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The controversial role of the Polycomb group proteins in transcription and cancer: how much do we not understand Polycomb proteins?

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Polycomb group proteins (PcGs) are a large protein family that includes diverse biochemical features assembled together in two large multiprotein complexes. These complexes maintain gene transcriptional repression in a cell type specific manner by modifying the surrounding chromatin to control development, differentiation and cell proliferation. PcGs are also involved in several diseases. PcGs are often directly or indirectly implicated in cancer development for which they have been proposed as potential targets for cancer therapeutic strategies. However, in the last few years a series of discoveries about the basic properties of PcGs and the identification of specific genetic alterations affecting specific Polycomb proteins in different tumours have converged to challenge old dogmas about PcG biological and molecular functions. In this review, we analyse these new data in the context of the old knowledge, highlighting the controversies and providing new models of interpretation and ideas that will perhaps bring some order among apparently contradicting observations.

Introduction

Polycomb group proteins (PcGs) were first described in *Drosophila melanogaster* as important regulators of development and tissue morphogenesis, starting more than 60 years ago with the identification of Polycomb [1]. The PcG mammalian orthologues began to be described in the early 1990s, starting from the identification of *Bmi1* (*Psc* in *D. melanogaster*) and the discovery of its direct role in cancer development as a cooperative oncogene in a mouse model of Myc-induced lymphomagenesis [2,3]. These observations raised a large interest in PcG factors, which led to the identification of several other mammalian orthologues [4]. These studies characterized PcGs at a biochemical and functional level revealing that PcGs are present as two distinct multiprotein nuclear complexes [5] that, due to their repressive nature, were eventually named Polycomb repressive complex 1 and 2 (PRC1 and PRC2). These complexes are formed by several PcGs with different and still not fully understood functions [6].

The PRC1 is the complex with the largest number of reported subunits and recent studies highlighted the existence of at least five biochemically distinct subcomplexes with potentially different biological functions [7] (Fig. 1). All the PRC1 sub-complexes contain the core RING1A or RING1B E3-ligases (also known as RING1 and RNF2, respectively) that catalyse all

Abbreviations
AML, acute myeloid leukaemia; CpGi, CpG islands; DIPG, diffuse intrinsic pontine gliomas; Eed, embryonic ectoderm development; Ezh, enhancer of zeste; HSC, haematopoietic stem cell; KMT, lysine methyltransferase; KO, knockout; MDS, myelodysplastic syndrome; MEF, mouse embryonic fibroblast; mESC, mouse embryonic stem cell; ncRNA, non-coding RNA; PCGF, polycomb group ring finger; PcGs, Polycomb group proteins; PNS, peripheral nervous system; PRC, Polycomb repressive complex; PRE, Polycomb response element; Suz12, suppressor of zeste.

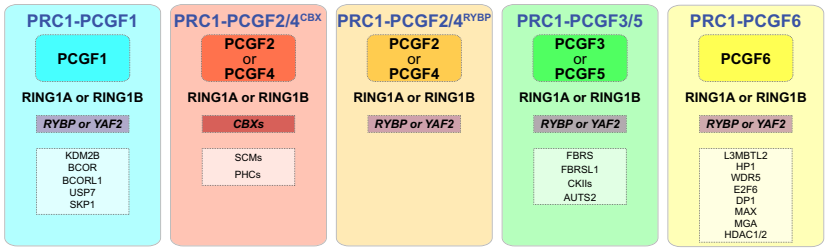

Fig. 1. Biochemical structure of the different PRC1 complexes. The picture summarizes the existence of functionally distinct PRC1 subcomplexes. The assigned nomenclature is from the original publication by Gao *et al.* [7] assuming that specific PCGF proteins, in association with either CBXs or RYBP/YAF2, define the functional and biochemical nature of the complexes. The definition of these complexes also assumes that PCGF2 and PCGF4 or PCGF3 and PCGF5 play redundant functions, as the biochemical composition of the PRC1 complexes formed by these proteins was identical.

mono-ubiquitination of the lysine 119 found on histone H2A (H2Aubq) [8,9]. Ring1a and Ring1b require stable interaction with different polycomb group ring finger (PCGF) proteins, which biochemically define the different forms of PRC1 (Fig. 1) [7]. The PRC2 composition is simpler than PRC1 and retains the ability to methylate (me) lysine 27 of histone H3 (H3K27). Such activity is exerted by the lysine methyltransferases (KMT) Ezh2 and Ezh1 [10] that control all the three states of H3K27 methylation (H3K27me1, H3K27me2 and H3K27me3) [11]. The catalytic activity of PRC1 and PRC2 is fully dependent on the formation of a core complex structure. While the RING1A/B ubiquitin-ligase activity largely requires the interaction with PCGF proteins *in vitro* and *in vivo* [12,13], EZH2/1 KMT activity requires interaction with the proteins EED and SUZ12 [14–16]. PRC1 and PRC2 are in large part associated together at chromatin sites enriched for genes involved in differentiation and proliferation processes [17,18]. Although PcG chromatin binding largely correlates with transcriptional repression [19], recent reports challenged this dogma and provided evidence that linked PcG activity also to active transcription [11,20,21].

Several components of both complexes are not essential for the intrinsic enzymatic activity of PRCs but seem to play fundamental roles in regulating the function and chromatin association of the two complexes. This is the case for RYBP, KDM2B (PRC1) or JARID2 (PRC2), which regulate stability and recruitment of PcG complexes to chromatin [22–25]. The complete understanding of the mechanisms that recruit PRC1 and PRC2 to chromatin in mammalian cells still remains a matter of debate that will be analysed in more detail in a following section.

PcG biological functions have been generally studied in two main directions: (a) the role of PcGs in differentiation and development and (b) the role in cellular proliferation and tumorigenesis [5,26]. While the genetic depletion of different PcGs results in mouse embryonic lethality or in distinct developmental defects, increased PcG activity is a negative prognostic factor for several tumours and PcG inhibition is generally considered a potential strategy for cancer treatment [5].

In this review we analyse the recent data that uncover new functions and molecular properties of PcGs, revisiting old and new dogmas and controversies linked to PcG biological activities.

