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Review

Polycomb proteins in mammalian cell differentiation and plasticity

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ABSTRACT

During development cell differentiation is accompanied by progressive restriction of the developmental potential and increased structural and functional specialization of cells. In this context, mechanisms of cell memory guarantee that cells maintain different identities previously determined by the integrated action of signalling and specific sets of transcription factors. Unraveling the molecular basis by which cells build and maintain their memory represents one of the most fascinating problems in biology. PcG proteins were originally identified as part of an epigenetic cellular memory system that controls gene silencing via chromatin structure. However, recent reports suggest that they are also involved in controlling dynamics and plasticity of gene regulation, particularly during differentiation, by interacting with other components of the transcriptional apparatus. In this review, we discuss the role of PcG proteins in pluripotent ES cells and in well known mammalian cell differentiation systems including skeletal muscle, epidermal, neuronal differentiation. The emerging picture suggests that indeed, plasticity and not rigidity is a fundamental aspect of PcG physiology and cell memory function.

1. Introduction

The ability to partition the genome into sets of active and quiescent genes and to subsequently maintain this partitioning through many cell divisions, underlies the process of cellular differentiation, which is essential for all multicellular life forms. Cellular differentiation is a natural event in every organism. Within an organism, hundreds of different cell types are defined by the unique subset of information they transcribe from a genome held in common. This cellular identity is maintained during development and in adulthood, by perpetuating patterns of gene expression through rounds of cell division. How is this cellular memory maintained and how can the cells with identical genetic patterns be different from each other? As such, a single genome may be modified to produce multiple epigenomes allowing for cellular diversity, a necessary criterion for the development of metazoans. The non coding "epigenome" contributes to the quality, stability and heritability of cell-specific transcription programs using different levels of control: DNA methylation, histone modifications, nucleosome mobility, domain organization, in trans interactions, nuclear organization that ensure the transmission of the epigenetic patterns and underlying transcription states from one cell to the daughter cells.

During cell differentiation or metabolic switch, cells undergo profound changes in gene expression. These events are accompanied by complex modifications of chromosomal components and nuclear structures, including covalent modifications of DNA and chromatin up to topological reorganization of chromosomes and genes in the nucleus. To various extents, all these levels of organization contribute to the stability and heritability of transcription programmes and define what is meant as the *epigenomic* level of gene regulation. Thus, epigenetic modifications influence gene expression patterns and provide a unique signature of a cell differentiation status.

Polycomb Group (PcG) genes code for chromatin multiprotein complexes that exert essential memory function during developmental and adulthood stages. Together with a battery of components including sequence-specific DNA binding/accessory factors, signalling pathway intermediates and non-coding RNA (ncRNA), PcG proteins maintain cellular identity homeostasis by setting heritable chromatin states to prevent changes in gene silencing programs [1]. The overall picture is that, in any given cell type, most alternative genetic programs are shut down by PcG proteins, except for the subset that is required in that cell type. Notably, PcG memory function is not merely conservative but also includes the ability of cells to dynamically respond to environmental cues. Indeed, current knowledge about the role of PcG proteins in the context of complex differentiation processes extends cell memory concept towards a more dynamic vision in which PcG appear to modulate silencing and also be involved in the establishment of transcriptional competence of promoter regions during differentiation. In this review, we focus on the role of the PcG proteins as epigenetic regulators of cell

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memory in the context of mammalian cell differentiation. In this perspective, we review the role of PcG proteins in pluripotent cells and in three well characterized differentiation systems, namely skeletal muscle, epidermal and neuronal cells aiming at a synthesis of the mechanistic principles governing chromatin regulation by this intriguing group of epigenetic functions. Finally, we address some of the apparent contradictions of the differentiation mechanisms, analyzing the opposite aspects of stability and plasticity of these processes, briefly focusing on the role of PcG during reversible somatic cell reprogramming.

2. Epigenome and cell identity diversity: some general aspects

During development cell differentiation consists of the progressive restriction of the developmental potential and increased structural and functional specialization of cells, leading to the formation of specialized cells, tissues and organs. In adult tissues, differentiation is a common process in which tissue specific adult stem cells guarantee organ homeostasis as well as tissue repair. This process involves highly controlled modifications in gene expression and selective repression of other cell type specific genes. With few exceptions, cellular differentiation almost does not involve a change in DNA sequence itself. Thus, different cell types display different morphological and functional characteristics despite having the same set of coding genome.

