

## STATE-OF-THE-ART REVIEW

# Chromatin folding by the Polycomb group proteins and its elusive role in epigenetic repression

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The Polycomb system epigenetically represses selected developmental genes to enforce gene expression programs of differentiated cells. The system requires the coordinated action of dozens of structurally unrelated proteins assembled in two evolutionarily conserved polycomb repressive complexes, PRC1 and PRC2. Genes repressed by the Polycomb system are enriched in histone H3 trimethylated at lysine 27 (H3K27me3), an epigenetic mark that propagates the repressed state after DNA replication. Despite the impressive progress in dissecting molecular functions of the Polycomb group proteins, the fundamental questions of how the Polycomb system represses transcription or how the H3K27me3 mark is translated to benefit the repression are still open. Multiple observations indicate that the binding of PRC1, PRC2, and elevated H3K27me3 correlate with changes in the chromatin structure of target genes, which may be integral for the associated epigenetic repression. In this Review, we summarize our current understanding of these observations. We discuss the chromatin folding inside the loci repressed by the Polycomb system, consider molecular processes causing it and reflect upon its possible impact on transcription and epigenetic memory of the repressed state.

**Introduction**

The Polycomb system is an essential regulatory mechanism to enforce specific gene expression programs when cells differentiate [1,2]. To this end, the system represses genes for transcriptional regulators and signalling molecules, which would activate alternative gene expression programs and convert the cell into a ‘wrong’ type if accidentally switched on. Importantly, somatic cells transmit the information that a gene has been repressed by the Polycomb system to the daughter cells when they divide. This epigenetic information

transfer does not involve changes in the nucleotide sequence of the DNA.

The Polycomb system consists of multiple structurally unrelated proteins, most discovered in genetic screens for repressors of *Drosophila melanogaster* homeotic genes [3–5]. Biochemical studies demonstrated that these proteins assemble in two kinds of evolutionarily conserved polycomb repressive complexes, PRC1 and PRC2 [6–9]. We refer the interested reader to recent reviews that describe the composition

**Abbreviations**

3C, chromatin conformation capture; FISH, fluorescent *in situ* hybridization; GFP, green fluorescent protein; H3K27me3, histone H3 trimethylated at lysine 27; HP1, heterochromatin protein 1; IDR, intrinsically disordered region; LLPS, liquid–liquid phase separation; Mb, million base pairs; O-GlcNAc, O-linked N-acetyl-glucosamine; PRC, polycomb repressive complex; PRE, polycomb response element; SAM, sterile alpha motif; TAD, topologically associated domain; TBP, TATA binding protein.

of PRC1 and PRC2 in detail [2,10]. Within the cell nucleus, DNA is wrapped around protein particles consisting of positively charged histone proteins (H2A, H2B, H3 and H4). The DNA–histone particles, known as nucleosomes, are critical for the orderly genome folding (for an extended discussion of the genome architecture see: [11]). PRC2 complexes can methylate histone H3 at lysine 27 (H3K27) [7,12] and one of the PRC1 subunits can specifically interact with histone H3, which carries three methyl groups at lysine 27 (H3K27me3). Both trimethylation of H3K27 by PRC2 and its recognition by PRC1 are required for epigenetic repression by the Polycomb system [13,14].

In addition to the ‘canonical’ complexes described above, one of the PRC1 subunits (represented by closely related proteins RING1 or RING2) is incorporated in several alternative complexes sometimes referred to as ‘non-canonical’ or ‘variant’ PRC1 [15–18]. These alternative RING1/2 complexes lack the subunit that can interact with H3K27me3. PRC1 and alternative RING1/2 complexes can monoubiquitylate histone H2A at Lysine 119 [15,19,20]. Multiple lines of evidence indicate that canonical PRC1 is critical for repressing developmental genes [3,21–25]. To what extent the monoubiquitylation of H2A or alternative RING complexes contributes to the repression by the Polycomb system is unclear [26–31]. Therefore, we will keep canonical PRC1 and PRC2 as the primary focus of this review.

Neither canonical PRC1 nor PRC2 incorporates sequence-specific DNA binding subunits. *Drosophila* genes regulated by the Polycomb system are equipped with specialized short (~1 kb) Polycomb Response Elements (PREs), which are thought to tether PRC1 and PRC2 via multiple sequence-specific DNA binding ‘adaptor’ proteins. In this view, the adaptor proteins combine their individually weak interactions with PRC1 and PRC2 to retain the complexes or facilitate their association with PREs (for a detailed review of PREs see [32]). The targeting of canonical PRC1 and PRC2 to mammalian genes is less studied, but the emerging picture appears principally similar to that in *Drosophila*. Thus, many human developmental genes contain DNA elements tethering canonical PRC1 and PRC2 [33]. However, in contrast to flies, DNA features associated with PRC1 and PRC2 tethering differ, and the binding of the two complexes to a regulated locus is less strictly linked. Therefore, the human genome contains various DNA elements that range from those that tether primarily PRC1 or PRC2 to ones that can tether both complexes [33].

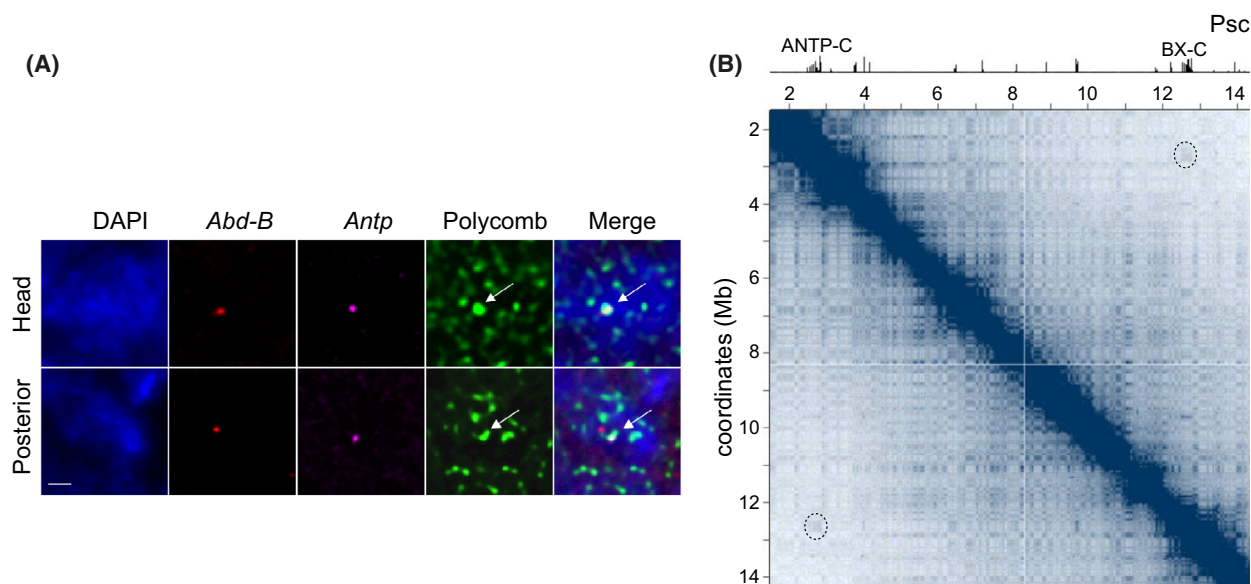
Despite three decades of fast progress in understanding the molecular workings of the Polycomb system,

