Review Article

Structural Basis of Polycomb Bodies

(Polycomb group proteins / Polycomb body / post-translational chromatin modifications / nuclear subcompartments / nuclear factories / chromatin domains)

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Abstract. The spatial organization of the cell nucleus into separated domains with a specific macromolecular composition seems to be the fundamental principle that regulates its functioning. Because of the importance of regulation at the nuclear level, the cell nucleus and its domains have been intensively studied. This review is focused on the nuclear domain termed the Polycomb (PcG) body. We summarize and discuss data reported in the literature on different components of the PcG body that could form its structural basis. First, we describe the protein nature of the PcG body and the gene silencing factory model. Second, we review the target genes of Polycomb-mediated silencing and discuss their essentiality for the structural nature of the PcG body. In this respect, two different schematic models are presented. Third, we mention new data on the importance of RNAs, insulator elements and insulator proteins for the structure of PcG bodies. With this review, we hope to illustrate the importance of understanding the nature of the PcG subcompartment. The structural basis of a subcompartment directly reflects its status in the cell nucleus and the mechanism of its function.

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Abbreviations: BMI1 – B lymphoma Mo-MLV insertion region 1 homologue; CBX – chromobox protein homologue; FISH – fluorescence *in situ* hybridization, FRAP – fluorescence recovery after photobleaching, H3K27me3 – trimethylation of histone H3 at lysine 27; IGC – interchromatin granule cluster, PcG body – Polycomb body, PcG proteins – Polycomb group proteins, PRC1/2 – Polycomb repressive complex 1/2, PRE – Polycomb response element, Xi – inactive X chromosome.

Polycomb group proteins and Polycomb-mediated gene silencing

Polycomb group proteins (PcG) are important epigenetic regulators that control transcription of their target genes. PcG target genes are mainly involved in the pathways related to cell cycle control, senescence, cell fate decision, stem cell differentiation and developmental segmentation (Sparmann and Van Lohuizen, 2006). PcG proteins execute their silencing function through binding to or in the vicinity of Polycomb response elements (PREs), the regulatory DNA elements that have so far been characterized only in *Drosophila melanogaster* (Fauvauque and Dura, 1993; Simon et al., 1993). PcG repressive functions are mainly associated with covalent post-translational modifications of histones (e.g. Cao et al., 2002, 2005; Czermin et al., 2002) followed by inhibition of chromatin remodelling and chromatin compaction (Francis et al., 2004; Grau et al., 2011). There are at least two main multiprotein complexes, the Polycomb repressive complex 1 (PRC1) and 2 (PRC2), that cooperate together in gene silencing (Martinez and Cavalli, 2006; Enderle et al., 2011). PRC2 is thought to be involved in the initiation of silencing by trimethylation of histone H3 at lysine 27 (H3K27me3) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). H3K27me3 serves as a docking site for PRC1 that is implicated in the stable maintenance of the repressed state of the genes (Fischle et al., 2003; Min et al., 2003; Lund and Van Lohuizen, 2004; Ringrose and Paro, 2004). PRC1 catalyses the monoubiquitination at lysine 119 of histone H2A that could trigger the compaction of chromatin (Wang et al., 2004; Cao et al., 2005; Martin-Perez et al., 2010). The compaction of chromatin by PRC1 proteins was shown by Francis et al. (2004) and by Grau et al. (2011). However, the initial clustering of the PcG target genes and the regulation of spreading of repressive marks was assigned to the DNA boundary elements called insulator elements (van der Vlag et al., 2000; Comet et al., 2011; Li et al, 2011, 2013).

The hierarchical network leading from the recruitment of PcG proteins to the gene silencing is widely accepted. However, there is growing evidence that the PcG-mediated silencing is a much more complicated and complex process. There are specific results showing that the repressive mark H3K27me3 is not essential for the maintenance of chromatin compaction (Chandra et al., 2012) or that the compaction of a large H3K27me3 domain on the inactive X chromosome (Xi) occurs in a PRC2-independent manner (Nozawa et al., 2013). Another report demonstrated that not all cells that exhibit a H3K27me3 mark on Xi show Xi enrichment of the PRC1 proteins (Plath et al., 2004). Further, it was shown that PRC1 recruitment to target genes in mammals could also be independent of PRC2 (Gao et al., 2012; Tavares et al., 2012). The results of Smigova et al. (2013) also indicate that PRC1 proteins are not essential for the maintenance of chromatin compaction.