Enzymatic activity of PRC2 and the role of its different partners

The best characterized activity of PRC2 is the trimethylation of H3K27 [16,27,28], which is preferentially deposited at CpG-dense genomic regions that largely correspond to gene promoters. Such deposition occurs simultaneously with the stable association of PcGs to the modified chromatin loci and correlates with transcriptional repression. The core composition of PRC2 is conserved from *Drosophila* to mammals, is maintained across cell types and implies the stable association of four core protein components: suppressor of zeste (Suz12), embryonic ectoderm development (Eed), retinoblastoma binding proteins 46 and 48 (RBBP4/7) and the two enhancer of zeste paralogues Ezh1 and Ezh2. The SET domain of EZH1/2 retains KMT activity specific for H3K27. The two different enhancer of zeste proteins are mutually exclusive within the PRC2 complex and display different *in vitro* KMT activities, cell type specific expression patterns and chromatin binding capabilities [29]. All core proteins are essential for Ezh1/2 enzymatic activity both *in vivo* and *in vitro* [15,30], which is consistent with the comparable developmental block observed in Eed,

Suz12 or Ezh2 knockout (KO) mice [14,31,32] and in line with the role of PRC2 in repressing the transcription of genes involved in cell differentiation and lineage specification [33–36]. Eed contains a WD40 domain that can directly bind H3K27me3, thus suggesting a self-sustaining mechanism for PRC2 activity during cell cycle progression [37,38]. In addition, H3K27me3 can establish an allosteric regulation that enhances the KMT activity of PRC2 [37]. *In vitro,* PRC2 KTM activity is boosted up to 7-fold in the presence of an H3K27me3 peptide, suggesting that allosteric regulation is not achieved via simple stabilization of PCR2 with its substrate (the nucleosome) but possibly through conformational changes in the complex. However, it remains unclear if Eed interaction with H3K27me3 is directly involved in such regulation since recent reports showed that a PRC2 complex, containing an Eed form mutated in its WD40 domain (Y365A) unable to bind H3K27me3, can be stimulated by H3K27me3 peptides to a similar extent as its wild-type counterpart [39], which is in contrast to what was previously reported [40]. These results leave the question about the physiological role of Eed recognition of H3K27me3 and the mechanism by which H3K27me3 can stimulate PRC2 activity in *cis* or *trans* in living cells open. *In vitro*, the PRC2 K_m is not altered by the addition of an H3K27me3 peptide, while the reaction V_{max} becomes strongly enhanced [40]. This demonstrates that allosteric H3K27me3 stimulation of PRC2 activity does not act by improving the affinity of the enzyme for its substrate but by increasing the rate of product conversion. Interestingly, the same study showed that the enzymatic kinetics and H3K27me3 allosteric stimulation were identical on mononucleosomes containing H3K27me0:H3K27me3 heterodimers with respect to fully unmethylated nucleosomes, demonstrating the lack of in *cis* H3K27me3 stimulation of PRC2 activity [40]. Moreover, the same study reported (but did not show) that PRC2 activity could not be stimulated in *trans* by fully methylated nucleosomes, leaving open the question whether pre-deposited H3K27me3 can stimulate PRC2 activity *in vivo*.

The H3K27 residue is modified post-translationally by EZH1/2 in a stepwise manner from the mono-methylated to the tri-methylated form (H3K27me1/2/3). Each different H3K27 methylated form can have different deposition patterns along the genome and functional outcomes [11]. *In vivo*, PRC2 activity regulates the deposition of H3K27me2 and H3K27me1 without stable association with target genomic loci [11]. Such deposition is linear with DNA synthesis [11], suggesting a rapid methylation of H3K27 upon the replication-dependent incorporation of new histones, in accordance with the localization of PRC2 at sites of ongoing DNA replication [38,41]. Proteomic studies performed in mouse embryonic stem cells (mESCs) showed that more than 80% of H3K27 is methylated and that the large majority (~ 70%) is in the di-methylated form. This suggests that the main enzymatic product of PRC2 in living cells is H3K27me2, which covers large genomic regions [11]. H3K27me3, which in ESCs accounts for approximately 7% of whole H3K27, is preferentially deposited at specific loci in correspondence to CpG-rich DNA regions that largely correspond to TATA-less gene promoters [11,42]. While both H3K27me3 and H3K27me2 correlate with transcriptional silent genomic regions, H3K27me1 (accounting for approximately 4% of all H3K27) is deposited throughout the gene bodies of actively transcribed genes in correspondence to H3K36me3 enrichment, a histone post-translational modification directly controlled by transcriptional elongation [11,43–46]. *In vitro* studies showed that H3K27me0 and H3K27me1 are better substrates for PRC2 then H3K27me2 [47]. This is consistent with the *in vivo* distribution of the H3K27 methylation pattern where H3K27me2 is the major product of PRC2 activity [11]. We have proposed that H3K27me1 domains are formed via H3K36me3-mediated in *cis* inhibition of PRC2-dependent H3K27me1 conversion to H3K27me2, while H3K27me3 deposition is achieved only upon stable interaction of PRC2 with chromatin, a condition necessary to compensate its low enzymatic efficiency in methylating H3K27me2 [47,48]. This model becomes even more evident when EZH2 Y641 hyperactive mutations (recently discovered in human lymphomas) are analysed *in vivo* and *in vitro* (these mutations will be discussed in detail in a following section). EZH2 Y641 mutations probably induce changes within the EZH2 SET domain that greatly enhance the ability of the enzyme to methylate H3K27me2. *In vivo* this results in a dramatic increase of H3K27me3 deposition with respect to the H3K27me1/me2 levels [47]. The diffused deposition of H3K27me2 in intra- and inter-genic domains has a protective function in preventing the aberrant activation of enhancer-like elements counteracting H3K27 acetylation (ac) [11]. This is consistent with previous reports showing that global H3K27ac levels are increased upon loss of PRC2 activity [24,49].

