC2 or 3 in embryonic stem cells [7,11,42], raising the possibility that Polycomb chromatin interactions in mESCs may not be primarily driven by polymerization. In addition to the PHC proteins, mammals express three SCM proteins (SCMH1, SCML1/2), which also each contain a SAM domain. While this domain appears capable of polymerizing [39,43], these proteins are poorly studied, and their contribution to 3D chromatin interactions remains unknown.

Of the five CBX paralogs in mammals (Figure 1a), all except CBX7 have a compaction and phase separation (CaPS) domain, which can mediate condensate formation and nucleosome compaction *in vitro* [41,44–47]. In mESCs and in other multipotent cell types, CBX7 is the most highly expressed paralog, while other paralogs are expressed at higher levels in more differentiated cell types [7,42,48,49]. Despite the low expression of CaPS domain-containing CBX proteins, CaPS-mediated chromatin compaction is important for Polycomb function: Inducing wildtype CBX2 expression in *Cbx2*<sup>-/-</sup> mESCs results in stronger gene repression than inducing a compaction-deficient CBX2 mutant [50], mESCs endogenously expressing chimeric CBX7-CaPS protein

Table 1

## Summary of cPRC1 protein subunits, their domains, and their mutant phenotypes.

Protein	Notable domains	Impact of deletion or mutation in mammalian development	Impact of deletion or mutation in cell culture
PCGF2, 4	<ul style="list-style-type: none"> <li>Dimerizes with RING1A/B to form a catalytic dimer <i>via</i> RING domain [1]</li> <li>Ubiquitin-like domain allows homooligomerization and interaction with PHC2 [36]</li> </ul>	<ul style="list-style-type: none"> <li><i>Pcgf2<sup>-/-</sup></i> or <i>Pcgf4<sup>-/-</sup></i> knockout mice exhibit posterior transformations [76]</li> <li><i>Pcgf2<sup>-/-</sup>; Pcgf4<sup>-/-</sup></i> double knockout mutation is lethal at 10.5 dpc (days post coitum) [76]</li> <li>Individuals with a missense <i>PCGF2</i> mutation exhibit impaired growth, intellectual disability, and skeletal, cardiovascular, and neurological abnormalities [77]</li> </ul>	<ul style="list-style-type: none"> <li>Conditional <i>Pcgf2/4</i> double deletion leads to decreased PRC1 occupancy but minimal changes to gene expression in mESCs [9,74]</li> </ul>
PHC1, 2, 3	<ul style="list-style-type: none"> <li>Head-to-tail polymerization <i>via</i> their sterile alpha motif (SAM) domain [38–40]</li> </ul>	<ul style="list-style-type: none"> <li><i>Phc1<sup>-/-</sup></i> mice are perinatal lethal [10]</li> <li><i>Phc2<sup>-/-</sup></i> mice are viable and fertile [78]</li> <li>Severity of developmental defect scales with gene dosage: <i>Phc2<sup>-/-</sup></i>; <i>Phc1<sup>+/-</sup></i> mice are perinatal lethal [78]</li> <li>Severe defects by 9.5 dpc in <i>Phc2<sup>-/-</sup></i>; <i>Phc1<sup>-/-</sup></i> double knockout embryos [78]</li> <li>Mice with <i>Phc2</i> SAM-domain polymerization mutation exhibit posterior transformations [10]</li> </ul>	<ul style="list-style-type: none"> <li>PHC2 SAM-domain polymerization mutation results in decreased PcG occupancy and increased gene expression in HCT116 cells [11] and mouse embryonic fibroblasts [10]</li> <li>Differential gene expression in <i>Phc1<sup>-/-</sup></i> mESCs [11]</li> </ul>
CBX2, 4, 6, 7, 8	<ul style="list-style-type: none"> <li>All except CBX7 have CaPS (compaction and phase separation) domain, which mediates chromatin compaction and condensate formation [22,26]</li> <li>Chromodomain allows recruitment to H3K27me3 [5,22–24]</li> </ul>	<ul style="list-style-type: none"> <li><i>Cbx2<sup>-/-</sup></i> mice exhibit homeotic transformations and postnatal lethality [79]</li> <li><i>Cbx2</i> c-terminal mutant mice exhibit homeotic transformations and sex reversal [52]</li> <li>Report of sex reversal in an individual with <i>CBX2</i> mutations [80]</li> <li>Mice expressing compaction-deficient <i>CBX2</i> exhibit anterior-to-posterior transformations [50,52]</li> </ul>	<ul style="list-style-type: none"> <li>Differentiating <i>Cbx2<sup>-/-</sup></i> mESCs to neuronal progenitor cells (NPCs) yields fewer cells compared to wildtype mESCs [63]</li> <li>Differentiating <i>Cbx2<sup>-/-</sup></i> mESCs to embryoid bodies (EBs) results in smaller EBs [42,63].</li> <li>Defective EB differentiation from <i>Cbx7<sup>-/-</sup></i> mESCs [42,51]</li> </ul>