some basic questions like ‘How does it repress transcription?’ or ‘How is the H3K27 methylation mark translated into transcriptional repression?’ still lack clear answers. It was proposed that H3K27me3 enables the repression by directing the recruitment of PRC1 [34]. However, experimental observations contradict this model. First, genomic mapping shows that PREs or analogous elements in mammalian cells are the sites where PRC1 is stably bound [33,35], not the H3K27me3-rich chromatin domains around them. Second, PREs are depleted from nucleosomes and are often the least methylated parts of the repressed genes [35,36]. Third, PRC1 continues to bind PREs when PRC2 and H3K27 methylation are removed by mutation [37].

The simultaneous binding of PRC1, PRC2 and elevated H3K27 methylation correlates with changes in chromatin structure. It is customarily assumed that such changes antagonize transcription, but the evidence supporting this notion is largely circumstantial. In the following pages, we review our current understanding of the chromatin configuration at loci repressed by the Polycomb system. We attempt to summarize the insights gained from experimental observations and computational modelling, focusing on molecular processes that cause chromatin folding and speculate on possible connections between chromatin folding and epigenetic repression.

## Long-range interactions

In experiments with immunostaining or GFP-tagged Polycomb group proteins, it was noted early on that Polycomb group proteins form distinct foci inside *Drosophila* and mammalian nuclei [38,39]. Sometimes referred to as Polycomb bodies, these foci are fewer than the number of repressed loci detected by genomic mapping. PRE-mediated repression of reporter genes is noticeably stronger in *Drosophila* with two copies of a transgene [40,41]. For this so-called ‘pairing-sensitive silencing’, the two transgenes must be in spatial proximity, which is usually the case in homozygous flies due to the somatic pairing of homologous chromosomes. However, pairing-sensitive silencing is not limited to transgenic insertions in the same genomic site. It can happen between two transgenes inserted in different chromosomes if, in these transgenes, PREs are coupled to genetic elements that promote *trans*-interactions [42]. The two notions combined suggest that Polycomb bodies may represent nuclear micro-compartments where multiple loci, regulated by the Polycomb system, cluster to achieve robust repression. Alternatively, the bodies may correspond to



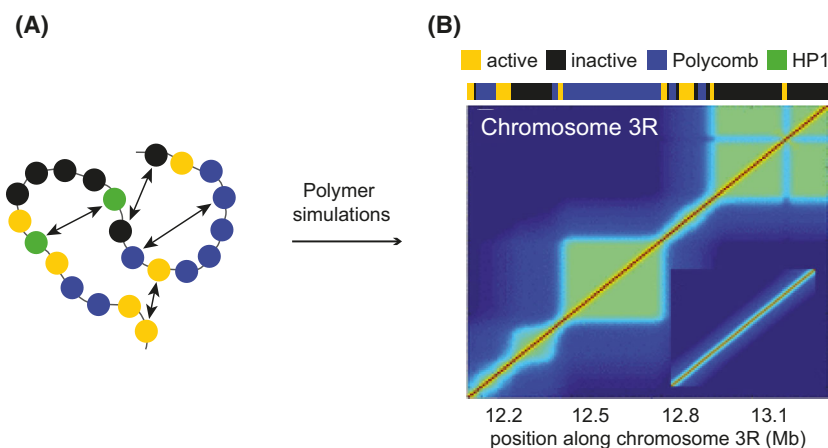
**Fig. 1.** Genes repressed by the Polycomb system are in closer proximity than expected. (A) Combined immunodetection and FISH of Polycomb, *Abd-B* (the gene from the bithorax complex), and *Antp* (the gene from the Antennapedia complex). Note the three signals colocalizing in the nuclei of the anterior part of the *Drosophila* embryo (head) where the Polycomb system represses both *Abd-B* and *Antp*, but not in the posterior part of the embryo, where *Antp* is repressed but *Abd-B* is transcriptionally active. The arrows point to the Polycomb bodies encompassing *Antp* and *Abd-B* in the head, but just *Antp* in the posterior part. The scale bars represent 1  $\mu\text{m}$ . The image is adapted from Figure 1D in [44]. (B) Heatmap representation of the chromatin contacts along a segment of *Drosophila* chromosome 3R (dark blue – more contacts, light blue – fewer contacts) [80]. The profile above the heat map shows Chromatin immunoprecipitation sequencing (ChIP-seq) signals for the PRC1 subunit Psc [37]. Clusters of Psc ChIP-seq signals mark locations of the Antennapedia (ANTP-C) and bithorax (BX-C) complexes. The heatmap shows elevated contact frequency between ANTP-C and BX-C (marked with dashed circles). Here and in Fig. 3 the heatmaps were plotted using Juicebox.js [112].

Polycomb complexes bound to multiple tethering elements of the same locus clustered together.

These hypotheses can be tested by simultaneous immunodetection of the Polycomb group proteins and fluorescent *in situ* hybridization (FISH) of DNA probes against the repressed loci or by the chromosome conformation capture (3C) assays. The latter is based on the idea that the spatial proximity of any two genomic fragments can be deduced from the frequency with which these fragments are joined together by the DNA ligase (for an in-depth review of the approach see: [43]). In the unbiased genome-wide version of the 3C assay called Hi-C, live cells are crosslinked by treatment with formaldehyde, their genome digested with a restriction endonuclease and the resulting fragments ligated under conditions favouring the ligation of fragments in close spatial proximity. The fragment junctions are then identified and counted by sequencing. Both approaches concur (Fig. 1) that loci repressed by the Polycomb system tend to be closer to each other in the nuclear space than expected by chance [44–50]. However, the overall spatial colocalization between distant repressed loci appears to be rare (less than 10% of

the time), at least for *Drosophila* where this question was systematically investigated by multiplexed chromatin imaging, and involves predominantly just a pair of genes [51]. In turn, the amount of Polycomb group proteins within specific bodies seems to correlate with the linear size of the incorporated repressed locus and the number of embedded tethering elements (PREs) [52]. These observations argue that most Polycomb bodies inside the nucleus encompass Polycomb complexes bound to multiple tethering elements of a single locus. The low number of Polycomb bodies compared to the number of repressed loci, as identified by genomic mapping, likely stems from the lower sensitivity of microscopy-based techniques that miss weaker signals emitted by short loci with one or two tethering elements. Infrequently, but with a likelihood greater than expected by chance, single-locus Polycomb bodies form pairs [51].