H3K27me3 deposition seems to occur only in the presence of a stable association of the PRC2 complex with chromatin. This is achieved by the association of PRC2 accessory proteins that are dispensable for the intrinsic KTM activity but important for the stabiliza-

tion of the complex at target sites. These proteins have been suggested to differentially regulate PRC2 activity among cell types and across developmental stages [10]. These accessory proteins include (a) AEBP2 [15], a zinc finger protein that enhances KTM activity *in vitro* and shows about 70% of co-localization with PRC2 (Suz12) genomic target loci [50]; (b) the three mammalian homologues of *Drosophila* Polycomb-like (Pcl1-3, also known as PHF1 [51,52], MTF2 [53,54], PHF19 [55,56]) which display some tissue specific expression and present a TUDOR domain capable of binding H3K36me3; such affinity has been suggested to be a triggering mechanism to initiate silencing of actively transcribed genes [57,58]; (c) the Jumonji and ARID domain containing protein Jarid2 (which is able to bind GC-GA rich DNA elements) required for PRC2 recruitment at target genes and for proper ESC differentiation [24,59–62]. Importantly, Pcl and Jarid2 proteins incorporate in the PRC2 complex in a mutually exclusive manner suggesting that the interaction with different accessory subunits could contribute to targeting the PRC2 complex to specific promoters in a context restricted manner [57]. Recent findings also revealed a physical interaction in mESCs and in human cancer cell lines between PRC1 and Eed (a specific member of the PRC2 complex) providing an additional layer of regulation and complexity. Eed can associate more stably with PRC1 proteins that belong to the CBX-containing PRC1 sub-complex ($PRC1^{CBX}$). In particular, Eed interacts with PCGF4 (BMI1) and PCGF2 (MEL18). In this work, the authors suggested that Eed mediates the recruitment of this PRC1 variant to PcG target loci in prostate cancer cell lines [63].

Biological and biochemical complexity of PRC1

PRC1 is present in different sub-complexes that are biochemically distinct from each other (Fig. 1) [7,64]. All PRC1 sub-complexes contain the RING1A or RING1B subunit, which determines PRC1 catalytic outcome [65]. These different sub-complexes are defined by the presence of one of the six PCGF subunits which are crucial for RING1A/B E3 ligase activity [7]. Although PRC1 sub-complexes were previously divided in six different complexes based on the presence of specific Pcgf proteins [7], such classification did not take into account the presence of CBXs or Rybp/YAF2 proteins, or considered that some of these complexes have redundant biochemical composition. Therefore, we decided to provide a new classification of the different PRC1 sub-complexes that is based on their interacting proteins as shown in Fig. 1. The so-called 'canonical PRC1' is defined by the presence of the PCGF proteins 2 or 4 (BMI1 and Mel18, respectively) and by the association of other subunits like PHC and CBX ($PRC1\text{-}PCGF2/4^{CBX}$) [66]. This complex seems to be recruited to chromatin through the ability of the CBX proteins to bind the H3K27me3 deposited by PRC2 [5]. Such mechanism is consistent with the large overlap in the target genes between PRC2 and RING1B [36] and with the global loss of RING1B chromatin association at those target sites in the absence of PRC2 activity [22]. However, upon loss of PRC2 activity, the global H2Aubq levels remain largely unaffected suggesting that PRC1 enzymatic activity does not depend on PRC2 [22]. Indeed, the residual PRC1 containing RYBP remains associated with the target genes to sustain the H2Aubq levels in the absence of PRC2 [22]. RYBP and its paralogue YAF2 are present in all the PRC1 sub-complexes and consequently are mutually exclusive with CBX proteins when associated with PCGF2/4. This forms the PRC2-dependent $PRC1\text{-}PCGF2/4^{CBXs}$ and the non-canonical $PRC1\text{-}PCGF2/4^{RYBP}$ complex (Fig. 1) [7,22]. Overall, the intricate biochemical structure of RING1A/B-associated proteins generates sub-complexes with potentially different biological functions. Surprisingly, CBX- and RYBP-containing complexes share a large degree of overlap in target genes [7,67] although they seem to bind adjacent regions separately [7]. In addition, a recent work demonstrated that PRC1-PCGF2/4 complexes are unable to deposit H2Aubq when forcibly recruited on chromatin [68] suggesting that (a) pre-existing H3K27me3 could be essential for PRC1-PCGF2/4 activity and (b) deposition of the H2Aubq is largely dependent on the activity of RYBP/YAF2-containing complexes. However, the fact that PRC1-PCGF2/4 complexes (which can also contain RYBP instead of CBXs) do not contribute to H2Aubq deposition in these conditions may suggest that PRC1-PCGF2/4 complexes are not a major source of H2Aubq in living cells [68].

The mechanisms by which the PRC1 and the specific sub-complexes are recruited on chromatin still remain an important open issue. Recent findings uncovered a role for Kdm2b in recruiting PRC1 on chromatin [69]. Kdm2b is specifically associated with the PRC1-PCGF1, which controls a large part of H2Aubq present in mESCs [7]. Together with its paralogue Kdm2a (which does not associate with PRC1), Kdm2b is a histone H3K36me3/2 specific demethylase [70]. Both demethylases contain a CXXC domain with high affinity towards CpG-rich DNA regions, consistent with their diffuse localization to CpG-rich promoters [71]. Kdm2b depletion in mESCs leads to premature differ-

entiation [25] similarly to *Ring1a/b* loss of function [9]. Furthermore, Kdm2b forced recruitment on chromatin leads to the recruitment of endogenous components of PRC1-PCGF1 and to the co-recruitment of PRC2, which can establish *de novo* H2Aubq and H3K27me3, respectively [68]. Another non-canonical PRC1 sub-complex that is probably involved in the regulation of ESC identity is PRC1-PCGF6 [7,72]. This complex contains the proteins L3mbtl2 and Wdr5, which are essential to maintain the pluripotent state of ESCs [73,74]. However, PRC1-PCGF6 is formed by promiscuous subunits: Wdr5 is also an essential component of all COMPASS complexes that control all H3K4 methylation states in different cell types [75]; Max is the dimerization partner of Myc that is essential for its transcriptional activity [76]; E2f6, a non-transactivating member of the E2F transcription factor family, can also associate with members of PRC2 and G9a/GLP complexes in proliferating cells [77,78]; Hdac1/2 are partners of several different repressive complexes [79]; and L3mbtl2 is stably present also in the NuRD complex [73]. The specific role of these proteins in the PRC1-PCGF6 is still poorly characterized. While the purified L3mbtl2-PRC1 complex is able to deposit H2Aubq on recombinant nucleosome [72], *L3mbtl2* KO mESCs do not show any significant change in the levels of this histone modification [73]. It is possible that L3mbtl2 contributes to the deposition of H2Aubq only at specific loci [72], even though L3mbtl2 does not seem to regulate classical PcG targets in mESCs [73]. The PRC1-PCGF1 and PRC1-PCGF3/5 are even more poorly characterized; however, the forced recruitment of these specific sub-complexes to chromatin is sufficient to deposit H2Aubq and to induce PRC2 recruitment [68]. In general, the current knowledge about the biological and molecular functions of the different PRC1 sub-complexes is still largely not understood and a more comprehensive characterization of their functions is absolutely required to decrypt the multifaceted activity of PRC1 and PRC2 complexes.

