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Role of PRC2-associated factors in stem cells and disease

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The Polycomb group (PcG) of proteins form chromatin-binding complexes with histone-modifying activity. The two main PcG repressive complexes studied (PRC1 and PRC2) are generally associated with chromatin in its repressed state. PRC2 is responsible for methylation of histone H3 at lysine 27 (H3K27me3), an epigenetic mark that is linked with numerous biological processes, including development, adult homeostasis and cancer. The core canonical complex PRC2, which contains the EZH1/2, SUZ12 and EED proteins, may be extended and functionally manipulated through interactions with several other proteins. In this review, we focus on these PRC2-associated proteins. As PRC2 functions are diverse, the variability conferred by these sub-stoichiometrically associated members may help to understand specific changes in PRC2 activity, chromatin recruitment and distribution required for gene repression.

Introduction

The basic unit of DNA storage in the nucleus is the nucleosome, which consists of a tight yet flexible complex comprising DNA and histones. Post-translational modifications of histone tails are a major mechanism by which gene expression is regulated. Functionally, histones provide a conformation to the chromatin, and specific post-translational modifications of histones appear to be linked with the transcriptional state of genes. This gene regulatory control via post-translational modifications is achieved by modifying the physico-chemical properties of the chromatin architecture, which affects the interaction between DNA and histones or nucleosome occupancy, and by generating docking sites for effector proteins or complexes with enzymatic activity [1,2].

Polycomb group (PcG) proteins represent a prime example of epigenetic modifiers that influence the chromatin state by acting on histone tails, and are therefore considered as key transcriptional regulators, controlling many fundamental cellular processes [3]. First discovered in *Drosophila* as crucial regulators of developmental processes, PcG proteins have recently received attention as modulators of stem cell differentiation and identity in mammals [4]. Notably, they have also been found to be involved in cell-cycle control and tumorigenesis [5,6].

PcG proteins exert their functions within complexes known as Polycomb repressive complexes (PRCs) that have enzymatic activities – ubiquitination at lysine 119 on histone H2A (H2AK119ub) by PRC1, and methyla-

Abbreviations
EED, embryonic ectoderm development protein; ES, embryonic stem; EZH, enhancer of zeste protein; PcG, Polycomb group; PCL, Polycomb-like gene; PRC, Polycomb repressive complex; SUZ, suppressor of zeste protein.

tion at lysine 27 on histone H3 (H3K27me1/2/3) by PRC2 – that are linked with a repressive transcriptional state of the target genes [3]. PcG proteins function as complexes in *Drosophila*, and homologues are found in most metazoans. Moreover, a higher degree of diversity is achieved in mammals by the presence of paralogues [3]. A clear example of this diversity is observed for PRC1, where each member of the canonical complex from *Drosophila* has between two and six paralogues in mammalian cells [6]. Together with the presence of the non-canonical complex PRC1, this confers the potential for great functional variety for PRC1, and recent efforts have been made to elucidate any non-overlapping roles of individual complexes [7].

PRC2 methylates histone H3 at lysine 27, which is associated with gene repression [8]. Although initial purification of the complex and structural reports revealed that the complex comprised five members [9,10], just three of these are strictly required for methyltransferase activity of the complex: (a) enhancer of zeste (EZH2 or EZH1), which is the catalytic subunit with a SET domain; (b) embryonic ectoderm development (EED); (c) suppressor of zeste 12 (SUZ12) [10]. In contrast to PRC1, the core PRC2 members are not represented by a great variety of paralogues in mammals; in fact, only EZH2 may be replaced by its closely related family member EZH1. However, diversity is achieved by the presence of other proteins that associate sub-stoichiometrically with this core complex [11].

Here we summarize current knowledge about the functional roles of PRC2-associated members in the context of PRC2-mediated gene regulation. The distinction between PRC2 core components and the associated factors is not always straightforward but instead is somewhat arbitrary. For instance, RBAP46/48 and AEBP2 were initially characterized as 'full' core PRC2 members with important functions in increasing catalytic activity without being strictly required for it; indeed, stable trimeric complexes with residual activity may be reconstituted [10]. The association of additional factors with PRC2 is highly context-dependent, and may reflect their different biological roles. In addition to the above-mentioned RBAP46/48 and AEBP2, which has DNA-binding capacity, we focus on JARID2, which also binds DNA and is the most-studied PRC2-associated factor. We also discuss the three mammalian orthologues of the *Drosophila* Polycomb-like gene (PCL), namely PHF19/PCL3, PHF1/PCL1 and MTF2/PCL2; all of these contain Tudor and PHD domains, which function within PRC2. Finally, we discuss the protein C17orf96, which to date has no precise function attributed and has few remarkable structural characteristics, but has recently been found as part of PRC2 (Fig. 1).