Notable domains describe those that are important for establishing Polycomb chromatin domains or mediate 3D chromatin interactions. Impact of deletion or mutation columns describes the consequences of deleting or mutating the subunit in mammalian development or in cultured mammalian cells.

Abbreviations: mESCs, mouse embryonic stem cells.

exhibit reduced differentiation capacity [51], and overexpressing CaPS domain-containing CBXs in hematopoietic stem cells results in reduced self-renewal ability [49]. Additionally, introducing a compaction-deficient mutation in *CBX2* in a mouse model results in anterior-to-posterior transformations similar to those observed in *Cbx2<sup>-/-</sup>* mice [50,52], suggesting that *CBX2*-dependent chromatin compaction is important in axial patterning during development. Recent *in vitro* work indicates that the combination of PHC and CBX paralogs determines the physical properties of cPRC1 [41]; future studies are still necessary to dissect the cell-type dependency of these proteins and how their polymerization and compaction or condensate-forming properties contribute to gene repression in various contexts.

### Polycomb-mediated interactions observed by genomics and imaging

Early evidence for higher-order Polycomb-mediated chromatin interactions came from studies in *Drosophila*. Imaging *Hox* gene clusters with FISH (3D fluorescence

*in situ* hybridization) revealed physical proximity between Polycomb binding sites that correlated with gene repression [53–55]. Chromosome conformation capture experiments further revealed that the *Hox* gene clusters form a network of contacts spanning more than 10 megabases (Mb) that are dependent on the protein polycomb (mammalian CBX) and H3K27me3 [54,55]. Deleting a binding site within one *Hox* cluster decreased chromatin contacts and resulted in derepression of interacting genes, supporting a functional role for these 3D interactions in gene repression [54]. With the development of Hi-C, these contacts were revealed to occur genome-wide at repressed gene promoters bound by PcG proteins [16,56].

Interactions between Polycomb-occupied chromatin regions are also observed in mammalian cells (Figure 2). PcG proteins compact *Hox* gene clusters [10–12,17,20,57] and mediate long-range interactions between target genes [14–16,18,21,35]. Impressively, these long-range interactions can span upwards of

several tens of megabases [15,16,18,21]. Comparison of Hi-C maps in mESCs, neural progenitor cells, and neurons reveals that these interactions weaken during differentiation and largely disappear in terminally differentiated cells [16], potentially indicating a link between loss of chromatin interactions and gene derepression. However, the relationship between 3D Polycomb-mediated chromatin interactions and gene repression is not a simple correlation: unlike primed state mESCs grown in serum, in which most studies have been conducted, naïve pluripotent state mESCs cultured with two small molecule inhibitors (2i) do not exhibit *Hox* compaction or long-range interactions despite Polycomb-target genes being repressed [35,58]. As naïve 2i mESCs represent an earlier stage of development than serum-grown mESCs [35,58], these chromatin contacts appear to transiently form during early development prior to lineage commitment, raising a possibility that they may be important for poising genes for expression.

In mammalian cells, chromatin interactions are believed to be driven by loop extrusion and affinity-based interactions [59]. Block copolymer modeling subject to affinity interactions or protein-mediated interactions effectively recapitulates the experimentally observed chromatin organization of Polycomb-occupied regions, supporting the concept that Polycomb chromatin interactions rely on protein-mediated affinity between the interacting sites. Modeling and experimental data suggest that loop extrusion antagonizes affinity-mediated interactions [59,60]. Consistent with this idea, weakening loop extrusion by depleting cohesin strengthens Polycomb interactions [61], while strengthening loop extrusion weakens Polycomb interactions [62]. Collectively, these studies point to protein-mediated affinity as the organizing principle for Polycomb chromatin interactions and further underscore the importance of understanding how Polycomb proteins drive such affinity.