Functional interplay between PcGs and non-coding RNAs

In the last few years an increasing number of reports have highlighted that PcGs can functionally interact with RNA molecules. Besides protein-coding RNA transcripts, the large fraction of PcG interacting RNAs have non-coding properties (ncRNAs). Such interaction can involve both small ncRNAs of a few tens of nucleotides and long ncRNAs hundreds of kilobases in length that can fold into secondary structures and form sequence specific DNA interactions [80]. These findings made PcG-lncRNA interaction an exciting mechanism by which PcGs can be recruited at target loci. This mainly involves (a) PRC2 recruitment to the inactivating X-chromosome via direct Xist interaction; (b) PcG recruitment to a Hox gene cluster by the HOTAIR ncRNA; (c) PcG recruitment to the *Ink4a-Arf* locus by the ANRIL ncRNA. Both PRC1 and PRC2 complexes decorate the inactive X-chromosome (Xi) in mammals depositing H3K27me3 and H2Aubq, respectively. While PRC1 recruitment at Xi is poorly characterized, PRC2 coating of the Xi is mediated by its ability to interact with different antagonistic ncRNAs transcribed from the X-chromosome inactivation centre (RepA and Tsix): this generates a complex mechanism of regulation resulting in the full transcriptional activation of one *Xist* allele. The resulting *Xist* transcript coats the Xi in *cis* and mediates PcG recruitment. However, it is still not clear if direct *Xist* binding is mediated by Ezh2 [81,82] or by the PRC2 subunit Jarid2 [83]. Similarly, the PRC1-PCGF2/4^{CBX} subunit Cbx7 can also be recruited to the Xi in an RNA-dependent manner [84].

A similar mechanism was shown to recruit PRC2 at the *HOXD* in *trans* via direct interaction of PRC2 with the ncRNA *HOTAIR*, a 2.1 kb transcript originating from a non-coding region of the *HOXC* cluster [85]. It was further proposed that different regions of the *HOTAIR* RNA could bind multiple repressive complexes functioning as a recruitment platform for epigenetic repressors to specific loci. Indeed, both PRC2 and the LSD1/CoREST/REST complex bind *HOTAIR* simultaneously to its 5′ and 3′ end, respectively, to form a super-repressive complex [86]. *HOTAIR* is highly expressed in cancer cell lines and is linked to enhanced tumour progression. However, such mechanism seems to be a specific feature of human cells, as murine *Hotair* deletion has no effect on HoxD expression during mouse development [87]. Finally, the PRC1 complex has been shown to repress the *Ink4a/Arf* locus in part via its interaction with *ANRIL*, a long antisense non-coding transcript originating from the *Ink4a/Arf* locus. *ANRIL* is highly expressed in prostate cancer tissues and mediates *INK4b/ARF/INK4a* epigenetic silencing by stabilizing in *cis* PRC1 activity to the locus [88].

High-throughput and *in vitro* approaches, aiming to identify additional ncRNAs associated with PcGs, have highlighted a promiscuous affinity of PRC2 for binding RNA molecules. More than 9000 different PRC2-associated RNAs were identified in mESC by RNA immunoprecipitation analysis [89]. The recombinant PRC2 complex is able to bind *in vitro* with good affinity a variety of RNA molecules of different

lengths (up to 300 bp) with no preference for a particular sequence. This suggests that PRC2 association with RNA could be dictated by affinity for RNA secondary structures rather than by recognition of particular binding motifs [90]. The transcription of short RNAs (50–200 bp in average length) has been reported at a fraction of PcG target loci in primary T cells and mESCs. These short Pol-II-dependent transcripts fold in secondary structures that are able to bind SUZ12, to recruit PRC2 and silence target genes [91]. Crosslinking immunoprecipitation analysis performed to identify PRC2-associated RNAs highlighted that PRC2 binds nascent RNA transcripts originating from active regions with low PcG enrichment and H3K27me3 deposition [90,92]. In the first report, the authors propose that PRC2 senses nascent RNA expression as an 'escape' from repression. PRC2 binding to nascent transcripts would therefore stimulate PRC2 activity and deposition of H3K27me3 to re-establish gene repression [90]. Differently, the second report suggests that contact between nascent RNAs and PRC2 prevents the accumulation of H3K27me3 allowing low transcription levels [92]. Further investigation is still needed to properly comprehend the role of PRC2 in binding nascent RNA transcripts. Interestingly, while PRC2 nascent RNA binding regions were devoid in JARID2 association [90,92], crosslinking immunoprecipitation assays for JARID2 mostly identified interaction with lncRNAs, suggesting different functional properties for PRC2 association with RNA molecules [93].

Chromatin recruitment of Polycomb group proteins

The investigation of the mechanisms underlying PcG targeting at specific genomic loci is one of the most debated and still undefined issues. Such mechanisms, ultimately leading to gene repression and establishment of correct transcriptional programmes, are better characterized in *Drosophila* but seem to retain a low level of conservation in vertebrates where alternative recruitment mechanisms have been proposed. However, such differences could be smaller than what they seem, as most of the knowledge related to PcG recruitment and transcriptional control in *Drosophila* is based on genetic screens and studies performed before the era of ChIP and high-throughput sequencing. Such genetic approaches led to the identification of distal *cis*-regulatory elements bound by PcGs, defined as Polycomb response elements (PREs), that are involved in the coordinated spatio-temporal regulation of homeobox gene transcription during development [94–96]. Differently, all available knowledge related to PcG transcriptional activity in mammalians is largely based on correlative studies aimed at mapping sites of enrichment for PcGs along the genome. PREs are almost devoid of nucleosomes and present consensus sites for a number of DNA binding proteins that are able to recruit PcG repressive complexes [97,98]. Such DNA binding factors are not conserved in mammals with the exception of PHO (YY1 in mammals). However, genome-wide and biochemical studies have demonstrated that Yy1 does not play any role in PcG recruitment in mammalian cells [99]. Indeed, retrospective analyses performed in *Drosophila*, with a similar approach to that used for mammalian cells, highlighted that PcG enrichment and H3K27me3 deposition are not restricted to PREs and can be frequently found also at gene promoters [97,98]. This suggests that the general mechanisms of PcG recruitment might retain a certain degree of conservation. In mammals, PcGs preferentially associate with CpG-rich promoters [42]. Although in the *Drosophila* genome CpG islands (CpGi) do not exist, the 'broad' features of mammalian CpG-rich promoters are highly conserved also in the *Drosophila* system (CpG-rich promoters do not have a TATA box and an initiator signal, and do not display a precise transcription start site) [100], suggesting that the PcG recruitment mechanism could be linked to the underlying nature of CpG-rich promoters rather than directly to the CpG-rich DNA elements. Indeed, no clear-cut data have been published about the role of the direct recognition of these DNA elements by PRC1 and/or PRC2 complexes and their recruitment to target promoters.