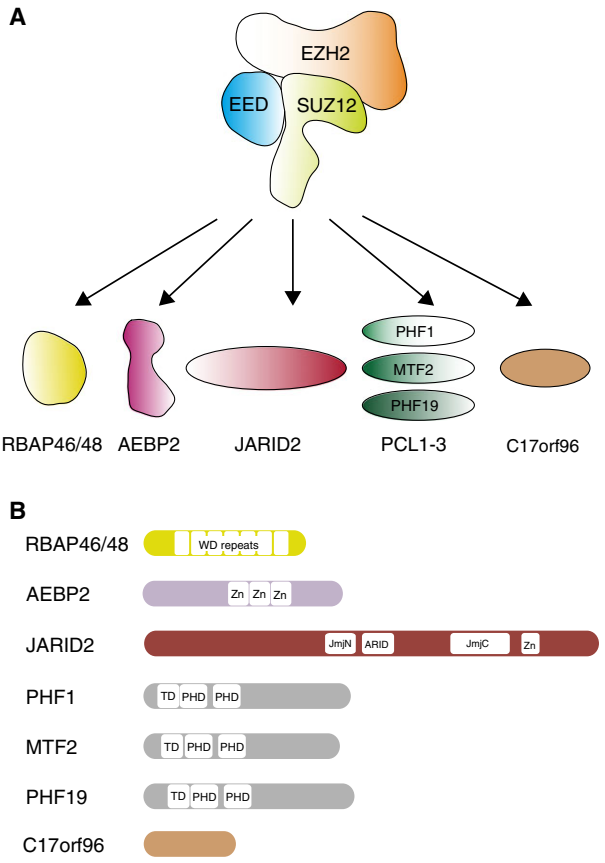

Fig. 1. Core PRC2 and associated factors. (A) Schematic representation of the PRC2 core, RBAP46/48, AEBP2 based on [9], JARID2, the three PCL orthologues (PHF1, MTF2 and PHF19) and C17orf96. (B) Protein domains of PRC2-associated factors.

RBAP46/48

Mammalian RBAP46 and RBAP48 are two highly similar proteins (90% amino acid identity) that are orthologues of *Drosophila* NURF55. They belong to the WD40 repeat family of proteins, and fold into a characteristic β-propeller [12–14]. NURF55 and RBAP46/48 form part of several complexes through protein–protein interactions, and play a central role in chromatin-related processes as histone chaperones [15]. They not only form part of histone deacetylase complexes, such as NuRD and NuRF, but are also involved in replication-related nucleosome assembly. This diversity complicates enormously elucidation of their contribution to individual events, because phenotypes observed in genetic experiments cannot be attributed to a specific complex. Therefore, *in vitro*

biochemical approaches have so far yielded most insight to their function.

Although the p46/p48 proteins are not required for the histone methyltransferase activity of EZH2 and do not appear to stimulate it, they have an important PRC2-related function. First, through their affinity for histones, they probably contribute to PRC2 binding to chromatin [16,17]. p48 binds to the H3–H4 dimer, and crystal structures have been individually reported for binding to the relevant H3 and H4 peptides, which, interestingly, have different binding sites on NURF55 [12,13,15,18]. Second, NURF55 may be important for the proper integration of active and repressive histone marks: in the context of PRC2, SUZ12 occupies the H4-binding site of NURF55. Moreover, H3 peptides that are methylated at lysine 4 (H3K4me1/me2/me3) are bound to NURF55 with decreased affinity compared to unmodified peptides; additionally, the modified H3 tails appear to inhibit the histone methyltransferase activity of PRC2 on oligosomes [18]. Therefore, PRC2 associated with NURF55/p46/p48 is less likely to interact with the H3 peptide when it is methylated at lysine 4, a mark that is found at transcriptionally active regions, resulting in a decrease in PRC2 activity at these sites. This is an attractive model for limiting the deposition of H3K27me3 at genomic regions that must be maintained in an active state. Importantly, the PRC2 histone methyltransferase activity is inhibited only when both copies of the H3 peptide are methylated at lysine 4, therefore allowing bivalent modified nucleosomes (K4me3 and K27me3) to be generated on different H3 tails on the same nucleosome [19].

Finally, it is not known whether p46 and p48 possess different functions in the context of PRC2. Both proteins appear to form part of PRC2 [20], but it is possible that they have slightly different functions or genomic localizations, with as yet unknown biological consequences.