What molecular process drives this pairing? Any answer to this question needs to consider the polymer nature of the chromatin [53]. Here computational modelling provides a useful ground framework. Regardless of specific details, most computational



**Fig. 2.** Illustration of the four-state block copolymer model. (A) Schematic representation of the model by Jost et al. [57]. The chromatin is represented as a self-avoiding bead-spring chain where each monomer corresponds to a 10 kb chromatin stretch characterized by its epigenetic state: yellow (active), blue (loci repressed by the Polycomb system), black (transcriptionally inactive), green (loci repressed by HP1). The arrows represent attraction between beads with the same epigenetic state. (B) Simulated Hi-C contact map for the chromatin region between 12.16 and 13.36 Mb of chromosome 3R. This region lacks HP1 but contains a stretch repressed by the Polycomb system. The positions of chromatin stretches with different epigenetic states are indicated at the top. The inset shows the starting polymer configuration. Adapted from [57].

models represent chromatin as a linear chain of equally sized monomers corresponding to a short chromatin segment, typically 1–10 nucleosomes long. Therefore, 575 to 5750 monomers are required to simulate the folding of 1 million base pairs (Mb) of chromatin. Applying such a model to entire chromosomes with lengths of more than 10 Mb becomes computationally challenging. This may be solved by increasing the monomer size to include longer chromatin stretches (coarse-graining). State-of-the-art computational models could be grouped into two primary categories: equilibrium and nonequilibrium [3,11,54]. The former focuses on passive binding and unbinding associated with pairwise interactions, such as reversible chemical reactions. These interactions are characterized by binding energies (or dissociation constants) between different polymer sections representing distinct chromatin types (e.g. transcriptionally active genes versus loci repressed by the Polycomb system). In contrast, the nonequilibrium models include active processes that require additional energy (e.g. ATP-ADP conversion). In such models, the monomers may move due to forces that consume energy and continuously push the system out of equilibrium. The best-known example of a nonequilibrium model is the chromatin loop extrusion [55,56].

To date, only equilibrium models have been applied to understand the proximity of loci repressed by the Polycomb system. In one prominent example, Jost and co-authors designed a block copolymer model that

represented four distinct epigenetic chromatin states, one of which corresponded to that of the genes repressed by the Polycomb system [57]. In such a model, a self-avoiding polymer chain is composed of blocks with different properties that interact with each other according to stipulated rules (Fig. 2). The model faithfully reproduced many general chromatin folding patterns observed in chromosome conformation capture experiments (i.e. Hi-C). However, it generally did not recover increased proximity between genes repressed by the Polycomb system. To achieve observed spatial proximities, the polymer simulations had to start from a non-random polymer configuration that mimicked experimental Hi-C maps. In a more recent attempt, Gurgo and colleagues reported recapitulating experimental proximity frequencies between distant loci repressed by the Polycomb system using a simpler, course-grained two-state self-avoiding block copolymer model [51]. In this model, the authors represented one *Drosophila* chromosome arm as a polymer consisting of 866 beads (20 kb each) with two possible identities: ‘Polycomb’ or ‘not Polycomb’. The distribution of self-attracting ‘Polycomb’ beads within the simulated polymer mirrored the location of repressed genes along the chromosome arm, while all intervening beads were of the inert ‘not Polycomb’ type. By keeping the attraction between the ‘Polycomb’ monomers as a free parameter, the authors found a range of attraction energies where simulated proximity frequencies matched those detected in the

experiment. Of note, at those attraction energies, the polymer reached the folding regime known as the equilibrium globule [58]. A striking feature of this polymer state is that the contact frequency between monomers becomes nearly constant at long distances (>1 Mb), which is at odds with chromatin contact decay profiles measured by Hi-C [49,59].

What proteins or molecular interactions may be responsible for attraction between the repressed loci? Two proteins from the Polycomb group were suggested as candidates: Ph (human orthologues PHC1, PHC2, PHC3, component of PRC1) and Scm (human orthologues SCMH1 and SCML2). The *Drosophila* Scm is incorporated as a sub-stoichiometric subunit into both PRC1 and PRC2 [60]. Similarly, mammalian SCMH1 and SCML2 were detected in biochemical purifications of PRC1 [15] and SCML2 was found to co-immunoprecipitate with EZH2 (core PRC2 subunit) from protein extracts of the germline cell nuclei [61]. Both Ph and Scm contain the sterile alpha motif (SAM) domain that can form homo- and heteropolymers *in vitro* [62,63]. Some authors proposed that the polymerization of Ph drives the formation of the Polycomb bodies [45,64]. Supporting this hypothesis, the size of the Polycomb bodies in *Drosophila* [64] and mouse cells [45] was reduced after the overexpression of truncated Ph lacking the SAM domain and thus cannot polymerize. However, the SAM domain of Ph is critical for the repression [45,65], which is consistent with the hypothesis but also complicates the interpretation of the results. In the experiments above, a substantial reduction of the Polycomb body size led to increased transcription of the investigated genes. As transcription is known to unfold the chromatin [66,67], it is uncertain whether the reported body size changes are mechanistically independent of transcription or are the consequence of it. There are additional confounding factors. Thus, the Ph polymerization appears under strict control *in vivo*. In *Drosophila*, the glycosyltransferase Ogt adds O-linked N-Acetylglucosamine (O-GlcNAc) moieties to Ph, which prevents its aggregation [65]. Impairing O-GlcNAcylation of Ph by either removing Ogt [68] or truncating Ph [65] impairs the repression. This implies that, in the cell, Ph has a limited ability to polymerize.

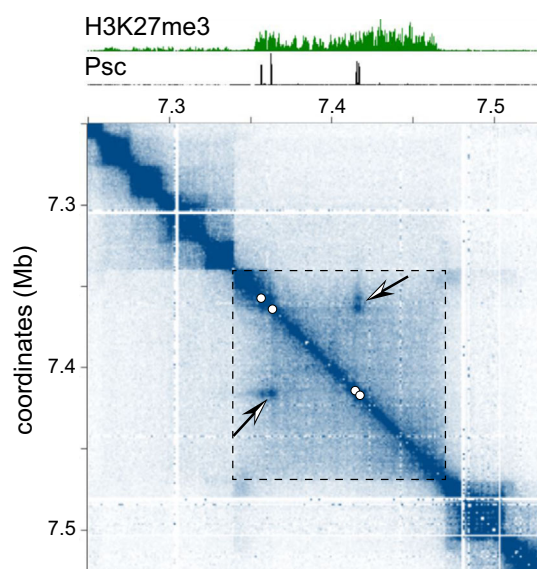
In conclusion, we note that both FISH and Hi-C assays used to study contacts between distant repressed loci rely on chemical crosslinking of live cells. The crosslinking 'freezes' all processes inside the nucleus. Therefore, we know very little about the time it takes for two loci to pair or how long the pair exists before drifting apart to find new mates. Emerging live imaging techniques, like those based on CRISPR/

Cas9-mediated locus tagging [69], may help to answer these questions.