Polycomb proteins have also been observed to form into nuclear foci, termed Polycomb bodies, in imaging experiments [10,34,47,63–66] (Figure 1b). Polycomb bodies differ in size and morphology, and have been speculated to be sites where Polycomb-mediated interactions occur [10]. Supporting this idea, a higher proportion of RING1B molecules is stably bound in Polycomb bodies than in the nucleoplasm [34,67], suggesting that they represent sites of high PcG protein occupancy at target chromatin that can form 3D interactions. Further, using FISH and immunofluorescence staining, *Hox* genes have been shown to colocalize with Polycomb bodies in both *Drosophila* and mammalian cells [10,54]. A caveat with these experiments, however, is that they were performed with fixation and denaturing FISH protocols, which can alter genomic structure [68]. Future studies to understand the composition of

Polycomb bodies and their relevance to genome-wide 3D chromatin interactions will provide important insights regarding their function.

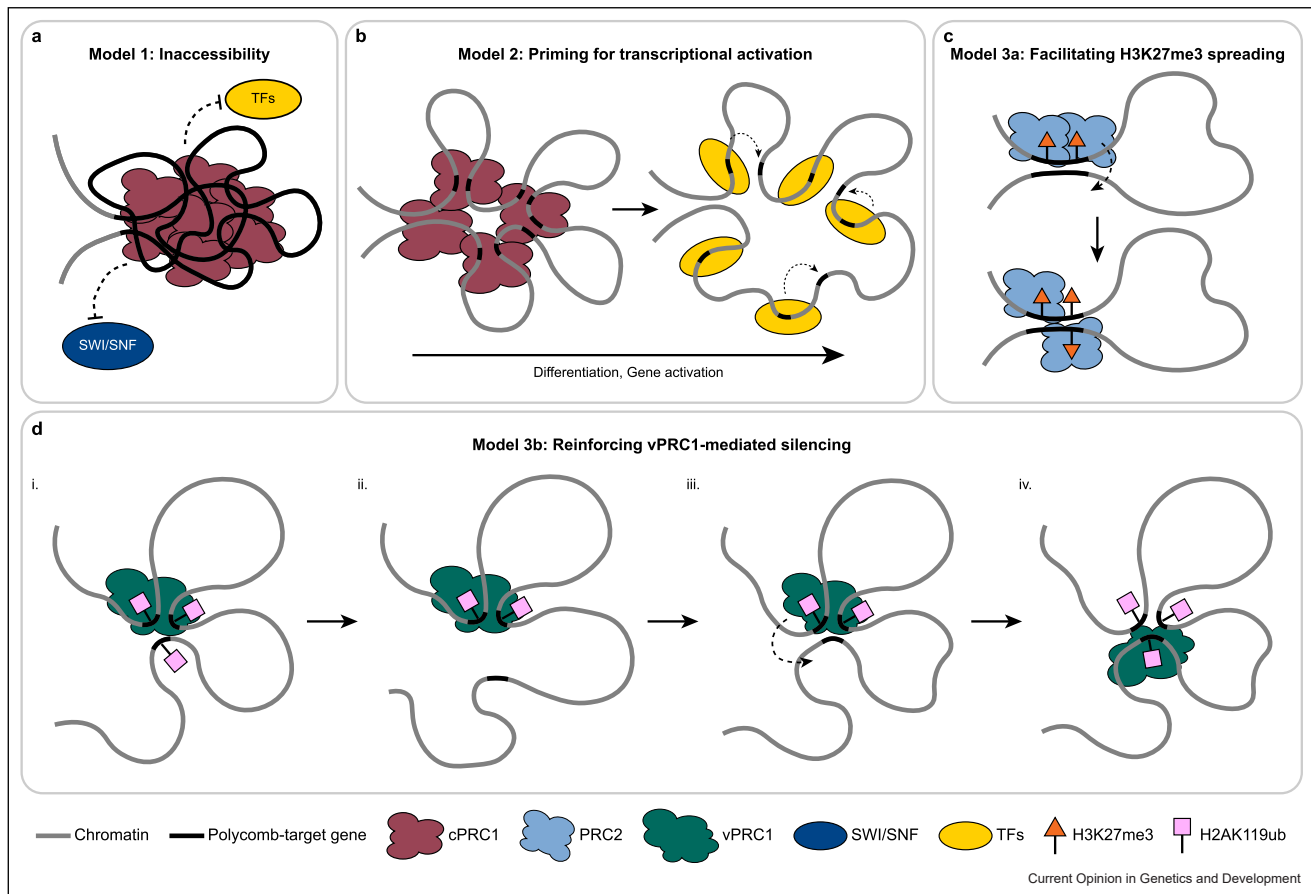
### Models for how 3D chromatin interactions contribute to Polycomb system function

Many studies have proposed ways in which 3D chromatin interactions may contribute to Polycomb function. In this section, we discuss four potential, non-mutually exclusive models (Figure 3). Although not discussed as a model, we also note that these chromatin contacts may form due to the propensity of cPRC1 subunits to form self-interactions without serving a functional role.