AEBP2

One of the main questions regarding PRCs in mammals is how are they recruited to the specific loci where they exert their enzymatic activity. An attractive mechanism for PRC2 is that proteins with DNA-binding domains confer this specificity. AEBP2 was first characterized as a zinc-finger protein with repressive capacity in mice [21]. Later, it was identified during purification of a complex with H3 methyltransferase activity in HeLa cells, which turned out to be PRC2 [22]. Whereas SUZ12 is strictly essential for PRC2 histone methyltransferase activity, AEBP2 is required for optimal enzymatic activity of the complex [10]. The stimulation of PRC2 activity by AEBP2 was suggested to be related to its capacity to interact with nucleosomes [10]. Later, it was confirmed that the activity of PRC2 containing AEBP2 was increased in the presence of nucleosomes, but a direct interaction between nucleosomes and AEBP2 was not demonstrated [23]. It is noteworthy that the cryo-electron microscopy structure of PRC2 was deduced in the presence of AEBP2, which stabilizes the complex and may function as an allosteric cofactor bridging DNA-binding and enzymatically active parts of the complex [9].

AEBP2 has two isoforms, with the shorter one mainly being expressed in the embryo. Both isoforms contain the zinc finger as well as a bipartite, variable DNA-binding sequence that is highly GC-rich (CTT $(N)_{15\text{-}23}$cagGCC with the lowercase bases being less critical for binding) [24]. No further genome-wide characterization or molecular analysis after AEBP2 depletion has been reported, leaving open the question as to what extent AEBP2 serves as a recruitment factor for mammalian PRC2. An AEBP2 gene-trap mutant mice line has been generated [25], and embryonic lethality was reported for homozygous mice. Further analysis of the heterozygous mice revealed defects related to neural crest development.

JARID2

Among the PRC2-associated factors, JARID2 is the most widely studied. JARID2 is the founder member of the Jumonji family group of proteins [26]. Initially, the Jumonji protein was suggested to be essential for neural tube formation, and, in later human studies, was found to be involved in embryonic development of the central nervous system [27]. All members of the Jumonji family contain a JmjC domain, which is characterized by α-ketoglutarate-dependent histone demethylase activity, although the JmjC domain of JARID2 does not appear to be active, as it lacks the amino acids required for enzymatic activity [28,29]. In addition to the JmjC domain, JARID2 contains a JmjN domain, as well as two domains with DNA-binding capacity: an AT-rich interaction domain and a zinc finger. This capacity implicates JARID2 as a good candidate for PRC2 targeting, but no specific binding motifs have been reported, and DNA binding of JARID2 appears to be independent of its PRC2-related functions [30].

Since its discovery, JARID2 has been studied in an embryological context. Various studies have reported embryonic lethality, although with differences depending on the genetic background of the mutant strain

[31]. The pleiotropic function of JARID2 has been confirmed by the various phenotypes reported, especially regarding the neural and cardiac systems. Jumonji was originally found to be expressed in the brain of developing mice, and several recent reports have linked JARID2 and intellectual disability in human patients: deletions in chromosome 6p22-p24, which include JARID2, are clinically linked with neurodevelopment disorders [32–34]. Further characterization is required to understand whether these clinical observations may be explained at a molecular level by association of PRC2 with JARID2. During cardiac development, JARID2 appears to control cardiac cell proliferation [35]. Indeed, the mouse phenotypes observed in both neurogenesis and heart formation have been linked to repression of the cyclin D1 gene [36–38]; however, cyclin D1 repression appears not to depend on PRC2 but on methylation of another lysine residue linked with repression, i.e. lysine 9 of histone H3 (see below).

The relationship between JARID2 and PRC2-driven repression has been mainly studied with respect to embryonic stem (ES) cell biology. In 2009/2010, five reports appeared that identified JARID2 as part of PRC2, and studied its role in PRC2-mediated repression in ES cells [29,30,39–41]. Several discrepancies existed between these reports, for example regarding the impact of JARID2 on the enzymatic activity of the complex and therefore the levels of H3K27 methylation at target genes [42], but some common conclusions were drawn: for example, JARID2 is highly enriched at PRC2 targets on chromatin, its depletion causes a reduction of PRC2 binding to target genes; recruitment of JARID2 and PRC2 is inter-dependent, as depletion of several members of PRC2 also reduced enrichment of JARID2 at chromatin, and JARID2, as well-known for the other members of the core PRC2, is required for proper ES differentiation. This still leaves several questions unanswered: (a) although two of these reports used JARID2$^{-/-}$ cells, PRC2 recruitment was not completely abolished [29,41], indicating that this cannot be the exclusive recruitment mechanism, and (b) how JARID2 confers target specificity was not resolved, as its DNA-binding capacity was not studied in depth [30,40] and no other domain of JARID2 was proposed to be able to confer specific targeting.