## Local chromatin folding

Like many other models explaining the inner workings of the Polycomb system, the popular ideas regarding its impact on the chromatin structure of individual repressed genes emerged from studies in *Drosophila*. The cloning of the *Polycomb* gene and the discovery that its eponymous product (the fly ortholog of mammalian CBX2, CBX4, CBX6, CBX7 and CBX8 proteins) contains the structural motif called Chromodomain shared with the Heterochromatin Protein 1 (HP1) led to the idea that the Polycomb and HP1 systems repress genes via a common mechanism [70]. Strengthening the apparent similarity, transgenes integrated into the vicinity of genes repressed by the Polycomb systems or transgenes equipped with polycomb response elements (PREs) display stochastic inactivation of reporter genes [41,42]. This resembles the variegated silencing of genes brought by chromosomal rearrangements in the vicinity of HP1-bound pericentromeric regions [71]. The HP1-bound regions appear compact in the diploid chromosomes of embryonic cells [72] and in giant polytene chromosomes of salivary glands [73]. Likewise, on polytene chromosomes, the homeotic gene clusters repressed by the Polycomb system appear shrunk and compact. These observations combined led to a view that HP1 and Polycomb group proteins repress transcription by compacting the chromatin of a target gene to a point where it becomes inaccessible to transcriptional activators and transcriptional machinery [74].

Hi-C may be used to investigate chromatin folding inside the loci repressed by the Polycomb system. When applied to *Drosophila* or human cells, Hi-C indicates that the repressed loci correspond to broad domains (up to a million base pairs) with similar chromatin contact frequencies, which slowly decay with the distance until the edge of the domain [49,75]. Such chromatin contact patterns are often referred to as topologically associated domains (TADs) [76]. TADs encompassing the repressed loci are often (but not always) coextensive with stretches of chromatin enriched in H3K27me3 (Fig. 3) [75,77]. Although these TADs can, at times, be split by chromatin insulators [78]. Strikingly, the Hi-C contact maps of *Drosophila* chromatin display spots of elevated contacts corresponding to some of the PREs within H3K27me3-enriched TADs [77,79,80]. The contact spots suggest that PREs of some (but not all) repressed loci end up in closer spatial proximity compared to neighbouring



**Fig. 3.** Chromatin contacts within the repressed *invected-engrailed* locus. The heatmap representation of the chromatin contacts along a segment of *Drosophila* chromosome 2R (dark blue – more contacts, light blue – fewer contacts) gives an example of elevated contacts between Polycomb Response Elements (PREs) and the relation between H3K27me3 and Topologically Associated Domains (TADs) [80]. The profiles above the heatmap show ChIP-seq signals for the PRC1 subunit Psc [37] and H3K27me3 [110]. The elevated ChIP-seq signal for H3K27me3 delimits the repressed *invected-engrailed* locus (also highlighted in the heatmap with dashed box edges). The four PREs of the locus (marked by the ChIP-seq signal peaks for Psc and white circles on the heatmap) appear as dots (indicated with arrows) on the heatmap, which suggests their spatial proximity. Note that the right edge of the H3K27me3-enriched *invected-engrailed* locus corresponds to the TAD border, while the left edge of the locus does not.

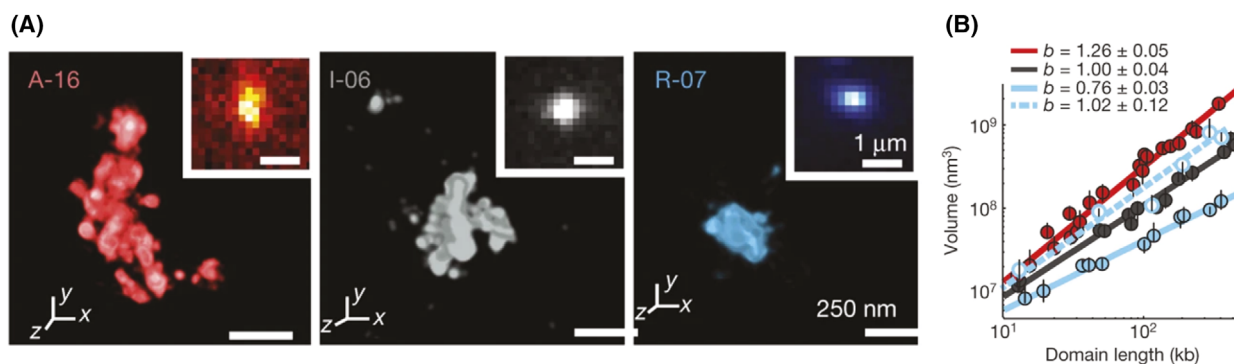
stretches of chromatin located at comparable linear distances.

Super-resolution microscopy combined with *in situ* hybridization of fluorescently labelled oligonucleotide probes provides alternative means to trace the 3D organization of chromatin inside the loci repressed by the Polycomb system. Applied to *Drosophila* embryos and cultured cells, such optical reconstructions indicate that the repressed loci are on average more crumpled compared to both transcriptionally active [66,81,82] and transcriptionally inactive, but not repressed, genes [66,81]. The chromatin density inside the volume occupied by the repressed loci appears to increase with their length (Fig. 4) [66]. Moreover, the chromatin of the repressed loci seems to have a higher degree of chromatin intermixing [66,81] and excludes the neighbouring transcriptionally active chromatin more than the chromatin of regular transcriptionally inactive genes [66]. Optical reconstruction of chromatin from

murine *Hoxa* cluster repressed by the Polycomb system revealed essentially the same properties [83]. Although the loci repressed by the Polycomb system appear more compact, the corresponding volume occupied by a repressed locus is only about half of that for the similar-sized transcriptionally inactive chromatin stretch.

Hi-C assay requires pools of hundreds of thousands to millions of cells, so its estimate of the contact frequencies is an average over a large cell population. Optical reconstructions are inherently single-cell techniques. But when averaged over many cells, these reconstructions show excellent correspondence with contact frequencies measured by Hi-C [81–83]. However, they indicate that chromatin folding within loci repressed by the Polycomb system varies substantially between different cells. In particular, they argue that the chromatin of the repressed loci frequently assumes an unfolded state partially mixed with the neighbouring chromatin [81,83]. Overall, the picture emerging from Hi-C and super-resolution microscopy is that of dynamic folding. In this view, the average more folded state of the chromatin within the repressed genes may arise from infrequent folding into an ultra-compact conformation or in a lower propensity to assume a very unfolded state. Technical advances enabling high-resolution live tracing of the chromatin motion inside loci repressed by the Polycomb system may allow us to discriminate between these possibilities.