The solid genetic data published on *Drosophila* about the role of DNA binding transcription factors mediating PcG recruitment favoured models where PcGs are selectively recruited at specific target genes via direct interaction with cell type specific transcription factors [101]. For example, both PRC1 and PRC2 have been shown to interact and to be potentially recruited at promoters by the transcription factor REST [102,103]. Bmi1 (PRC1) was biochemically purified along with Runx1/Cbfβ, which is able to recruit PRC1 in a PRC2-independent manner [104]. Similarly, PRC2 can be recruited by Snail1 to play a role in epithelial to mesenchymal transition [105] or, aberrantly, via interaction with leukaemic DNA binding fusion proteins such as PML-RARα [106] and PLZF-RARα [107].

The discovery of the affinity of PRC1 and PRC2 complexes for RNA binding molecules also favoured a different model in which ncRNAs could drive

promoter specific recruitment of PcG activities, as described in the previous section. However, the promiscuous binding affinity of PcGs for RNA species, and the large number of RNA molecules to which PcGs can associate *in vivo,* favour the existence of an in *cis* mechanism that senses transcribed RNA molecules (independently of their nucleotide sequence) rather than an in *trans* mechanism of active recruitment, such as those proposed for HOTAIR at the HOXD locus.

The model by which in *cis* transcribed RNAs, that can be either small, nascent or lncRNAs, mediate PcG recruitment is in contrast with another model in which PcGs would tend to bind by default CpG-rich promoters, and this association is simply excluded by either active transcription or DNA hyper-methylation at the CpGi [42,108–110]. Indeed, recent evidence reported that chemical inhibition of RNA Pol II activity doubled the amount of PcG bound promoters (together with H3K27me3 deposition) within a few hours from the block of transcription. Such accumulation still occurs at CpGi-containing promoters that were previously annotated as PcG targets in differentiated cells [108]. This result implies that PRC2 association to CpGi occurs as a default mechanism and that transcription is sufficient to exclude PcG association from promoter elements. This observation is in line with the known role of PcGs during *Drosophila* development, where PcGs or TrxGs are recruited to PREs to maintain a pre-established transcription status [111]. Consistently with this model, the induction of transcription from an ectopic PRC2-bound CpG-rich promoter was sufficient to prevent PRC2 association *in vivo* [109]. Nonetheless, the model leaves the mechanism by which PRC2 and non-canonical PRC1 preferentially associate to CpG-rich DNA elements still an open question.

An additional mechanism that could mediate or contribute to PcG recruitment to specific loci is the ability of different PRC1 and PRC2 subunits to bind specific histone modifications. All Cbx proteins of PRC1-PCGF2/4 can bind H3K27me3 [112,113]. HP1 proteins are stable partners of PRC1-PCGF6 and can bind H3K9me3 [113]. Similarly, the Wdr5 present in PRC1-PCGF6 is also an essential subunit of the COMPASS complexes and is involved in the recognition of H3K4me3 [7,114]. In addition, the PRC2 complex stably includes PCL proteins (PCL1-3) containing a Tudor domain that can directly bind H3K36me2/3, a histone modification linked to active transcription. For instance, it is possible that Wdr5 could favour the association of PRC1 complexes at bivalent promoters (which simultaneously contain H3K4me3 and H3K27me3) in ESCs. However, it is more likely that the recognition of histone post-translational modifications by PcGs contributes to stabilizing their chromatin association rather than dictating cell type specific promoter association.

Regardless of the mechanisms dictating PcG promoter selection in specific cell types, the hierarchy and the interdependence between PRC1 and PRC2 association at target loci is an additional important issue of investigation and debate. Once the different PRC1 sub-complexes were clearly defined, it immediately became clear that the old hierarchical model in which PRC2 mediates PRC1 recruitment by the recognition of H3K27me3 with CBX proteins was inadequate (Fig. 2) [112,113]. Although the stability of Ring1b at the target promoter is in large part dependent on PRC2, a residual amount of Ring1b (~ 10%) is still associated with target loci in PRC2-null mESCs. This is sufficient to maintain normal levels of H2Aubq [22]. Such residual binding is dependent on RYBP, which is a mutually exclusive subunit in the canonical PRC1 (PRC1-PCGF2/4^{CBXs} versus PRC1-PCGF2/4^{RYBP}) and a constitutive subunit of non-canonical PRC1 complexes (PRC1-PCGF1, PRC1-PCGF3/5 and PRC1-PCGF6) (Fig. 1) [22]. Therefore, the deposition of H2Aubq seems fully dependent on RYBP-containing PRC1 complexes. The fact that KDM2B (Fbxl10) loss of function results in a global reduction of H2Aubq deposition strongly suggests that H2Aubq levels are largely under the control of PRC1-PCGF1^{RYBP}. Kdm2b retains a zinc finger CXXC domain that confers high binding affinity to CG-rich elements suggesting a direct mechanism that links non-canonical PRC1 to CpGi [23,115]. However, Kdm2b is not an exclusive partner of PRC1-PCGF1^{RYBP} and is found to be localized at almost all CpGi present in ESCs. This could serve as a potential platform for PcG recruitment as some of these genes can become PcG targets at later differentiation stages [23]. Recent reports have further challenged the previous model showing that the PRC2 complex can be recruited to chromatin by non-canonical PRC1 sub-complexes. While PRC1-PCGF1^{RYBP} and PRC1-PCGF3/5^{RYBP} are able to deposit H2Aubq and induce the recruitment of PRC2 activity, the forced recruitment of PRC1-PCGF2/4^{CBX} to the same genomic loci failed to deposit H2Aubq and to recruit PRC2 activity [68]. Furthermore, PRC2 was recently shown to bind *in vivo* and *in vitro* H2Aubq [116]. Together, these observations suggest a novel mechanism (Fig. 2) in which non-canonical PRC1 complexes are first recruited to establish H2Aubq domains, which mediate PRC2 association and H3K27me3. The establish-