Polycomb body as a microscopically visible nuclear subcompartment

The distribution pattern of PcG proteins and their associated histone marks is cell type-dependent (Sparmann and Van Lohuizen, 2006). Mostly, PcG proteins are diffusely distributed in the cell nucleus. However, in some cell types they also form foci visible by fluorescence microscopy, whether imaged using GFP fusion proteins or conventional immunofluorescence. These local accumulations of PcG proteins were termed Polycomb (PcG) bodies (Gunster et al., 1997; Satijn et al., 1997; Schoorlemmer et al., 1997; Saurin et al., 1998). The conspicuous PcG bodies were found in embryonic cells and in some cancer cell lines. The most obvious PcG foci are encountered in U-2 OS human osteosarcoma (Fig. 1) and 2C4 human fibrosarcoma cells, where their relative sizes differ from 0.2 to 1.5 μm and from 1 to 1.5 μm, respectively (Saurin et al., 1998). Besides mammalian cells, PcG foci have been described in cells of a number of other species including *Drosophila* and *Caenorhabditis elegans* (Buchenau et al., 1998; Zhang et al., 2006). In the nuclei of *Drosophila* embryo cells, PcG bodies are clearly distinguishable at the end of early embryogenesis and they progressively increase in size and number during stages 5–11 of late embryogenesis (Cheutin and Cavalli, 2012).

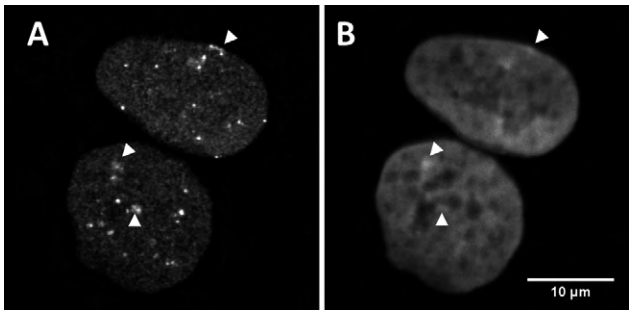

Fig. 1. Polycomb foci in U-2 OS cells stably expressing BMI1-GFP protein. (**A**) The fixed BMI1-GFP signal is localized in the cell nuclei in a diffuse form and in the form of discrete PcG bodies. The nuclei are counterstained with distamycin A/DAPI (**B**), which represents better quantitative DNA staining than DAPI alone. The DA/DAPI staining shows that PcG foci are DNA-rich structures (arrowheads).

The size and number of PcG foci reflect the expression level of PcG proteins (Saurin et al., 1998; Cheutin and Cavalli, 2012). In transformed cells, it was shown that these variations in expression result from karyotypic differences (Saurin et al., 1998). However, PcG foci also occur in non-transformed primary cell lines (MRC-5, CS22F) with known and normal karyotypes and in keratinocytes from human tissue sections, suggesting that they are not themselves a consequence of cellular transformation and subsequent overexpression (Saurin et al., 1998). However, PcG proteins are known to be associated with cancer as they control some aspects of neoplastic development (Sparmann and Van Lohuizen, 2006).

The nuclear positioning of PcG bodies is not completely random, as the bodies appear to be preferentially associated with some loci on particular chromosomes (Saurin et al., 1998; Voncken et al., 1999). However, colocalization experiments showed no association between PcG foci and Cajal bodies, gemini of Cajal bodies and probably also PML bodies (Saurin et al., 1998).

Components of the PcG body

1. Proteins of the PRC1 complex

The Polycomb body is primarily considered to be a protein-based structure, a distinct nucleoplasmic body formed by an accumulation of PcG proteins (e.g. Bantignies and Cavalli, 2011). Essential for the existence of PcG bodies are considered to be proteins of the PRC1 complex. The PRC1 core complex in *Drosophila* is composed of equimolar amounts of Polycomb (PC), Posterior sex combs (PSC), Polyhomeotic (PH) and Sex combs extra (SCE) proteins (Shao et al., 1999). However, in mammals, the PRC1 complex has undergone considerable expansion during evolution, resulting in the existence of multiple orthologues of each PRC1 member. Human cells thus encode five HPC (CBX), six PSC, three HPH and two SCE orthologues (Levine et al., 2004; Vandamme et al., 2011).