What molecules and molecular forces lead to the local chromatin folding within the repressed loci? Here, the insights come from genetic, biochemical and computer simulation studies. The *in vitro* reconstituted PRC1 compacts nucleosomal arrays even when those contain no tethering elements (e.g. PREs) [84]. Consistent with this observation, multiple studies indicate that chromatin of the loci regulated by the system becomes less folded when one of the Polycomb group proteins is ablated by mutation [64,66,83,85,86]. The latter is customarily taken as proof that Polycomb group proteins are required for folding. However, the observed unfolding of a locus is often accompanied by an increase in transcriptional activity, which is known to unfold chromatin [66,67]. Hence, unless this is considered, it is impossible to conclude whether the change in the chromatin structure precedes transcription or is the consequence of it. Two studies attempted to separate the effects of disrupting the Polycomb system and transcription. In one of them, the folding of *Drosophila* homeotic gene clusters was assayed in the early *Pc* or *Ph* loss-of-function embryos (mutants for one of the PRC1 subunits) when the clusters were still repressed by maternally supplied regulators



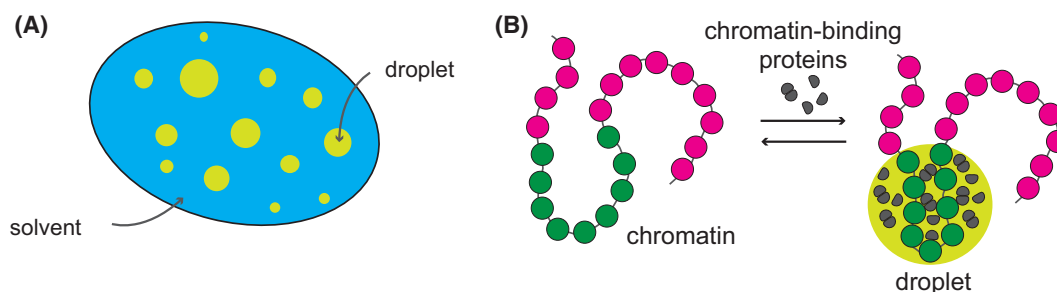
**Fig. 4.** Optical reconstruction of local chromatin folding. (A) 3D-STORM images of chromatin stretches (domains) in three distinct transcriptional states: 'active' (red), 'inactive' (grey), and 'repressed' (light blue). The insets show the corresponding conventional images. The scale bars represent 1  $\mu\text{m}$  (insets) and 250 nm (main panels). (B) log-log plot of the median chromatin volume as a function of domain length for active (red solid circles), inactive (black solid circles), and repressed (light blue solid circles) domains, as well as for repressed domains in Ph-knockdown cells (light blue hollow circles). Error bars represent 95% confidence intervals derived from resampling ( $n \approx 50$  cells). The lines indicate power-law fits, with the scaling exponent  $b$  shown in the legend. Figure reproduced from Ref. [66].

independent of the Polycomb system [85]. In the other the chromatin folding of the *Hoxa* locus was reconstructed after simultaneous degradation of RING1B (PRC1 subunit) and EED (PRC2 subunit) in mouse embryonic stem cells, where this did not cause an increase of *Hoxa* transcription [83]. Both reported that chromatin unfolds without a detectable increase in transcription, which argues that the Polycomb group proteins are indeed the primary agents of the folding.

How could then the Polycomb group proteins fold chromatin? As discussed in the previous section, Polycomb group proteins Ph and Scm can form homo- and heteropolymers *in vitro* [62,63]. It was hypothesized that the polymerization of Ph drives the chromatin folding within genes repressed by the Polycomb system [64]. The same may be suggested for Scm. It was also proposed that polymerization of Ph might mediate PRE clustering [77,80]. Consistent with these hypotheses, reduced Ph concentration in the nucleus [66] led to reduced chromatin folding within genes regulated by the Polycomb system. However, the SAM domains of Ph and Scm are required for the repression [65,87] and chromatin unfolding triggered by Ph removal or truncation was accompanied by increased transcription of the investigated genes. Therefore, from the above experiments alone, it is impossible to discriminate whether the changes in the chromatin structure occurred because of impaired Ph polymerization or due to transcription. Likewise, genetic evidence suggests that, in the cell, Ph has a limited ability to polymerize [65].

Liquid-liquid phase separation (LLPS) was hypothesized as another mechanism to aid local chromatin folding by the Polycomb system. LLPS is the process

where macromolecules separate into a dense phase that resembles liquid droplets, which coexist with a dilute phase. LLPS is driven by the exchange of macromolecule/solvent interactions for macromolecule/macromolecule and solvent/solvent interactions when this becomes thermodynamically favourable (Fig. 5). The most thoroughly studied example of chromatin segregation by LLPS is the nucleolus, the membraneless nuclear organelle for ribosome subunit assembly [88]. LLPS provides an elegant way of increasing the local concentration of macromolecules which, unlike regular aggregation, does not sequester the mobility of the molecule inside the phase. The ability to undergo LLPS may be a universal property of proteins and nucleic acids under specific conditions, many of which may never be encountered in a cell [89]. Therefore, rigorous characterization of a macromolecule's behaviour *in vitro* and in the cell is required before one can confidently discriminate between LLPS and other mechanisms that may lead to a high local concentration of the molecule inside the nucleus. We point the reader to excellent reviews that discuss guidelines for experimental validation of LLPS [89,90]. One of the mammalian PRC1 subunits (Cbx2) was reported to phase separate and drive phase separation of associated PRC1 and nucleosomal arrays *in vitro* [91–93]. However, the current evidence falls short of recommended guidelines [89,90]. The reported LLPS-like behaviour requires an intact intrinsically disordered region (IDR) of Cbx2, and mutations of the corresponding residues within IDR lead to homeotic transformations in mutant mice [94]. It remains to be tested whether Cbx2 undergoes LLPS *in vivo*, and it is not immediately clear how the presumed high local concentration