The specificity of the targeting has since been studied further. Two previously uncharacterized close regions of the protein have been shown to be required for interactions of JARID2 with nucleosomes [23] and non-coding RNA [43]. This hypothesis is especially attractive as the plethora of non-coding RNAs may provide specificity at various target genes. Further investigation and determination of the interaction structures of JARID2, PRC2 and non-coding RNAs may shed light on this issue. Another study examined the PRC2- and JARID2-dependent repression of specific regulatory sites of various *hox* genes in a reporter construct. The results demonstrated that PcG-dependent repression is complex: although all the elements showed a partial decrease of PRC2 and/or H3K27me3 upon JARID2 depletion, not all showed a parallel de-repression of transcription [44]. Identification and isolation of more of these elements may offer a functional answer to how JARID2 mediates PRC2 recruitment. Finally, JARID2 has also been studied in ES cells in the context of PRC2-dependent inactivation of the X chromosome. In this case, JARID2 recruitment is not inter-dependent with that of PRC2 (i.e. it is still recruited in the absence of functional PRC2), but is highly dependent on Xist RNA [45].

JARID2-dependent recruitment of PRC2 has been also observed in adult tissues, such as muscle cells. In an elegant study, PRC2 was shown to mediate an inflammation-dependent block of myogenesis via muscle-specific gene repression that is dependent on JARID2 [46]. The authors even elucidated the order of events, starting with class II transactivator, which is recruited to muscle-specific genes in response to the inflammatory cytokine interferon-γ. JARID2 interacts with the class II transactivator and is recruited to these sites, leading to enrichment of other PRC2 members. Another study has reported the role of JARID2 in epidermal homeostasis, again in relation to its capacity for regulating PRC2 activity [47]. In this case, depletion of JARID2 in mice keratinocytes led to an increase in postnatal differentiation, and this was molecularly linked to reduced PRC2 genomic occupancy, lower H3K27me3 levels, and increased expression of PRC2 epidermal targets. Many further such studies are necessary to fully understand the pleiotropic functions of JARID2.

JARID2 is also involved in other mechanisms and pathways. For instance, it has been recently reported that JARID2 is part of a specific PRC2 (also involving AEBP2) that recognizes the mono-ubiquitination of histone H2A at lysine 119 (the mark deposited by PRC1) [48]. Also, as mentioned above, JARID2 is associated with the repression of cyclin D1 through methylation of lysine 9 of histone H3 (H3K9). In this case, JARID2 interacts with the methyltransferases G9a/GLP [49] and SETDB1 [50], although the composition of these JARID2/methyltransferase complexes has not been reported.

JARID2 mRNA is a target of microRNA 155 (miR-155), with several functional consequences.

miR-155 induces pathological cardiomyocyte hypertrophy, and miR-155-null cardiomyocytes display increased expression of JARID2 [51]. These findings may explain the cardiac defects observed in JARID2 mutant heart development [36]. Moreover, miR-155 is best known as an oncogenic factor [52,53], suggesting that JARID2 down-regulation occurs during carcinogenic transformation. Nonetheless, JARID2 has been implicated in maintaining the undifferentiated state of rhabdomyosarcomas through changes in H3K27 methylation of target genes [54].

PCLs

The *pcl* gene was first reported in *Drosophila* in the early 1980s [55], and its protein was shown to be involved in recruiting the PcG homologue of EZH2 [56]. PCL is required for H3K27 trimethylation at PRC2 targets [57]. *Drosophila* PCL contains two PHD domains and a Tudor domain that, in contrast to other Tudor domains, lacks the capacity to bind to trimethylated lysines. Three orthologues of PCL exist in mammals: PHF19/PCL3, PHF1/PCL1 and MTF2/PCL2. All of these have been hypothesized to interact with trimethylated lysines, as they each have a complete Tudor domain [58]. Thus, PCLs have been proposed to target specific histone modifications, which may help to recruit PRC2, as discussed below.