#### Model 1: inaccessibility model

The earliest proposed mechanism for how Polycomb-mediated chromatin interactions contribute to gene repression is through PcG-bound chromatin compaction, blocking access of transcription factors and chromatin remodelers to gene promoters [1,37]. This model is supported by *in vitro* work using purified PcG proteins and nucleosome arrays, which showed that cPRC1 compacts nucleosomes [13,41,44,46,47]. A recent study showed that SWI/SNF (SWItch/sucrose non-fermentable) is unable to bind nucleosome arrays occupied by cPRC1 *in vitro*, and that knocking out PHC2 in mammalian HCT116 cells results in increased SWI/SNF binding at Polycomb targets [47], consistent with previously reported SWI/SNF and PRC antagonism in mouse embryonic fibroblasts [69]. Furthermore, experiments where CBX2 or a CBX7-CaPS domain fusion was expressed in mESCs resulted in silencing of Polycomb-target genes, linking compaction to gene repression [50,51]. However, several studies argue against a simple inaccessibility model, at least in mESCs. While Polycomb-target gene promoters exhibit reduced accessibility compared to non-Polycomb genes, mESCs lacking PRC1, PRC2, or both complexes exhibit similar accessibility to wildtype mESCs [70], and increased Polycomb occupancy does not correlate with accessibility [71], indicating that PcG proteins do not directly confer decreased accessibility. Super-resolution chromosome tracing recently revealed that Polycomb-bound *Hoxa* genes are frequently in decompacted configurations despite being repressed in mESCs [20], directly arguing against the formation of a stably compacted chromatin structure. Additionally, a recent study showed that the Polycomb system reduces the frequency with which promoters enter a transcriptionally permissive state by countering the formation of the pre-initiation complex rather than constitutively blocking transcription [72]. Abolishing cPRC1 by rapidly depleting PCGF2/4 does not impact pre-initiation complex formation or transcription, demonstrating that transcription factor access to cPRC1-bound promoter is not blocked [72].

Figure 3



Putative models of how 3D chromatin interactions contribute to Polycomb function. **(a)** Model 1: Polycomb-bound loci may form compacted domains that are inaccessible to transcription factors (TFs) or chromatin remodelers such as SWI/SNF to drive gene repression. **(b)** Model 2: Polycomb-mediated chromatin interactions may bring target loci together to facilitate transcriptional activation during differentiation. In the pluripotent state, Polycomb-target genes form a hub of repression. During differentiation, TFs may be able to efficiently initiate transcription of genes that are in proximity to each other. **(c)** Model 3a: Interactions between distal chromatin loci may facilitate H3K27me3 spreading by facilitating PRC2 catalysis of H3K27me3 modifications on nearby nucleosomes. **(d)** Model 3b: Transient interactions may reinforce gene repression by ensuring vPRC1-mediated H2AK119ub deposition at all target sites despite low RING1B occupancy. i) Three Polycomb-target sites have H2AK119ub while one vPRC1 molecule is bound. ii) One of the sites loses H2AK119ub. iii) Due to the proximity of the sites, vPRC1 can unbind its current site, instead binding the site that has lost the H2AK119ub. iv) H2AK119ub is re-established.

An explanation for the conflicting data is that the role of compaction in Polycomb gene repression is cell-type specific. The aforementioned *in vitro* nucleosome array studies focus on CBX2 and PHC2, which are more highly expressed in differentiated cell types than in mESCs [42,48], in which the above studies have been conducted. *In vitro* studies estimate a ratio of one PRC1 complex per three nucleosomes for compaction [13]. Measurements of PRC1 abundance in mESCs suggest it is well below this level (0.1 molecules per 1 kb of RING1B-enriched chromatin) [34], and levels of CBX2 are even lower (~3 molecules of CBX2 per Polycomb body) [63]. Although total RING1B levels do not drastically change during differentiation, levels of CBX2/4/6/8 and PHC2/3 increase, as does the number of CBX2

molecules in Polycomb bodies (from ~3 to ~15) [7,48,63]. This raises the possibility that regulating chromatin accessibility may be a viable mechanism of repression at sites with high PcG protein occupancy in differentiated cells. Additionally, while these data argue against cPRC1-mediated compaction and resulting inaccessibility as a main mechanism of gene repression in mESCs, sparse PcG binding may still be sufficient to antagonize the recruitment of chromatin remodelers.