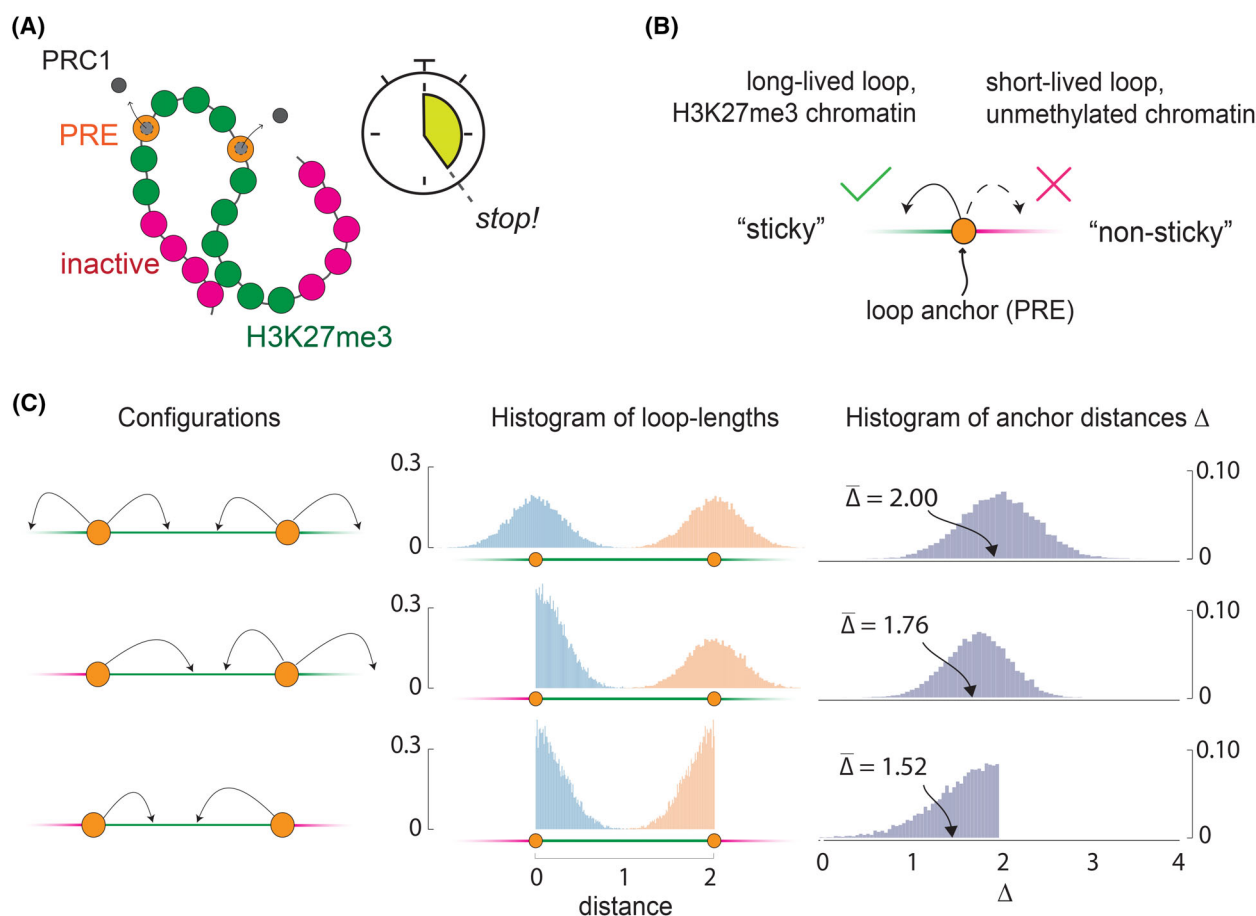
**Fig. 5.** Liquid–liquid phase separation (LLPS) may aid chromatin folding. (A) Protein–protein or protein–chromatin interactions lead to phase separation into liquid-like droplets containing macromolecules of high concentration. LLPS is driven by the exchange of macromolecule/solvent interactions for macromolecule/macromolecule and solvent/solvent interactions when this becomes thermodynamically favourable. (B) Certain chromatin regions (green) may fold when bound by self-interacting proteins (grey) when the latter phase separate and form droplets.

of phase-separated PRC1 translates into increased local chromatin folding. Further complicating the issue, of the closely related mammalian orthologues of *Drosophila* Polycomb protein (Cbx2, Cbx4, Cbx6 Cbx7 and Cbx8), only Cbx2 appears to phase separate *in vitro* by itself [92,95] and Cbx8 as part of the PRC1 complex and in the presence of chromatin [96]. This implies that most paralogues have lost the ability for LLPS or that this ability evolved after the paralogues diverged from their common ancestor (i.e. after the Polycomb system evolved to epigenetically repress developmental genes). Whether *Drosophila* Polycomb protein can phase separate *in vitro* has not been tested.

Lastly, it appears possible that the chromatin of genes repressed by the Polycomb system is folded just by stochastic interactions of PRE-bound PRC1 with histone H3 trimethylated at lysine 27. A recent computational study from our laboratories used a biochemically grounded Monte Carlo – Molecular Dynamics simulation framework to model the chromatin folding within *Drosophila* PRE-equipped genes [97]. Unlike traditional block copolymer models discussed earlier, this pseudo-nonequilibrium model introduced a ‘new’ PRC1–H3K27me3 interaction to a chromatin fibre that has been in equilibrium, proceeded with molecular dynamics simulations but stopped them after a short time comparable to that for PRC1 residence at PREs (Fig. 6A). By scanning a range of binding constants for PRC1 – PRE and PRC1–H3K27me3 interactions, the study demonstrates that stochastic interactions of PRE-tethered PRC1 with H3K27me3 are sufficient to fold the methylated chromatin, if PREs are occupied by PRC1 most of the time. In line with the results of optical reconstructions [81,83], simulations indicate that the extent of folding varies substantially between individual chromatin fibres. The model makes a testable prediction. In cells where PRC1 is truncated so it

can no longer interact with H3K27me3 or where H3K27 methylation is removed by PRC2 mutation, PRC1 will continue to bind PREs, but the chromatin folding will be lost. Such genetic and imaging experiments have not yet been performed. However, genomic studies in PRC2 knock-out cells do show that, in the absence of H3K27me3, PRC1 continues to bind PREs while its interaction with the surrounding chromatin becomes less stable [37].