The typical PRC1 complex contains a single representative from each gene family (Sanchez et al., 2007; Maertens et al., 2009; Vandamme et al., 2011; Gao et al., 2012), but the final number of possible variants of PRC1 complexes is fairly high. The reason for the expansion of PRC1 families is unclear but intensively explored. For example, the recent study by Pemberton et al. (2014) was designed to determine whether the multiple orthologues that are co-expressed in human fibroblasts act on different target genes and whether their genomic location changes during cellular senescence. Surprisingly, the results showed that multiple variants of PRC1 associate with the same DNA target. However, the authors

observed distinctive PRC1 subnuclear localizations in different types of fibroblasts, and the representative patterns were preserved at senescence (Pemberton et al., 2014).

The structural nature and function of PcG bodies and the heterogeneity among the PcG foci composed of different orthologues were studied by various experiments focused on the kinetics of PcG proteins. Generally, PcG proteins were shown to exchange rapidly (Ficz et al., 2005). However, there is a difference between the dynamics of PcG proteins localized to PcG bodies and those localized outside the foci. Fluorescence recovery after photobleaching (FRAP) analysis revealed that whereas the PcG dynamics outside the foci is fast, perhaps because it is governed by diffusion as complexes and transient binding to chromatin, their kinetics inside the foci is mostly slower and exhibits large variability (Hernandez-Munoz et al., 2005; Sustackova et al., 2012; Vandenbunder et al., 2014). Vandenbunder et al. (2014) showed that there are three different populations of PcG proteins in PcG foci: fast, slow and immobile during 300 s. The fast fraction showed similar recovery time as measured outside the foci, i.e. 2–4 s, and should represent proteins moving by diffusion. The slow fraction was detected to have an average recovery time of about 20–80 s. The immobile fraction, also observed by Saurin et al. (1998) and Hernandez-Munoz et al. (2005), is thought to result from the tight binding of PRC1 proteins to chromatin and/or from recycling of these proteins within the foci.

The different kinetic pools measured in individual foci could be indicative of stochastic seeding events followed by random self-assembly of the Polycomb body (Yao et al., 2007; Vandenbunder et al., 2014), as hypothesized for the biogenesis of typical nuclear bodies such as Cajal or histone locus bodies (Dundr and Misteli, 2010; Dundr, 2011). The results from other than FRAP experiments also led to the conclusion that PcG bodies are proteinaceous nuclear bodies or factories (e.g. Cavalli, 2007; Bantignies et al., 2011). Using a combination of high-resolution fluorescence *in situ* hybridization (FISH) and immunostaining to PcG proteins, Bantignies et al. (2011) showed the co-localization of PcG target genes into the PcG body only when the genes are silenced. In this respect, PcG bodies have been termed gene-silencing factories (Hodgson and Brock, 2011). The silencing factory is thought to be formed by local accumulation of PcG proteins and non-coding RNAs, localized in the interchromatin compartment inside which the genes are looped to be co-silenced (Cavalli, 2007; Bantignies et al., 2011; Comet et al., 2011; Hodgson and Brock, 2011, see Fig. 2A). There are several models according to which PcG target genes shuttle between PcG bodies when repressed and to transcription factories when transcriptionally active (Bantignies et al., 2011; Pirrotta and Li, 2012). Moreover, Yang et al. (2011) showed shuttling of the PcG target genes between PcG bodies and interchromatin granule clusters (IGCs) in response to a growth signal followed by methylation/demethylation of the PC2 protein. They also found that the movement is the consequence of binding of methylated and unmethylated PC2 to non-coding RNAs located in PcG bodies and IGCs, respectively.

On the other hand, experiments on kinetics demonstrating the complete recovery of PcG proteins outside the PcG foci rule out the hypothesis that PcG bodies are structures where PcG proteins locally recycle (Vandenbunder et al., 2014). Conversely, these data rather indicate the chromatin nature of the PcG body. A similar conclusion was obtained by analysis of fluorescence recovery in PcG foci composed of different orthologues. Vandenbunder et al. (2014) detected variability between CBX4-GFP and CBX8-GFP foci. More stable and larger CBX4-GFP bodies suggested a more abundant im-