PHF19/PCL3

Although PHF19 was the last PCL homologue identified, its relationship with PRC2 has been widely studied. It was identified as PCL3 in humans, and its isoforms and expression pattern have been described [59]. In that study, the Tudor domain and the two PHD domains present in *Drosophila* PHF19 were identified, as well as a C-terminal region with weak homology to a chromodomain and two putative nuclear localization signals. It has two isoforms in humans, PHF19/PCL3-long and PHF19/PCL3-short, the latter of which contains only the Tudor domain and one of the PHD domains. A function of PHF19 in transcription repression has been reported [59]. PHF19 was later shown to associate with PRC2, and it was found that over-expression of the two PHF19 isoforms in HEK293 cells led to interaction of EZH2 with their Tudor and PHD2 domains [60].

The most relevant studies regarding PHF19 have been performed using ES cells. In 2012, three reports confirmed the importance of PHF19 in the context of PRC2-associated repression [61–63]; PHF19 is part of a specific PRC2 that does not contain JARID2, and, by studying its genome-wide localization, most PHF19-binding sites were found to coincide with PRC2-binding and H3K27me3 regions [61–63]. Interestingly, PHF19 depletion leads to a substantial reduction in SUZ12 binding in 82% of PHF19/SUZ12/H3K27me3 co-targets. Moreover, 71% of the target genes completely lost their H3K27me3 mark upon PHF19 depletion [62]. Notably, this regulation of PRC2 function is independent of the stability of the complex or the protein levels. As for JARID2, the binding capacities of PHF19 and PRC2 appear to be inter-dependent, as PRC2-depleted cells also showed a clear decrease in PHF19 at target sites [62].

Due to the importance of the repressive functions of PRC2 in self-renewal and differentiation of ES cells, PHF19 was studied in this context. With regard to self-renewal, it was reported that over-expression of PHF19 promoted colony formation in medium containing a reduced level of leukemia inhibitory factor (LIF) [61], and, accordingly, depletion of PHF19 led to an increase in spontaneous differentiation and a decrease in pluripotency markers [61–63]. However, the function of PHF19 upon differentiation has created further debate. PHF19 expression is strongly reduced upon differentiation, but due to its considerable influence at early stages, it could exert an important control on lineage commitment. Two of the studies found defects in embryoid body formation in PHF19-depleted cells [62,63]. Moreover, Ballaré *et al.* [62] found that the teratomas produced were substantially smaller than the controls, and contained tissues representative of the three germ layers but with an over-representation of ectoderm tissue. However, Hunkapiller *et al.* [61] explicitly reported no differences in embryoid body formation; moreover, they did not find any alteration in formation of the three germ layers within the teratomas.

The genome-wide localization of PHF19 and its PRC2-inter-dependent presence on the chromatin makes it a very interesting candidate as a PRC2-recruiting factor. PHF19 contains a complete Tudor domain that, although not necessary for SUZ12 interaction, requires an intact aromatic cage to control the deposition of H3K27me3 by PRC2 [61]. Interestingly, this Tudor domain is able to bind to H3K36me3, a well-known mark linked to gene transcription [62–65]. In addition to classic peptide-binding assays, structural evidence for such binding was revealed using NMR spectroscopy [62]. Moreover, it was confirmed that recognition of H3K36me by the PHF19 Tudor domain is required for Polycomb function and for H3K27me3 deposition [62–64]. Strikingly, the H3K36me3 mark antagonizes PRC2 and H3K27me3 [18]. Accordingly,

although the two marks coincide at some specific loci [64], the overlap between PHF19 and H3K36me3 *in vivo* is very poor in general [62]. In fact, genomic areas with high H3K36me3 enrichment, i.e. the intragenic regions, lack PHF19, which, as expected based on its interaction with PRC2, localizes mainly at promoters. This counter-intuitive finding may be explained by the fact PHF19 also recruits the specific H3K36 demethylase NO66 to chromatin in ES cells, which may remove methyl groups from H3K36me3 [63]. Accordingly, KDM2b, another H3K36 demethylase, also co-localizes with PHF19 and PRC2 at target genes, suggesting potential redundancy of these demethylases [62]. It has been hypothesized that PHF19 acts as a reader of the H3K36me3 mark in promoters of transcriptionally active genes, and mediates recruitment of PRC2 together with H3K36 demethylases, which then remove the histone mark. PRC2 mediates the deposition of repressive marks (H3K27me3), leading to gene silencing (Fig. 2). This may explain why very few loci are simultaneously decorated with both marks and enriched with PHF19. It is worth mentioning that the presence of H3K36me3 at transcribed intragenic loci has been independently linked with PRC2 activity in mouse ES cells [66]: there is a correlation between H3K36me3 and PRC2-dependent H3K27 mono-methylation (H3K27me1).