Remarkably, chromatin folding by PRC1–H3K27me3 interactions leads to spatial clustering of PREs, noted in Hi-C experiments [77,79,80], without a need for specific protein–protein interactions. The clustering appears to have a geometric/probabilistic explanation, which we briefly discuss below and refer the interested reader to the original study for details [97]. Let us consider PRC1-bound PREs as points on a line (Fig. 6B) that may touch surrounding line segments and form loops. Depending on the segment type, which could be ‘sticky’ (e.g. H3K27 trimethylated chromatin) or ‘non-sticky’ (e.g. unmethylated chromatin), the loop will be long-lived or short-lived. If we consider the behaviour of two PRC1-bound PREs, they can be found in three basic configurations (Fig. 6C). In the first configuration, the ‘sticky’ segments surround both PREs. As a result, those may form stable loops to the left and right with the same probability (the probability to touch a specific segment point declines with its distance from a PRE, and the exact dependence is not essential for the argument). As illustrated by Fig. 6C, this configuration yields a symmetric distribution of loop lengths and absolute distances ( $\Delta$ ) between the two anchor points. As a result, the average distance between the PREs ( $\bar{\Delta}$ ) equals 2, which is identical to their linear separation. In the second configuration, the leftmost segment is ‘non-sticky’, while the other segments remain the same as in the

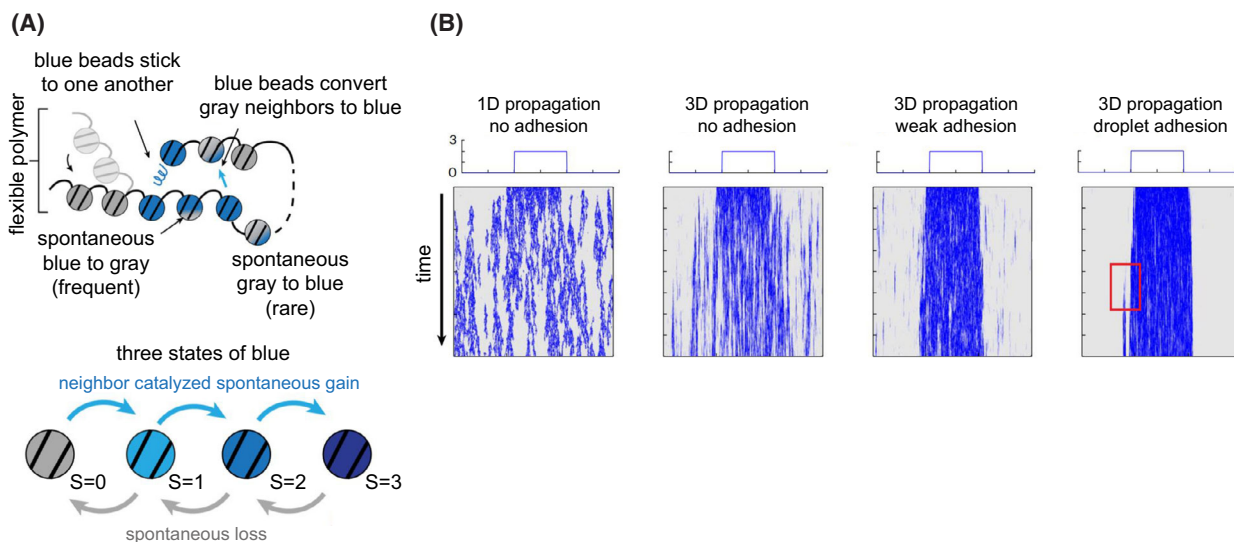


**Fig. 6.** Chromatin folding by PRC1-H3K27me3 interactions leads to spatial clustering of Polycomb Response Elements (PREs). (A) Schematic of the pseudo-nonequilibrium block copolymer model of Lizana et al. [97]. PRE monomers (orange) attract PRC1 complexes (grey), and stochastic interactions between PRC1 and H3K27me3 fold the methylated chromatin. The simulation stops after a short time, comparable to the PRC1 residence time at PREs. (B) Simple looping model. It considers anchor points (orange circles) on a line that may touch surrounding line segments and form loops. Depending on the segment type, "sticky" (green) or "non-sticky" (red), the loops will be long or short-lived. The model postulates that only long-lived loops are stable enough to be detected. (C) Three basic configurations of anchor points and chromatin types. Arrows indicate the directions in which the anchor points may form long-lived loops. Shown to the right are the corresponding histograms of the simulated loop lengths and anchor distances. The average anchor distance ( $\bar{\Delta}$ ) depends on the relative arrangement of anchor points and the "sticky" chromatin segments. Panel C is reproduced from Ref. [97].

first configuration. Since, in this arrangement, no long-lived loops form towards the leftmost segment, the corresponding loop length histogram is asymmetric, and the average distance between the PREs shortens to  $\bar{\Delta} = 1.76$ . Compared to the first configuration, PREs become statistically closer. Lastly, we consider the most extreme configuration where interactions with only one segment between the two PREs can yield stable loops. In this arrangement, the average distance between PREs shortens even more to  $\bar{\Delta} = 1.52$ .

To summarize, Polycomb group proteins appear to increase local chromatin folding. However, the resulting chromatin compaction is modest (no more than 2-fold on average) and dynamic. The

chromatin of genes repressed by the Polycomb system remains free to explore unfolded states and periodically mix with the chromatin of neighbouring genes. This folding behaviour is incompatible with the model where Polycomb group proteins repress transcription by compacting the chromatin to a point when it becomes inaccessible to transcriptional activators and transcriptional machinery. In line with this view, attempts to correlate the repression of a locus by the Polycomb system to the loss of DNA accessibility yielded conflicting results. Thus, transgenic reporters repressed by the Polycomb system are harder to methylate by exogenously expressed Dam DNA methylase [98] or excise with FLP recombinase [99]. Yet, DNA



**Fig. 7.** Polymer modelling argues that chromatin folding by the Polycomb system helps to propagate the epigenetic memory. (A) Schematic illustrating key features of the model of Murphy and Boettiger [83]. (B) Kymographs from representative simulations. The X-axis reports monomer position (genomic coordinate), and the Y-axis reports simulation time (arbitrary units). The starting state of the simulation is shown above the kymograph, along with the degree of adhesion between blue monomers. Note the propagation of epigenetic memory (e.g. a stretch of chromatin enriched in methylated H3K27) without stable compaction on the second kymograph from the right. The red box in the rightmost kymograph highlights a monomer outside the repressed locus that acquired methylated H3K27 by accident. Figure reproduced from Ref. [83].

of homeotic genes is equally accessible to restriction nucleases in transcriptionally active and repressed states [100], and although 5' ends of the genes occupied by PRC1 and PRC2 in mouse embryonic stem cells exhibit reduced DNA accessibility compared to those that do not bind the complexes, the accessibility does not increase after PRC1 or PRC2 are ablated [101]. Likewise, PREs of genes repressed by the Polycomb system are DNase I hypersensitive [36] and display elevated nucleosome exchange [33,102,103]. The repression does not prevent the binding of TATA binding protein (TBP), RNA polymerase II, and the heat-shock factor to a reporter gene driven by the hsp26 promoter [104], and does not hinder insulator proteins from binding to insulator elements [78].