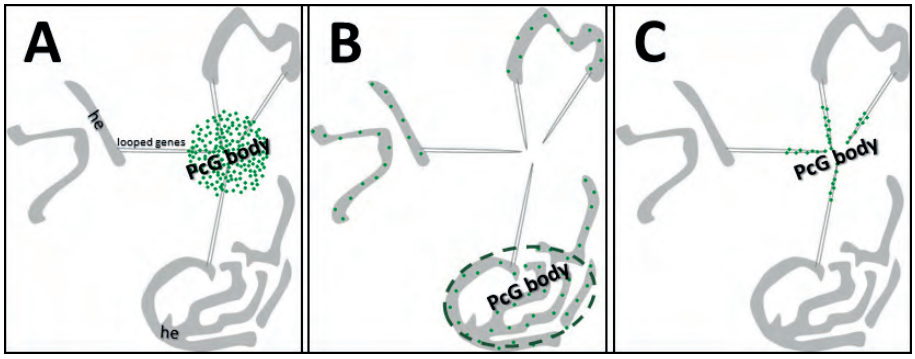

Fig. 2. Different models of a PcG body. (**A**) A Polycomb body as a typical nuclear body or a nuclear factory. It is formed by a local accumulation of PcG proteins and is localized into an interchromatin compartment. The model was drawn according to results of e.g. Bantignies et al. (2011). (**B**) A PcG body represents a DNA-rich chromatin domain. Accordingly, the appearance of a PcG body corresponds to a local accumulation of condensed chromatin fascicles coated with PcG proteins. Drawn according to results of Smigova et al. (2011). (**C**) In this model, which in fact represents a revised model A, a Polycomb body is thought to be formed by an accumulation of PcG proteins bound on their target genes. The genes are looped out from their chromatin context. The PcG body is localized into euchromatin. According to results of e.g. Cheutin and Cavalli (2012). Abbreviations: Condensed chromatin fascicles (he, heterochromatin, grey), looped genes (grey loops), PcG proteins (green dots), a circumference of a PcG body (dashed green ellipse).

mobile fraction and a longer recovery time for the slowly exchanging fraction than in CBX8-GFP foci. The authors suggest that the differences between CBX4 and CBX8 foci could result from an increase of the same type of binding sites. As they hypothesize, the increase could be caused by self-SUMOylation of the CBX4 protein and its subsequent spreading along the target genes.

2. PcG target genes

PcG target genes are expected to be another component of the PcG body. However, the importance of PcG target genomic loci for the structural basis of this subcompartment is fairly controversial. Some studies state that PcG bodies serve as host sites for PcG target genes. PcG target loci are looped out from their chromosomal context and localized into the protein-based PcG body in order to be co-silenced (Cavalli, 2007; Bantignies et al., 2011; Bantignies and Cavalli, 2011; Comet et al., 2011). In this respect, Bantignies et al. (2011) showed co-localization of two silenced genes, the *Antennapedia* (*Antp)* gene and the *Abdominal-B* (*Abd-B*) gene, with each other inside PcG bodies in the head region of the *Drosophila* embryo. In contrast, in the posterior parasegment 13 of the embryo where the *Abd-B* gene is active, the active gene was found to be localized outside the PcG foci. These studies suggest that looped PcG target genes use the PcG bodies as silencing factories, rather than structurally constitute them (see Fig. 2A).

On the other hand, there are studies that emphasize the importance of PcG genomic regions for PcG bodies' structure. Smigova et al. (2011) directly visualized the fine structure of the PcG body by using correlative light electron microscopy. The immunolabelled BMI1 Polycomb protein was detected to be specifically enriched within condensed chromatin fascicles (large-scale heterochromatin fibres) throughout the nucleus. The accumulation of the label in PcG foci was shown to be generated by the local accumulation of condensed chromatin fascicles in space. In a subsequent study, Smigova et al. (2013) demonstrated that under conditions of changed macromolecular crowding, the behaviour of PcG bodies vastly differs from the behaviour of typical nucleoplasmic bodies. This study also indicates that PRC1 protein accumulations do not represent a genuine nuclear subcompartment. Both studies showed that the so-called PcG body is rather a chromosomal domain than a typical protein-based nucleoplasmic body or a nuclear factory (see Fig. 2B, Fig. 3). The importance of chromatin