Regarding the involvement of PHF19 in human diseases, each isoform of PHF19 has been found to be over-expressed in a number of human cancers [59] and cancer cell lines [60]. Tagged over-expressed versions of both isoforms were shown to interact with PRC2 members, but no further characterization of target genes controlled by PHF19 and PRC2 has been described in any of these tissues or biological systems. PHF19 has also been reported to play a role in controlling the balance between growth and invasiveness of melanoma cell lines, as PHF19 silencing reduces the cell proliferation rate and increases the trans-endothelial migration capacities [67]. Further analyses are required to understand how PHF19 controls these processes at the genomic level, especially in the context of the repressive functions of PRC2.

PCL1/PHF1

PHF1 was first identified in humans on the basis of sequence similarity with *Drosophila* PCL, and was also found to share conserved domains with MTF2 [68]. The interaction with PRC2 was confirmed in another study that analysed the interaction between *Drosophila* PCL and E(Z) (a homologue of EZH2), which demonstrated that this interaction is conserved in humans. Strikingly, MTF2 was not found to interact with EZH2, although only the PHD fingers were used to test the interactions [69]. Two studies addressed the role of PHF1 in PRC2 function and found that: (a) PHF1 localizes to PRC2 targets (especially HoxA loci), (b) PHF1 depletion reduces methylation of H3K27 in target genes, with a concomitant increase in expression, and (c) PHF1 increases the enzymatic activity of PRC2 in *in vitro* assays [70,71]. However, PHF1 does not appear to be a main recruiter of PRC2, and was suggested to act instead as an enhancer of PRC2 repression [71]. However, no genome-wide occupancy of PHF1 has been reported, hindering our understanding of which PRC2 targets are shared with PHF1 and whether this correlates with the K27 methylation state and gene repression.

Similar to PHF19, the Tudor domain of PHF1 also recognizes H3K36me3, a well-known mark linked to gene transcription [72]. This interaction was suggested

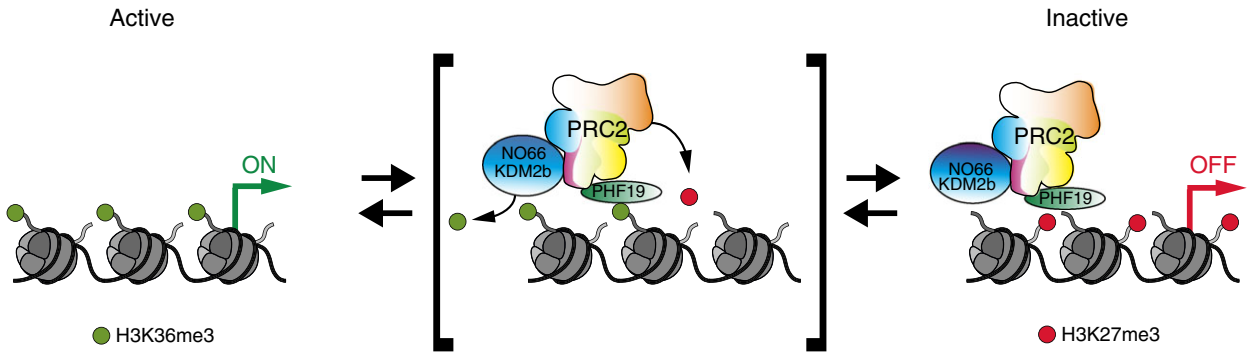

Fig. 2. Molecular mechanism of Phf19-mediated gene repression. PHF19 binds to the H3K36me3 mark in transcriptionally active promoters, and facilitates recruitment of PRC2 together with H3K36 demethylases NO66 and KDM2b. PRC2 mediates deposition of the repressive mark H3K27me3, leading to gene silencing.

to inhibit PRC2 activity (i.e. H3K27 methylation) on isolated nucleosomes and bulk histone marks after over-expression of PHF1 in HEK293 cells. Again, the lack of genome-wide data prevents systematic analysis of PHF1 occupancy and correlation with these two marks under various physiological conditions. However, these results do agree with the concept that repressive and activating methylation of histone H3 (K27 and K36, respectively) are antagonistic, and that the capacity of PHF1 to bind to H3K36 may mechanistically explain this finding. However, another rationale for PHF1 (and PHF19) binding to H3K36me3 has been proposed: it may be a mean by which PRC2 intrudes into active regions, thus spreading its repressive function [64]. This was shown in a subset of HoxA genes that were initially decorated with H3K36me3 in HeLa cells. Finally, a recent report showed that the Tudor domain of PHF1 binds not only to H3K36me3 but also to H3K27me3 [73]. The fact that this was observed but mainly ignored in previous reports [65] may be due to the fact that H3K27 binding is strongly enhanced specifically in the histone H3 variant H3.4. Therefore, PHF1 was suggested to have a dual specificity [73]. PHF1 does not appear to be expressed in ES cells, in which most of the genome-wide studies of other PRC2-associated factors have been performed [74]. Further studies are required to understand when and where PHF1 overlaps with H3K36 methylation marks, and the specific impact on PRC2 function under these particular conditions.