### Model 2: priming for transcriptional activation

Chromatin interactions between Polycomb target genes have been proposed to prime them for derepression during differentiation. In this model, genes can be derepressed simultaneously by allowing transcription

machinery to easily access multiple gene promoters in proximity. Many Polycomb-repressed genes exhibit H3K4 methylation, referred to as a bivalent chromatin state, which is proposed to mark these genes as poised for activation upon differentiation [2]. Polycomb was found to mediate contacts between gene promoters and poised enhancers (defined as having H3K27me3 and H3K4me1), and genes that form these contacts became derepressed upon PRC1 deletion as the associated poised enhancers become active, leading to the proposal that Polycomb interactions may contribute to poising target genes for activation [15]. Observations that Polycomb genes form 3D chromatin interactions in serum-grown ‘primed’ state mESCs but not in naïve state (2i) mESCs or in differentiated cells further supported this model [16,35,58]. However, separation of function studies to directly address the role of Polycomb chromatin interactions challenge this model. Deleting PCGF2/4 to abolish cPRC1, and thereby diminish Polycomb interactions, has little effect on transcriptional activation during retinoic acid differentiation, suggesting that contacts themselves do not facilitate gene derepression [19]. Rather, gene activation depends on cyclin-dependent kinase module Mediator complex, a non-canonical Mediator complex frequently found at Polycomb loci, through a cPRC1-independent mechanism [19]. Additionally, Polycomb chromatin interactions do not exclusively form at poised promoters, but rather correlate with RING1B occupancy, and an active gene that becomes repressed during differentiation can form new Polycomb-mediated chromatin interactions despite the lack of need for transcriptional poising [16]. Collectively, though priming of specific genes during some cell fate transitions cannot fully be ruled out, these data argue against a widespread role for Polycomb chromatin interactions in transcriptional priming.

### Model 3a: facilitating H3K27me3 spreading

An alternative model proposes that 3D chromatin interactions contribute to the spreading of H3K27me3 histone modification to establish classical Polycomb domains (PRC1 and PRC2 occupied regions) genome-wide [21,73]. Supporting this view, genetically deleting Polycomb genes alters H3K27me3 patterns both locally and distally [21]. Using *Eed*<sup>-/-</sup> mESCs in which EED repression can be induced to track *de novo* formation of H3K27me3 domains reveals that H3K27me3 modifications are nucleated at specific sites and subsequently spread to neighboring chromatin [73]. Deleting nucleation sites delays H3K27me3 domain formation when no pre-existing H3K27me3 is present, while synthetic recruitment of EZH increases H3K27me3 at interacting loci. These data raise the possibility that a few nucleation sites can lead to thousands of H3K27me3 domains through multiple transient interactions [73].

While this model is plausible, further work is needed to establish a connection between Polycomb interactions, domain establishment, and gene repression. Direct evidence that deleting nucleation sites impacts distal H3K27me3 spreading is lacking, and the proposed order of events — in which cPRC1-mediated interactions precede H3K27me3 spreading — is opposite of the current understanding of Polycomb domain establishment where cPRC1 is recruited to H3K27me3 or PRC2. Indeed, inducing PCGF2/4 deletion to abolish cPRC1 and disrupt Polycomb-mediated chromatin interactions in mESCs does not impact SUZ12 occupancy or H3K27me3 [9,74], indicating that cPRC1-mediated Polycomb interactions are not required for maintenance of H3K27me3. Yet, whether these chromatin contacts facilitate H3K27me3 spreading during *de novo* establishment of Polycomb domains remains unknown. Future studies to track *de novo* Polycomb domain formation in the absence of cPRC1-mediated chromatin interactions may elucidate whether these interactions indeed facilitate H3K27me3 spreading.