At the chromosomal level, diversity appears to rely on different sets of epigenetic signatures that mark regulatory elements to specify the functional state of tissue specific genes and help to maintain cell type specific transcription programs. However, to date the complete dynamics and role of the epigenome during cell differentiation and possibly dedifferentiation is not understood. Both processes have profound implications also for diseases in which cell identity is compromised (e.g. cancer) as well as reprogramming as it may matter for tissue regeneration.

3. Memory of cell identity

During development, cells after acquiring different identities under the action of specific sets of transcription factors must *remain* different after the original combination of signalling and transcription cues responsible for cell diversification have left the stage. To unravel the molecular basis by which cells build and maintain their memory represents one of the most fascinating problems in biology.

In the context of cell determination, three broad categories of cell memory can be distinguished which may be called cytoplasmatic, autocrine and nuclear memory. The first mechanism is related to components encoded by a set of active gene products that are present in the cytoplasm and act back on the genome, directly or indirectly, to maintain the selective expression of that specific set of genes. The second one is a variant of the cytoplasmic memory as it depends on a positive loop, but with the special feature that its products are secreted into the extracellular environment and feed back on signalling components of the cell surface. In contrast, "nuclear memory" depends on self-sustaining changes that are intrinsic to the chromatin-changes that define the selection of genes to be expressed and yet leave the DNA sequence unaltered. By changing chromatin structure, chromatin modifiers alter the accessibility of a gene to the transcriptional machinery and promote either its activation or its silencing. At cell division, cells use the epigenome to fix memory of earlier determined transcription states and ensure their perpetuation after cell division. Chromatin modifications, hence the epigenome, play a key role in this process.

4. Polycomb group genes: master controllers of cell memory

Discovery and investigation of PcG gene silencing memory system, and its activators counterpart the Trithorax group (TrxG) substantially contributed to our current understanding of how cells remember who they are in time and space. Polycomb group genes were discovered in *Drosophila melanogaster* in the context of a large genetic screen aimed at the identification of anterior–posterior segmental regulators of Hox gene silencing [2,3]. The cloning of Polycomb protein revealed a region of homology with heterochromatin protein 1 (Hp1) hence coined "chromodomain" (chromatin-modifier-domain) [4]. For these reasons, PcG proteins were originally proposed to be part of a cellular memory system that stably locks Hox gene expression states via chromatin structure [5].

Recent genome-wide studies have shown that PcG proteins control several hundreds of genes regulating most signalling and developmental pathways [6–8], cell cycle [9], spermatogenesis [10], cytoskeleton [11], cellular senescence [12], X-chromosome inactivation [13,14], genomic imprinting [15–17], stem cell plasticity and cell fate determination, as well as tumor progression [18,19]. Of note, PcG proteins do not solely control silencing via chromatin structure but they are involved also in controlling dynamic and plasticity of gene regulation by interacting with other components of transcription apparatuses. Indeed, active PcG target genes can be re-repressed or be activated or exist in intermediate states. Thus, PcG memory function involves also the ability to respond to signalling and to regulate the balance between gene silencing and switch.

PcG proteins are found in multiprotein complexes, including the Polycomb Repressive Complexes PRC1 and PRC2. Two other PcG complexes were characterized in *Drosophila*, Pho-Repressive Complex (PhoRC) and Polycomb Repressive Deubiquitinase (PR-DUB), and their components have orthologues in mammals [20–23]. An important emerging theme is that there are multiple versions of PRC1 and PRC2, with mounting evidence that alternative subunit compositions may confer distinct target gene specificity [24–26]. Polycomb mediated gene silencing rely mostly on the regulation of chromatin structure, through post-translational modifications (PTM) of histones. PRC2 complex is responsible for the methylation (di- and tri-methylation) of Lys 27 of histone H3 (H3K27me2/3) [23,27] through its enzymatic subunits Ezh2 and Ezh1, whereas the PRC1 complex monoubiquitylates Lys 119 of histone H2A (H2AK119ub) via the ubiquitin ligases Ring1A and Ring1B. In addition, some PRC1 complexes can regulate gene expression by compacting chromatin in a manner independent of its enzymatic activity [28]. PRC1 component PC, known as Cbx in mammals, binds specifically to the product of PRC2 catalysis, H3K27me3, leading to the hypothesis that PRC1 functions downstream of PRC2. However some genes targeted by PRC2 lack H2AK119ub [29] and genes targeted by PRC1 may not contain PRC2 components [30,31]. These data suggest that PcG signature is complex and each gene may contain just part of it, depending on its history and also specific regulation [1,23,27,32–34].