Although the specific histone modifications recognized by PHF1 require further characterization, a very interesting fact derived from the H3K36me3 recognition has been proposed: PHF1 is recruited to DNA double-strand breaks [75]. Interestingly, this recruitment is partially dependent on its capacity to bind H3K36me3 [72]. A very attractive hypothesis is that PHF1 may recruit PRC2 to specific sites in response to DNA damage [76].

Another interesting feature of PHF1 is its capacity to interact with tumor suppressors: both PHF1 and PHF19 have been reported to interact with HIC1 (hypermethylated in cancer 1) and act as co-repressors, suggesting that this interaction may recruit PRC2 [77]. Moreover, PHF1 interacts with p53 to promote its stability [78] and hence p53-dependent cancer cell growth arrest and apoptosis. This fact has important implications in tumorigenesis; for example, the same study showed that the levels of PHF1 are reduced in breast cancer [78]. On the other hand, another interesting feature of PHF1 with respect to cancer has been described, namely, its involvement in chromosome rearrangements: chromosomal fusions between *phf1* and other genes, such as *jazf1* and *meaf6*, have been reported in endometrial stromal sarcoma [79,80]. Interestingly, SUZ12 rearrangements in this type of cancer have also been reported [81,82]. Apart from endometrial stromal sarcoma, this type of rearrangement has also been found in tumors of mesenchymal origin, such as ossifying fibromyxoid tumor [83,84]. Although a link with PRC2 has been suggested [83], no actual mechanisms involving these tumors and gene repression have been elucidated.

PCL2/MTF2

Among the three PCL orthologues, MTF2 is the most highly expressed in ES cells [74], but, paradoxically, is the least well-studied member. Its expression and role *in vivo* have been studied in *Xenopus* [85], chicken [86] and mice [87], by over-expression or gene depletion, and a plethora of phenotypes, some of them homeotic and PcG-related, have been reported.

However, as with other PRC2-related proteins, the molecular mechanisms and functions of MTF2 have been mainly analysed in ES cells. MTF2 depletion was reported to promote self-renewal and an undifferentiated state, as well as impairment of differentiation capacity, and was recognized as part of PRC2. Knockdown of MTF2 appeared to moderately increase the levels of bulk EZH2 and H3K27me3. However, when specific genes are studied, the scenario is different: upon MTF2 depletion, direct MTF2 targets showed a reduction of PRC2 members, as well as of the H3K27me3 mark [74]. Another study on the recruitment role of MTF2 in ES cells showed that PRC2 members were clearly reduced at gene targets upon MTF2 depletion (although the results regarding H3K27me3 levels were inconclusive) [88]. The authors of this study also demonstrated that MTF2 recruits PRC2 to inactivated X chromosome, as depletion by shRNA reduced the presence of PRC2 components in Xi foci independently of Xist RNA [88].

Finally, another report suggested that the role of MTF2 and PRC2 in ES cells and development is more complicated [89]: more than two isoforms of the protein were predicted, some of them without the Tudor domain. MTF2 was mutated using two strategies: a gene trap that impaired its expression, and a targeted deletion that removed the Tudor domain and the first PHD finger. Interestingly, while the mutants without the Tudor domain were viable, the gene-trap mutation led to death after birth. Both mutants showed homeotic phenotypes similar to those previously described [87]. With regard to molecular mechanisms in ES cells, a dual functional role for MTF2 has been described:

first, in Hox genes, it interacts with PRC2 (and PRC1) to promote repression, although the recruitment to these loci is not inter-dependent (i.e. MTF2 lacking the Tudor domain does not impair the binding capacity of PRC2). This is in apparent contradiction of a previous report [74]; however, the previous report used a general depletion of the whole protein, rather than specific depletion of the Tudor domain. Second, in embryonic fibroblasts, MTF2 activates expression of the Cdkn2a locus, which induces senescence and is normally repressed by PRC1 [89]. These results demonstrate the plasticity of PRC2-associated proteins.