### Model 3b: reinforcing vPRC1 repression

Instead of a direct role in mediating gene repression, chromatin interactions may serve to reinforce fidelity of vPRC1-mediated H2AK119ub deposition, which is the main mechanism of Polycomb gene repression in mESCs [9,27,28]. Due to the similarity to the previous model, which also links Polycomb interactions with the spreading of its histone modifications, we term this Model 3b. Combining chromosome tracing and polymer modeling, a recent study found that Polycomb domains are often in decompacted states that are not significantly different from non-Polycomb loci, and proposed that this decompacted state is important for ensuring that epigenetic states are correctly maintained [20]. Multiple transient contacts between Polycomb loci may ensure that the epigenetic state can be transmitted to many target sites, whereas a stably compacted domain would not be able to do so. For example, a Polycomb-target locus that is missing the H2AK119ub modification may have a higher probability of encountering vPRC1 *via* transient interactions with other Polycomb-bound loci, whereas a locus that is in a stable compacted configuration would rely on vPRC1 successfully ‘searching’ for the locus. Since H2AK119ub is rapidly turned over by deubiquitylation [28,75], chromatin interactions may reinforce H2AK119ub maintenance at Polycomb-target genes by facilitating contacts between vPRC1 and target loci. However, while plausible, there is currently no direct experimental evidence to support this model. One prediction would be that disrupting Polycomb-mediated chromatin interactions would result in increased cell-to-cell variability in H2AK119ub occupancy and gene repression. Degron-based experiments combined with

single-cell measurements may be helpful to further test this model.

### Concluding remarks

The Polycomb system mediates 3D chromatin interactions that are visible in chromosome conformation capture methods (Hi-C, Micro-C), and by imaging (FISH, chromosome tracing). Yet, how these interactions are formed by cPRC1 and their biological significance is unclear. The heterogeneity in PRC composition adds to the difficulty of dissecting the key subunits that mediate these interactions in living cells. Future studies to investigate PcG protein abundance and stoichiometry of PHC and CBX paralogs and their oligomeric states would help clarify whether these interactions are mediated by PHC polymerization, CBX compaction, a combination of both, or an alternative mechanism. Combining these studies with selective degradation of each component will be especially informative.

Here, we have discussed four potential models of how 3D chromatin interactions may contribute to Polycomb function. While a simple inaccessibility model is attractive and supported by *in vitro* assays, we believe that this model is unlikely in mESCs, and, even in differentiated cells, may only apply to genes that have high PcG protein occupancy. Current evidence also suggests that these interactions do not play a role in poisoning Polycomb target genes for activation, although whether PRC2 and vPRC1, which are known to be required for differentiation, could maintain a subset of interactions independently of cPRC1 has not been thoroughly explored. This leaves two less explored models, where multiple transient interactions play an indirect role in gene repression by facilitating H3K27me3 spreading or reinforcing H2AK119ub deposition, ensuring proper establishment and maintenance of these repressive histone modifications. Live-cell chromatin tracking imaging studies to directly observe these interactions in different cell types would help further address these models in cell-type-dependent contexts. For the first two models, interactions are expected to be stable to either block transcription factor accessibility (Model 1) or organize all poised genes in a hub (Model 2). In contrast, the latter two models (Models 3a and 3b) assume dynamic chromatin contacts in which target genes form multiple transient interactions. Although much of the work discussed here is in mESCs, varying PRC composition in different cell types could support alternative models of Polycomb-mediated interactions contributing to Polycomb system function. Therefore, expanding beyond mESCs to characterize PRC composition, expression levels, and 3D chromatin interactions in other cell types would be valuable to understand whether Polycomb chromatin interactions contribute differentially to gene repression in other contexts.

While Polycomb bodies have long been speculated to be sites of PcG-mediated gene repression, most studies have relied on fixed-cell denaturing chromosome tracing and FISH, which have recently been shown to disrupt fine-scale 3D genomic structure [68], and have focused mainly on the *Hox* loci. Much remains unexplored, including the composition and function of foci of various sizes and brightness, whether other Polycomb target genes also localize to Polycomb bodies, and how stably PcG proteins interact with target genes within these foci. Future studies to investigate the identity and composition of Polycomb bodies would provide further insight regarding how the Polycomb system is spatially organized with respect to target chromatin.

### Data Availability

Genomics data presented were generated in prior studies and are publicly available on the NCBI Gene Expression Omnibus under the accession numbers GSE119619 and GSE130275.

### Declaration of Competing Interest

All authors declare no competing interests.

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