### Implications for epigenetic repression

If the chromatin folding by the Polycomb system does not hinder the access of transcriptional activators and transcriptional machinery to chromatin, is it critical for epigenetic repression, and if so, how does the folding promote it? Using computational modelling, two recent studies argue that chromatin folding by the Polycomb system is critical to propagating epigenetic memory [83,105]. The model of Murphy and Boettiger represents chromatin as a flexible polymer where each

segment (monomer) can switch epigenetic states (acquire methyl groups on H3K27) dynamically over time—from unmethylated to mono-, di-, or trimethylation (Fig. 7). The switching happens at some constant rate but only if other methylated monomers are brought into spatial proximity. The loss of methylation (demethylation) occurs randomly. Furthermore, each methylated state is associated with an adhesion parameter that represents the interaction between the Polycomb group proteins bound to methylated histones, presumably PRC1 and PRC2. This parameter determines how tightly methylated monomers stick together if meeting in 3D space. From simulations, the model predicts a delicate balance between chromatin folding and flexibility. If the adhesion is too weak, a locus repressed by the Polycomb system loses H3K27 methylation because the methylation spreads only in 1D, which is slow, and eventually gets lost due to random demethylation. But if the adhesion is sufficiently high, methylated monomers are on average closer to each other, leading to more efficient methylation spreading and tighter folding in a self-reinforcing feedback loop. This process results in a long-lived epigenetic state with bi-stable characteristics. It can restore erroneous loss of epigenetic states for individual monomers and recover from cell division that reduces the density of methylated monomers by 50%. However, having a too

high adhesion parameter comes with a cost. If a monomer outside the repressed locus acquires methylated H3K27 by accident, it fuses with the other methylated monomers and its methylated state is perpetuated indefinitely. To summarize, the model predicts that the chromatin folding by the Polycomb system must remain balanced. It should be efficient enough to maintain a long-lived methylation state of the repressed locus, but not too strong so it sustains erroneous methylation of the flanking chromatin [83]. Qualitatively similar conclusions follow from a more general model of Owen and colleagues [105]. In this case, the authors emphasize that locus-specific, yet epigenetically stable repression requires that the marking enzyme (e.g. PRC2) is limited relative to its substrate (nucleosomes).

While coherent, neither of the above models considers that PRC2 (the marking enzyme) and PRC1, which enables chromatin folding, are tethered to specific genes by DNA elements (PREs in *Drosophila* or CpG-islands and PTEs in mammals). Experimentally induced deletion of tethering elements leads to the rapid loss of H3K27 methylation and de-repression [106,107]. Thus, models that explain the propagation of epigenetic memory through self-attraction and 3D spreading of H3K27 methylation are considerable simplifications. Perhaps, they imply tethering as part of molecular processes that enable the self-attraction and/or methylation spreading. Conversely, the probabilistic model grounded in the enzymatic properties of PRC2 in the solution that accounts for H3K27 methylation by PRE-tethered complexes can forecast genomic H3K27 methylation with the accuracy of current experimental techniques [108]. This model incorporates chromatin folding around PREs implicitly when it converts the degree of PRC2 tethering at PRE-equipped genes into higher methylation probability.

To summarize, the local chromatin folding by the Polycomb group proteins is likely required for extensive trimethylation of H3K27 around tethering points (i.e. PREs) and the sufficient speed with which the H3K27me3 is regained after chromatin replication. Together with the allosteric stimulation by the pre-existing H3K27me3 [109], the folding makes PRC2 better at methylating H3K27 at sites where it is needed the most (i.e. developmental genes equipped with tethering elements) as opposed to indiscriminate methylation of the entire transcriptionally inactive genome [108,110]. It is tempting to speculate that the impact of local chromatin folding is not limited to the propagation of the epigenetic memory record. Exploratory computational modelling from our laboratories

suggests that stochastic interactions of PRE-bound PRC1 with the surrounding H3K27me3 slow down the transitions of the chromatin fibre from one folding conformation to another. If transcription initiation happens only in certain infrequent folding conformations, reduced chromatin fibre dynamics would inhibit the process. In line with this view, a recent live-cell transcription imaging study in mouse embryonic stem cells indicates that genes repressed by the Polycomb system alternate between transcriptionally active and inactive states [111]. While the system does not constrain the transcription in the active state, it makes this state extremely infrequent [111]. More work is needed to extend these observations and test their possible connection to local chromatin folding.

*Trans*-interactions between genes repressed by the Polycomb system can boost the repression. As discussed earlier, PRE-containing transgenic reporters exhibit ‘pairing-sensitive silencing’, a phenomenon where *Drosophila* homozygous for the transgenic allele express the reporter less than those heterozygous for the allele [40,41]. Even though, in the former case, the flies carry two copies of the transgene as opposed to one present in heterozygotes. Pairing-sensitive silencing requires that the two transgenes are in spatial proximity, which, in the cases above, arises from somatic pairing of homologous chromosomes. The pairing-sensitive silencing can happen even between two transgenes inserted in different chromosomes. In those cases, the transgenes must contain a chromatin insulator element to promote *trans*-interactions [42]. Overall, the spectacular and well-documented cases of pairing-sensitive silencing involve the side force helping to bring the repressed genes in spatial proximity. To what extent the long-range chromatin interactions mediated by the Polycomb group proteins contribute to the known cases of pairing-sensitive silencing is an open question. Likewise, it remains to be seen whether long-range interactions are important to repress genes at their native genomic locations. We are unaware of cases where a deletion or a translocation of one copy of a gene regulated by the Polycomb system led to the de-repression of the unaffected allele. Undoubtedly, more work is needed to gain a mechanistic understanding of how the long-range interactions between the repressed genes affect the transcription.

## Concluding remarks

Recent observations using Hi-C, super-resolution microscopy, and polymer modelling leave little doubt

that Polycomb group proteins increase the local chromatin folding. Stochastic interactions of tethered PRC1 with H3K27me3 or interactions between PRC1 complexes emerged as the prime candidates that enable the process. Remarkably, the resulting chromatin compaction is rather modest (no more than 2-fold on average) and dynamic. This behaviour questions traditional models where Polycomb group proteins repress transcription by restricting access for transcriptional activators and transcriptional machinery. How such dynamic chromatin folding enhances the epigenetic repression or whether the folding is just an ineffectual byproduct of molecular interactions within the Polycomb system are important open questions for future research. The emerging live imaging methods and computational modelling are the approaches most likely to bridge these gaps.

Likewise, genes repressed by the Polycomb system tend to be closer to each other in the nuclear space than expected by chance. However, this effect is even subtler than local chromatin folding. Whether such spatial proximity involves molecular interactions between the Polycomb group proteins bound to the 'paired' genes remains to be seen. In contrast to local chromatin folding, many lines of evidence indicate that the proximity between distinct genes promotes repression. Yet, the reported effects have been limited to transgenes and required an additional force to bring the repressed genes in spatial proximity. Testing the relative contribution of long-range interactions to the repression of genes at their endogenous locations is a challenging problem to solve in the coming years.

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## Conflict of interest

The authors declare that they have no competing interests.

## Author contributions

Conceptualization: LL and YBS. Writing original draft: LL and YBS. Review and editing: LL and YBS.

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