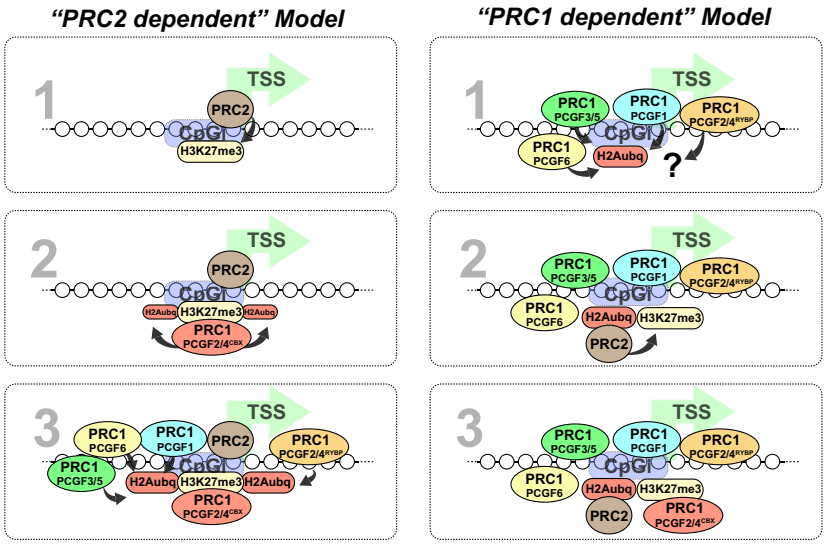

Fig. 2. Mechanisms of hierarchical PRC1 and PRC2 recruitment to chromatin. The picture highlights different interpretations of PRC1 and PRC2 association at target loci. A 'PRC2-dependent' model is based on the initial literature and implies that PRC2 mediates PRC1 recruitment via H3K27me3 recognition. This scheme introduces the existence of PRC2-independent PRC1 sub-complexes that bind the same genomic loci independently of H3K27me3 and play a major contribution to sustain H2Aubq levels. Differently, the 'PRC1-dependent' model puts in context the recent discoveries showing that PRC2 can be directly recruited to chromatin by non-canonical PRC1 sub-complexes, potentially by recognizing H2Aubq. PRC1-PCGF1 is placed at the centre of a hypothetical CpGi to stress the ability of its subunit KDM2B to bind directly CpG-rich DNA. The PRC1-dependent model also stresses the poor contribution of PRC1-PCGF2/4 in establishing and sustaining H2Aubq levels.

ment of H3K27me3 mediates the recruitment of PRC1-PCGF2/4^{CBX}, recreating the co-occupancy observed *in vivo* by ChIPseq analyses for these complexes to the same target loci. The high affinity of KDM2B for CpGi further suggests that this protein could serve as a direct docking site to 'nucleate' the recruitment of multiple PcG complexes to the same loci. However, the loss of function of Kdm2b is perinatal lethal in mice in comparison with the severe pre-implantation and post-implantation lethality observed in fully deficient PRC1 and PRC2 KO embryos, respectively [68]. This diminishes the central role of Kdm2b in regulating the recruitment of PcG activities at CpG-rich promoters. Indeed, loss of function of Kdm2b in mESCs resulted in a modest decrease in PRC2 association at target loci [68]. Similarly, the complete loss of function of PRC1, although with greater effect, was not sufficient to fully displace PRC2 from its target promoters [68], highlighting the existence of multiple mechanisms that stabilize PRC1 and PRC2 association at their target promoters. Finally, it is important to highlight that all these observations were generated under experimental conditions that only address the mechanism by which PcGs maintain a pre-established binding pattern, which could be very different from the mechanisms that establish *de novo* PcG chromatin association.

Polycomb in stem cells and cellular differentiation

PcGs are able to establish heritable chromatin states that preserve gene-silencing patterns in a cell type specific manner. *In vitro* and *in vivo* evidence has shown that Polycomb proteins are able to mediate gene repression through different processes. Two main mechanisms of PcG-mediated gene repression have been proposed: (a) chromatin compaction and (b) impairment of the functions of the transcription machinery. The ability of PRC1 in compacting nucleosome arrays was first described with the *Drosophila* PRC2 and this allowed the Posterior Sex Combs genomic region to be repressed [117]. Functional PcG domains of compacted chromatin were also observed as foci within nuclei and were named PcG bodies [118]. Although these data suggested that PRC1 ubiquitin E3-ligase activity is required for compaction, recent studies in ESCs showed that H2Aubq is dispensable for the condensation of the *Hox* gene cluster but indispensable for proper repression of the same targets [119]. In general, a more compacted state of

the chromatin is less accessible for chromatin remodellers (e.g. SWI/SNF complex) and transcription factors which eventually can lead to transcriptional activation [120]. Moreover, densely packed nucleosomes have been shown to stimulate PRC2 activity on H3K27, thus generating a positive feedback loop on PRC2 activity [121].

Another mechanism of PcG repression involves the direct inhibition of the transcriptional machinery. Although it was reported that PcG binding does not exclude RNA Pol II associations to promoters in *Drosophila* cells [122], genome-wide data showed a reduced RNA Pol II occupancy at bivalent promoters in ESCs [123]. RNA Pol II is associated at PcG target promoters in its poised form (phosphorylated at Ser5 at its C-terminal domain) and the loss of Ring1a/b activity induces a switch to the elongating form (phosphorylated at Ser2) [124,125]. This suggests that PcG occupancy at bivalent promoters is able to hold and stall RNA Pol II at transcription start sites.

In ESCs, self-renewal is controlled by the expression of pluripotency transcription factors (i.e. Oct4, Sox2 and Nanog) and probably by the repression of lineage specific genes [126]. PcGs are expressed at high levels in ESCs and are enriched at promoter regions of key developmental regulators [34,35,126]. While loss of PRC2 activity does affect the self-renewal capabilities of ESCs [127,128], complete loss of the entire PRC1 activity induces a gradual loss of ESC self-renewal [9]. This further suggests that the non-canonical PRC1 complexes, which are not dependent on PRC2 activity, play very important developmental roles. This is consistent with the block during embryonic development at the two-cell stage observed in Ring1a/b double null embryos [129] with respect to the early post-implantation lethality of PRC2 [128,130] or PRC1-PCGF2/4^{CBX} deficient mice (Bmi1 and Mel18 double KOs) [131].

Some controversies regarding the role of Cbx7-associated PRC1 complex in maintaining ESC pluripotency exist. Cbx7 is the major Cbx protein expressed in ESC. While its overexpression can enhance ESC self-renewal [132,133], Cbx7 loss of function (RNAi-based) was reported to either impair [132,133] or not impair [132,133] ESC self-renewal. The fact that Cbx7 KO mice develop normally and are viable [134] suggests that, if required, this property of Cbx7 must be restricted to its acute loss in ESCs.