Although, like PHF1 and PHF19, MTF2 contains a Tudor and two PHD domains, no information regarding histone recognition by MTF2 is currently available. Casanova *et al.* [88] showed that the PHD2 domain of MTF2 is required for its localization at target genes, but no further analyses regarding histone binding were described. Thus, the observations regarding gene expression reported in the various systems are difficult to correlate with functional epigenetics.

C17orf96

C17orf96 is a recently discovered PRC2-associated protein that is highly expressed in mouse ES cells and in the two human-derived cell lines HeLa and HEK293 [11,90–92]. Previous efforts to identify PRC2-associated factors overlooked C17orf96, presumably for several reasons: the protein was poorly annotated and does not contain any chromatin-related domains, it apparently does not have a homologue in *Drosophila*, and its mRNA expression during mammalian development or in adult tissue is largely unknown because of missing probes in microarrays.

A first indication that C17orf96 is involved in PRC2 function came from identification of the H3K27me3-binding proteome, in which C17orf96 (similar to other PRC2 subunits) appeared as a protein highly enriched on CpG-unmethylated nucleosomes [92]. Biochemical studies showed that C17orf96 (also called esprc2p48) is indeed present in a complex that contains the PRC2 core components SUZ12 and EZH1/2 [91]. The exact composition of this complex is still unknown, especially with respect to the presence of other PRC2-associated factors. While Zhang *et al.* [91] showed co-immunoprecipitation of C17orf96 with MTF2 and JARID2, Alekseyenko *et al.* [93] suggested that C17orf96 and at least JARID2 are mutually exclusive. Further studies may resolve whether these differences are due to the different cellular systems used (e.g. mouse ES (mES) cells versus human HEK293), and what functional consequences this may have. In this context, it is important to note that the genomic targets of C17orf96 have not been reported. Therefore, its function on chromatin and the possible implications of PRC2 binding are unknown.

C17orf96 stimulates the *in vitro* histone methyltransferase activity of PRC2, and this activity is reflected by decreased levels of global H3K27me3 levels in mES cells after depletion of C17orf96 [91]. As for most PRC2 components, the effects of C17orf96 overexpression or depletion on gene expression in mES cells appear to be modest; nonetheless, a considerable number of affected genes are positively regulated by C17orf96 [90]. However, it is probable that a substantial proportion of these effects are secondary, and chromatin immunoprecipitation data are required to separate direct from indirect effects. mES cells depleted of C17orf96 retain their self-renewal potential, and its expression decreases quickly upon *in vitro* differentiation [90,91]. This lack of phenotype in loss-of-function mutants is reminiscent of PRC2, which is also dispensable for self-renewal of mES cells. Whether C17orf96-depleted cells fail to initiate the differentiation process properly is difficult to determine at present, as the phenotype of mES *c17orf96*$^{-/-}$ knockout cells has not been reported, making it impossible to compare the effects with their eed$^{-/-}$ or *suz12*$^{-/-}$ counterparts. Interestingly, during murine *in vivo* embryonic development, there is considerable expression of C17orf96 at later stages when neuronal precursors differentiate either into GABAergic or glutamatergic neurons. C17orf96 appears to be involved in the choice between these lineages, but it is not known whether this is a PRC2-related function [90].

Concluding remarks

The findings described in this review explain many specific, context-dependent functions of PRC2 during development, homeostasis and disease states. However, further efforts are required not only to elucidate the function of every single PRC2-associated protein, which is of course of great significance in the field, but also to understand their dynamics with respect association with core PRC2 members and other associated factors. Although technically challenging, systematic studies that address the composition of PRC2, taking into account which factors are associated, and in what proportions, would be highly informative. It may also be relevant to determine which associated factors co-exist in a specific complex. Indeed, until now, most studies have tackled the function of a single factor. Simultaneous depletion of more than one

PRC2-associated protein in cell culture, or generation of double/triple mutant mice models, may reveal synergistic effects of these factors, and alleviate compensatory effects that have impeded clear characterization of their impact on gene repression.

Finally, it is worth mentioning that the involvement of these factors in human disease, for example in cancer, remains to be studied in detail. In particular, future studies should examine whether disease-related alterations linked to these PRC2-associated proteins are due to the repressive function of PRC2. Epigenetic characterization of pathological states represents a novel way to identify therapeutic targets, and thus members of PRC2 are under intense scrutiny. It is thus logical to propose that PRC2-associated factors that modulate the activity of the complex may also play a very important role during disease progression.

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Author contributions

All authors have contributed to this article.

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