A recurring question in the study of PcG mechanisms is how the complexes are recruited to their target genes. While several Polycomb Response Elements (PREs), defined as epigenetic DNA modules that silence promoters at distance in a PcG-dependent manner have been studied in detail in *Drosophila* [35–38], in mammals it remains unclear to what extent DNA sequence plays a role in PcG targeting. Search for mammalian PREs appears to be difficult and has only led to isolated successes [30,39]. Recently, it has been recognized that PcG can physically interact with non-coding RNA [40–43], providing another mechanism for sequence specific targeting. Since many issues remain unsettled about PREs and recruiters, more work is needed to define the rules for PcG target-

ing in mammalian cells. Therefore, future efforts will be necessary to better clarify the mechanisms that govern the dynamic states of PcG target genes, the recruitment of PcG complexes and the switches that occur when the transcriptional readout of these target genes changes.

5. Polycomb repressive complexes in embryonic stem cells

Stem cells, or more properly pluripotent cells, are classically defined as cells that can both self-renew and generate progeny able to enter multiple differentiation programs. In the case of embryonic pluripotent stem (ES) cells, the range of lineage options comprises every type of tissue that is found in the adult animal. For adult stem cells, this range is restricted; for example, as we described in the following paragraphs, neural stem cells generate various neuronal and glial populations, epidermal lineage originates from a single layer of multi-potent progenitors, the committed muscle cells, the myoblasts, differentiate and fuse into multinucleated myotubes to finally maturate into myofibers. Three transcription factors, Oct4, Sox2 and Nanog, cooperate to ensure the self-renewal and pluripotency of ES cells [44,45]. The careful balance of transcriptional regulators and epigenetic factors is responsible for the crucial genomic plasticity proper of ES cells. Polycomb group proteins are among the chief epigenetic regulators that maintain pluripotency and also set the stage for transition to diverse differentiation programs. Depletion of Ezh2, Suz12 and Eed, the three core components of PRC2 complex, results in early embryonic lethality in mice [46–48]. Interestingly, analysis of $Ezh2^{-/-}$ ESCs suggests existence of an additional enzyme(s) catalyzing H3K27 methylation. Shen et al. [49] identified Ezh1 as a PRC2 component able to mediate H3K27me3 and to complement Ezh2 in maintaining stem cell identity and executing pluripotency [50–52]. Conversely, PRC1 deficiency is associated with less severe phenotype as the progeny is in general viable but exhibits transformations or other developmental abnormalities [53–55]. Ring1B is the unique PRC1 component that shows embryonic lethality after its knockdown, due to gastrulation defects [28,56]. Interestingly, the components of both PRC1 and PRC2 are required for differentiation and lineage commitment but they are not necessary for ES cell self-renewal [57,58]. PcG mutant ES cells can still self-renew, maintain normal morphology and express Oct4, Sox2 and Nanog [57–59]. Moreover, although the PcG knockout ES cells do not differentiate efficiently into the three germ layers, they can still contribute to their formation, in vivo and in vitro [57–59]. However, loss of individual PRC components in ES cells does not lead to increased expression of lineage-affiliated genes and unscheduled differentiation [57,58,60], an effect that is even more pronounced in ES cells carrying targeted deletions of both PRC1 and PRC2 [59]. These data demonstrate an unexpected redundancy between PRC1 and PRC2 during the formation of differentiated cells, suggesting that PcG system is critical for fine-tuning gene expression and that epigenetic patterns required to progress through differentiation cannot be set up in the absence of PcG regulation.