Although the loss of PRC2 activity does not affect ESC self-renewal, it affects the differentiation capabilities of PRC2 deficient ESC. Upon induction of differentiation, PRC2 deficient ESCs fail to activate correct lineage transcription programmes. Such defect is consistent with the developmental block observed during early post-implantation stages when developmental lineages start to be established [31,32,128]. It is likely that PRC2 deficient ESCs are not able to maintain lineage specific repression of different sets of genes [135]. However, recent findings from our laboratory further suggested that lack of PRC2 activity can also influence the correct establishment of cell type specific enhancer activation as well as the transcription of specific genes in the absence of H3K27me1 deposition at highly transcribed gene bodies [11]. In addition, the depletion of PRC2 regulatory subunits, such as Jarid2 and Pcl2, was also reported to affect the establishment of proper differentiation programmes. Jarid2 depleted ESC cells fail to differentiate in cell culture in accordance with their essential role in embryonic development (Jarid2 deficient embryos die between E11.5 and E15.5) and with their essential function in recruiting PRC2 in ESCs [24,60,62]. Pcl2 depleted ESCs also fail to properly differentiate in cell culture; however, this is due to the maintenance of a high level of the pluripotency factors Nanog and Oct4 in differentiating cells [53]. Such effect seems to be restricted to cell culture experiments, as Pcl2 KO mice only display some growth defects but remain viable [136].

Although the single loss of Ring1b only mildly affected global H2Aubq levels in ESCs, it led to severe embryonic lethality (days *post coitum* 9.5) [137]. The PRC1 subunits RYBP and L3MBTL2 also have important roles in development and loss of their functions result in embryonic lethality due to an aberrant gastrulation [73,138]. RYBP and L3MBTL2 knockdown in ESCs does not allow correct cell culture differentiation, mirrored by an altered cell proliferation and deposition of H2Aubq [7,73]. All KO models for other PRC1 subunits can reach birth; however, they often display different types of developmental defects. Cbx2 KO male mice present female gonads, while female Cbx2 KOs do not develop ovaries [139]. Interestingly, in humans, a female individual with a male karyotype was found to carry a germline inactivating mutation in the *CBX2* gene [140]. Single inactivation of Pcgf2 (Mel18) or Pcgf4 (Bmi1) are viable, but the born mice displayed homeotic transformation of axial skeleton and immune deficiency [141,142]. However, the combined inactivation of Pcgf2 and 4 causes embryonic lethality at E9.5. Considering the role of PRC1-PCGF2/4^{CBX} complex in the deposition of H2Aubq discussed in the previous section, this result raised the need to reconsider the role of H2Aubq deposition in the regulation of embryonic development [131].

PcGs also retain fundamental roles in adult tissue homeostasis. Early B cell development strictly depends on Ezh2 activity to regulate *Igh* gene rearrangement [143]. Similarly, mutant *Eed* null haematopoietic stem cells (HSCs) are not able to give rise to mature blood cells, inducing exhaustion of the adult HSC pools [144]. Pcgf4 (Bmi1) is also needed for HSC maintenance via transcriptional repression of the *Ink4a/Arf* locus [145]. Cbx proteins tightly control HSC self-renewal and differentiation capabilities. Cbx7 is present at high levels in HSCs and its overexpression can induce leukaemia. The Cbx7 protein levels decrease through HSC differentiation in favour of Cbx2/4/8 that, if aberrantly expressed in HSCs, induces stem cell exhaustion [146]. Adipocyte formation is impaired in the absence of PCR2 activity due to the failure in suppressing the Wnt signalling pathway [147]. PRC2 is also required for proper myogenesis where a high level of Ezh2 expression in precursor cells prevents the premature transcription of muscle specific genes that can activate myogenesis [148]. Similarly, epidermis formation from the basal layer of multipotent progenitors requires PRC2 mediated repression of AP1 ensuring proper time-controlled activation of lineage specific genes [149].

The controversial role of PcGs in cancer

The role of PcGs in cancer is currently one of the most interesting topics that prompt an intense parallel work in drug discovery [150–152]. Despite the increasing attention on Polycomb proteins as potential therapeutic targets in cancer, the biological role of these proteins in tumours is becoming more and more controversial [153,154]. Of all results, the data related to the role of PcG haematological malignancies are puzzling [155,156]. While in adult haematopoiesis PRC2 activity clearly plays an essential role (i.e. *Eed* loss in the adult haematopoietic compartment results in long-term HSC exhaustion and pancytopenia [144]), inactivating mutations were found in *EED* and other PRC2 gene loci (i.e. *SUZ12, EZH2* and *JARID2*) in both myelodysplastic syndrome (MDS) and leukaemic patients [157–161]. Moreover, targeted *Ezh2* deletion in the haematopoietic compartment of adult mice resulted in the development of T-acute lymphoblastic leukaemias with an insurgence range between 152 and 281 days after deletion [162]. Unexpectedly, a transgenic model that overexpressed *Ezh2* in the haematopoietic compartment also induced the development of MDS [163]. This was a direct effect on HSCs as the serial transplantation assays with transgenic HSCs mirrored the original MDS phenotype [163]. PRC2 activity was shown to be required for the development of MLL-AF9 acute myeloid leukaemias (AML) [164–166]; however, loss of PRC2 activity promoted the development of MDS and leukaemias induced by ASXL1 mutations [167]. Recently it has been shown that the loss of EZH2 activity promotes the development of MDS induced by RUNX mutations; although, it prevents MDS to further develop into AML, thus highlighting a 'double-face' role for PRC2 in haematopoietic malignancies [168].

The PRC1 role in leukaemia also presents a certain degree of controversy. While Bmi1 is essential for AML1-ETO or PLZF-RARα induced leukaemias [107,169], it is dispensable for MLL-AF9 driven leukaemogenesis. This is due to the specific ability of MLL-AF9 to activate *Hoxa7* and *Hoxa9* expression that can maintain the *Ink4a-Arf* locus repressed in the absence of Bmi1 activity to promote leukaemia progression [169]. More recent reports showed that Cbx8, a known PRC1 subunit, is required for the development of MLL-AF9 driven leukaemias independently of Ring1b or Bmi1 [170]. However, such effect is probably independent of *Ink4a/Arf* expression as loss of Cbx8 expression fails to activate *Ink4a/Arf* transcription despite preventing leukaemogenesis [170]. To further complicate the role of Cbx8 in the haematopoietic compartment, it has recently been shown that Cbx8 overexpression in HSC and progenitors induces cell exhaustion and differentiation [146]. Understanding the CBX8 roles that are dependent or independent from PRC1 will certainly help to unravel the ambiguities on CBX8 function in cancer.