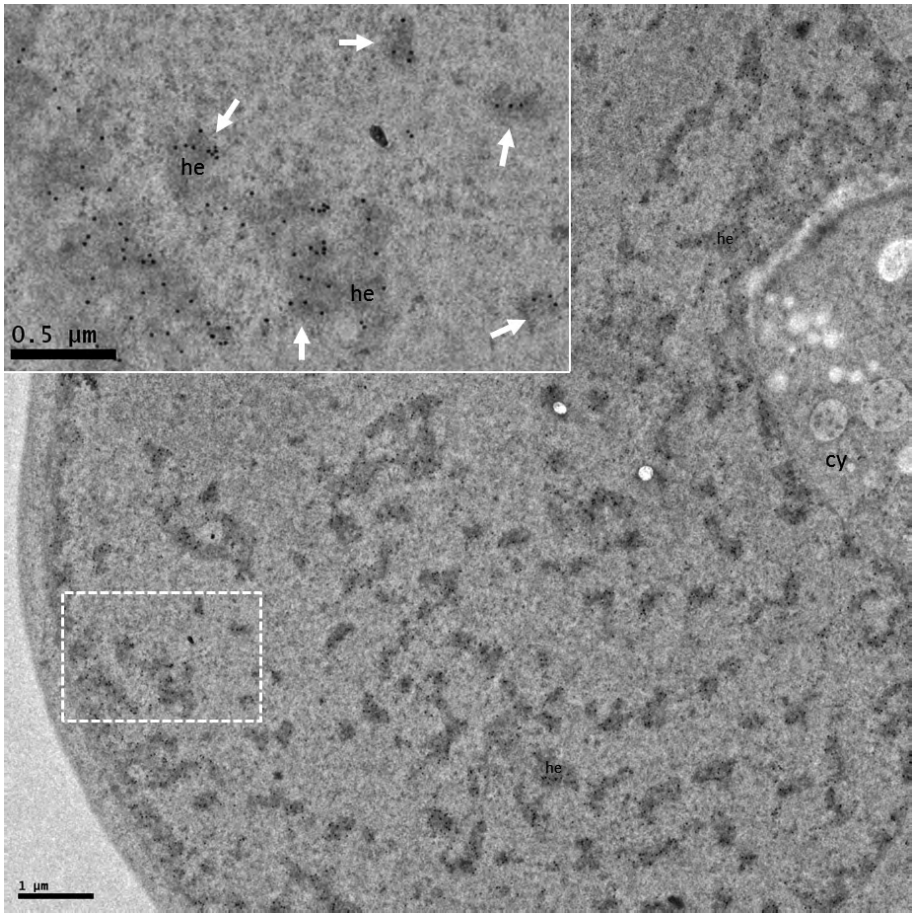

Fig. 3. Immunogold labelling of polycomb BMI-1 protein in U2-OS cell line. The immunogold label (15 nm gold particles) is specifically enriched within the electron-dense heterochromatin structures throughout the nucleus (arrows in inset). The cell is processed by high-pressure freezing followed by freeze substitution that allows preservation of the cellular fine structure and antigenicity. The image represents a 70 nm thick resin section through the unstained Lowicryl-embedded cell. He, heterochromatin, cy, cytoplasm.

architecture, especially the organization of chromatin into possible chromosome arm territories, for long-range PcG target sites interactions was suggested by Tolhuis et al. (2011) in *Drosophila* larval brain tissue. Using chromosome conformation capture on chip methods, they revealed that PcG target sites interact frequently with each other even when they are separated by megabases of sequence. However, these interactions occurred almost exclusively on the same chromosome arm. These authors also showed that the organization of chromosomes, rather than a sequence-based mechanism, is restricted for these PcG target sites' interactions.

Later, the correlation between the enrichment of PcG proteins and the enrichment of genomic regions was showed by Cheutin and Cavalli (2012). They calculated the ratio between the intensity of the immunolabelled PcG proteins measured within PcG bodies and the intensity of the DNA probes hybridized to genomic gene clusters coated with PcG proteins. Their measurements revealed that the amount of PcG proteins within the PcG body depends on the linear size of the genomic region. These results rather support the chromatin nature of the PcG bodies, although they were presented to support the model of gene-silencing factories (see Fig. 2C).

In contrast to "Model B", Cheutin and Cavalli (2012) localized PcG foci into euchromatin, according to weak DAPI staining and time-lapse chromatin motion experiments showing that PcG bodies move within volumes slightly larger than those of condensed chromatin domains. One possible explanations for this result may be the use of developing cells with undecided chromatin-containing bivalent domains, with coexistence of repressive and activating marks, which may be occupied by PcG proteins (Azuara et al., 2006; Bernstein et al., 2006; Bracken et al., 2006; Lee et al., 2006; Schuettengruber et al., 2009; Schwartz et al., 2010). As shown by Ahmed et al. (2009), chromatin marked by bivalent domains has a structure similar to that of the 10 nm chromatin fibre. Compacted chromatin domains can be detected from two-cell stage embryos during mouse development. They disappear in eight-cell stage embryos and appear again at later stages (Ahmed et al., 2009). The looseness of the undecided PcG chromatin or lack of fully established chromatin domains could be inferred from the kinetic observations of Fonseca et al. (2012) showing that the plasticity of PcG proteins binding is higher in stem cells than in more differentiated cells. Moreover, Ren et al. (2008) showed that in stem cells there is no significant difference in the dynamics of CBX fusion proteins localized to PcG bodies and those localized to regions outside the foci.