The role of Cbx7 is also quite controversial. Differently from Cbx8, Cbx7 is preferentially expressed among all the other Cbx proteins in HSCs and its overexpression leads to increased self-renewal, immature blast-like morphology and leukaemia development [146]. Different studies showed that Cbx7 could act as an oncogene mainly by exerting its transcriptional repression on the *Ink4a/Arf* locus [171–173] while others demonstrated its tumour suppressive activity [134,174]. Although this could be explained by the different tissue ontologies presented in these studies, a general mechanism that distinguishes an oncogenic versus tumour suppressive role for Cbx7 is still missing. More generally, the role of PcGs in regulating tumour growth is frequently associated with their ability to repress the *Ink4a/Arf* locus, a well-known, non-cell-type specific tumour suppressor and negative regulator of cell cycle progression [171,173,175–182]. Such general mechanism has been proposed for years as the principal way through which PcGs favour tumour cell proliferation. However, our group has

recently demonstrated that the genetic deletion of either PRC1 or PRC2 activities strongly impairs mouse embryonic fibroblast proliferation and transformation capabilities in an Ink4a/Arf-p53-pRb independent manner [41], further showing that PcGs can supervise DNA replication by directly localizing at sites of ongoing DNA replication [41]. This finding opens up the possibility of treating cancer with EZH2 inhibitors despite the functionality of the pRb and p53 pathway, which is inactivated in nearly all human tumours [183,184].

PcG inhibition is indeed becoming an attractive strategy for cancer treatment. An EZH2 inhibitor is currently in clinical trial (#NCT01897571) as single agent treatment for lymphomas and solid tumours. Lymphomas are clearly the best candidates for PcG inhibiting compounds as they are characterized by a strong expression of PcG subunits and by high PcG activity [5]. Furthermore EZH2 is frequently mutated in diffused large B cell lymphomas and in follicular lymphomas [185] at Y641 within its SET catalytic domain. Although initially these mutations were considered a loss of function (supporting a tumour suppressor activity for EZH2 also in lymphomas), later studies demonstrated that these mutations confer a gain of function towards the accumulation of H3K27me3 [48]. Indeed, EZH2 Y641 mutants are unable to generate mono- or di-methylated H3K27 *in vitro* (H3K27me1 or H3K27me2), but acquire enhanced activity on H3K27me2 to generate H3K27me3 [48,186]. Lymphomas expressing these mutations are addicted to the expression of EZH2 Y641 mutants and small molecules specific for EZH2 Y641 mutated forms were generated to kill specifically lymphoma cells that expressed these mutations [187–189]. The specific targeting of mutant EZH2 is very important since targeting wild-type EZH2 could have diffuse toxic effects. Ezh2 is essential for normal germinal centre (GC) formation [190,191] as well as for other physiological processes [10]. Studies aimed to investigate the oncogenic nature of EZH2 Y641 mutations showed that the activation of EZH2 Y641N in GC-B cells induced GC hyperplasia but was insufficient to generate lymphomas [191]. However, the ectopic expression of EZH2 Y641F cooperated with Bcl-2 in inducing diffused large B cell lymphomas in Bcl-2 overexpressing bone marrow transplanted cells [191]. The latter result suggests the existence of cooperating genetic events in which EZH2 Y641 mutations have a direct oncogenic effect. However, it still remains to be clarified if this oncogenic property can be recapitulated with specific mouse models and which are the molecular mechanisms behind the oncogenic activity of EZH2 Y641 mutations. Moreover, the EZH2 Y641N transgenic mouse consists of an extra copy of the *EZH2* gene that is expressed by an exogenous promoter resulting in an increased Ezh2 expression. It is therefore important to determine if the EZH2 Y641 mutations are directly inducing GC hyperplasia or if the simple EZH2 overexpression is *per se* sufficient to cause such phenotype. A comparison with a mouse model conditionally expressing a wild-type EZH2 extra allele or the generation of heterozygous EZH2 Y641 mutated mice will be required to clarify this issue.

Although EZH2 gain of function mutations in lymphomas positively support the proto-oncogenic role of PRC2, Suz12 heterozygous mice have an increased clonogenicity of B cell lymphoid progenitors and accelerate Myc-induced lymphomagenesis [192]. Such putative tumour suppressive role has recently been proposed also in glioblastomas. PcGs were (a) reported to be general negative prognostic factors in glioblastomas [193], (b) shown to be essential for the maintenance of glioblastoma cancer stem cells [194,195] and (c) shown to be essential for gliomagenesis [196]. Nevertheless, H3K27M mutations were recently discovered as frequent somatic mutations in one H3.3 variant in diffuse intrinsic pontine paediatric gliomas (DIPG) [197,198]. Such mutation was more recently shown to inactivate the global PRC2 enzymatic activity both *in vitro* and directly in DIPG tumours [39,199]. Although the causative role of these mutations remains to be addressed, the global loss of PRC2 activity in them suggests an enigmatic theory that considers the PRC2 as an oncogene and its enzymatic activity as a tumour suppressor. A possible explanation that reconciles such paradox could reside in additional PRC2 non-histonic targets. Indeed, it was shown that Ezh2 is able to control glioblastoma stem-like cells by methylating Stat3 to promote its oncogenic functions [200]. Whether the H3K27M mutation also inhibits non-histonic PRC2 activity still remains to be determined. A tumour suppressive role for PRC2 also came from the recent report of loss of function mutations in the *Suz12* locus in peripheral nervous system (PNS) tumours that cooperate with *NF1* mutations. Importantly, genetic mouse models seem to partially recapitulate the human malignancy and the increased levels of H3K27ac to synthesize PRC2-deficient PNS tumours to the treatment with BET inhibitors (BET inhibitors target a family of BROMO domain proteins that bind to acetylated histone lysines) [201]. Overall, understanding the molecular mechanism by which PcG genetic alterations can contribute to cancer development will not only provide important knowl-

edge for disease treatment but will also generate invaluable information to better understand the biological roles and functions of these complicated yet fascinating proteins.

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Author contributions

AS and AP wrote the manuscript. DP revised the manuscript. AS and DP made the figures.

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