Chromatin domains should be, due to the established chromatin, rather studied in differentiated cells. However, generally, the higher-order chromatin structure remains poorly understood to date. It is still unresolved whether the chromatin domains are formed by densely packed 10 nm chromatin fibres (van Holde and Zlatanova, 1995; Eltsov et al., 2008; Ahmed et al., 2009; Fussner et al., 2011) or by 30 nm chromatin fibres (Scheffer et al., 2011; Bian and Belmont, 2012; Song et al., 2014).

By electron microscopy, it was shown that PcG bodies correspond to areas consisting of separated condensed approximately 100 nm thick chromatin fascicles (Smigova et al., 2011). Moreover, they appear to represent the most dense chromatin domains in the nucleus of U-2 OS cells. However, the detailed architecture of the chromatin organized into PcG bodies remains to be elucidated. The uniqueness of the PcG chromatin was shown by van Steensel's group, who distinguished, according to the presence of unique combinations of histone marks and chromatin-binding proteins, five distinct chromatin types using the DamID technique in *Drosophila* (Filion and van Steensel, 2010; van Steensel, 2011). Future electron microscopy research should be focused on the phenotypes of different types of chromatin to reveal even more details about the organization of chromatin depending on its activity.

3. Insulator elements, insulator-binding proteins and RNAs

Recently, the role of chromatin insulators, the genetic boundary elements that are known to block enhancer-promoter interactions or to prevent heterochromatin spreading, in the clustering of the PcG target genes was discovered (van der Vlag et al., 2000; West et al., 2002; McElroy et al., 2014). It was shown that insulator elements are even more important than Polycomb response elements (PREs) (Pirrotta and Li, 2011). The insulator element interposed between a PRE and a PcG target gene prevents interaction between the PRE and the distal promoter, and thus blocks its silencing (Comet et al., 2011; Li et al, 2011). On the other hand, two spaced insulator elements change chromatin conformation by formation of a chromatin loop that is able to bring an upstream PRE in contact with a downstream gene, and thus facilitate the silencing (Comet at al., 2011). *Trans* interactions are thus not blocked by insulator elements (Comet et al., 2011; Li et al., 2011). Li et al. (2011) also showed that PcG complexes bound at different PREs (*bxd, Mcp*, and *Fab-7*) are neither necessary nor sufficient to mediate long-distance interaction. Therefore, the insulator-binding proteins rather than the PcG complexes are thought to be the principal determinants of the higher-order organization of PcG targets in the nucleus. In a subsequent study, Li et al. (2013) showed that the clustering occurs only between PcG genes with similar transcriptional competence, either both repressed and targeted to PcG bodies or both active and probably targeted to transcription factories. Thus, the same insulator-binding protein is able to direct the PcG target genes to different nuclear subcompartments (Li et al., 2013).

On the other hand, PcG proteins seem to contribute to the function of insulator proteins. For example, Polycomb protein CBX4 has been shown to have a SUMO E3 ligase activity (Kagey et al., 2003) and SUMOyation

was detected on the insulator protein CTCF that is known to be associated with PcG bodies (MacPherson et al., 2009; Pirrotta and Li, 2012). However, the direct SUMOyation effect of CBX4 on the CTCF protein and their relationship to gene silencing remains to be studied.

Further, RNAs also appear to have a significant role in PcG-mediated gene silencing. For example, RNAi components were shown to be important for clustering of PREs (Grimaud et al., 2006), functioning of insulator elements (Lei and Corces, 2006) or modulation of the overall nuclear architecture. Thus, RNAs seem to be the crucial messengers and regulators of structural components of the PcG body.

Conclusion

This review summarized the present knowledge of the Polycomb group proteins and Polycomb-mediated silencing, particularly in mammalian and *Drosophila* cells, including PcG target genes and insulator elements. Throughout this review, however, a focus is placed on the structural nature of the PcG bodies. In this respect, we conclude that PcG bodies correspond to local accumulations of PcG proteins. However, further work is necessary to establish whether PcG bodies represent a nuclear body in the interchromatin compartment or a nuclear heterochromatin domain.

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