Six independent research groups identified Jarid2 as a previously unreported component of PRC2 in ESCs [24–26,61–63]. Interestingly, Jarid2 contains a Jumonji C domain (demethylase) but it is devoid of detectable histone demethylase activity. Indeed, the role of this PRC2 component appears to be quite complex. It co-localizes with PRC2 and H3K27me3 on the chromatin and modulates the function of this PcG complex in ESCs. Genome-wide ChIP-seq analysis of Jarid2, Ezh2 and Suz12 binding reveals that Jarid2 and PRC2 occupy the same genomic regions [26,61]. Jarid2 is required for efficient binding of PRC2, indicating that the interplay of PRC2 and Jarid2 fine-tunes deposition of the H3K27me3 mark. However, evidence was reported for a different role of Jarid2 in pluripotent ES in regulating RNA Pol II recruitment at bivalent genes [24]. This novel function for PcG protein suggest that PcG memory is required not only to prevent changes in gene expression but also to set the stage and provide competence for switching by regulating RNA Pol II at promoters [24,64]. Thus, the precise role of Jarid2 in ES remains to be elucidated.

Recent advances suggest that non-coding RNAs play a key role in the recruitment of PcG complexes in ES cells. DNA microarray analysis showed that short ncRNAs ($\leqslant$ 200 nt) were transcribed from the 5′ end of several hundred PcG target genes in ES cells [65]. Interestingly, these ncRNAs interact with PRC2 and are involved in stabilizing PRC2 association with chromatin. Moreover, the ncRNAs were depleted from PcG target genes that are derepressed during differentiation [65]. This indicates that short ncRNAs might function as the interface between DNA and specific chromatin remodelling activities, though the importance of direct base pairing at specific sequence motifs is still unknown. Aside short RNA, also long ncRNA appears to be an integral component of PRC2 complex [40,43]. Deep-sequencing analysis of PRC2 associated RNA revealed an unexpected complexity with several hundreds candidate RNA moieties being potentially involved in PRC2 function [43]. An open question is whether these RNA act in cis or in trans. In the case of X-chromosome inactivation PRC2 binds Xist RNA [66]. Long intergenic non-coding RNA (lincRNA) encoded in the mouse HoxB clusters is required for PRC2 dependent silencing of HoxD cluster [67]. Notably, interaction with ncRNA appears to be cell cycle regulated [68].

Mendenhall et al. [69] demonstrate a causal role for GC-rich sequences in PRC2 recruitment and implicate a specific subset of CpG islands depleted of activating motifs as instrumental for the initial localization of this key regulator in mammalian genome. Several studies, included the four discussed above, reported the identification of a Pcl2/Mtf2 containing PRC2 complex in ESCs [24,25,62,70,71]. Pcl2, one of the three homologs of *Drosophila* Polycomb-like, functions as a bona fide PcG protein. Indeed Pcl2-PRC2 occupies a subset of PcG target genes in ESCs in similar pattern as PRC2 [25,70,71] and appears to promote H3K27 trimethylation [70,71]. On the basis of the gene expression effects upon Pcl depletion in ESCs, the authors speculate that Pcl2/PRC2 may function to regulate self-renewal to enable an appropriate response to differentiation cues. Considering these observations together, the overall picture is that diversity of Polycomb complexes could reflect functional consequences on gene expression as well as biological output.

Precise localization of PRC1 and PRC2 within the genome is necessary to facilitate those specific changes in chromatin and gene expression that accompany lineage commitment. Genome-wide studies of PRC1 and PRC2 in ES cells have shown that they target promoters of >2000 genes, of which a large subset overlaps with target genes of Oct4, Nanog and Sox2 [6,8]. PcG proteins co-occupy and regulate the expression of a large cohort of developmental and signalling genes in ESCs, such as the *Hox* gene cluster as well as members of the *Dlx*, *Fox*, *Irx*, *Lhx*, *Pou*, *Sox*, *Tbx*, and *Wnt* gene family [6,8,29]. Interestingly, it has been reported that H2AZ histone variant is enriched at PcG complex target genes in ES cells and is necessary for lineage commitment [72]. Moreover, a recent work showed that a subset of PcG-target genes is bound and regulated in response to altered c-Myc levels [73]. Bernstein and colleagues [74] identified a novel chromatin pattern in ESCs, the "bivalent domains", that harbors both the "repressive" H3K27me3 and the "activating" H3K4me3 modifications (Fig. 1) [75–77]. Bivalent domains are not, however, exclusive of pluripotent stem cells. They can also be found in cells of restricted potency, including T cells and fibroblasts, where genes are unlikely to be poised in preparation for subsequent activation [75,77–81]. This "contradictory" chromatin pattern is consistent with the idea

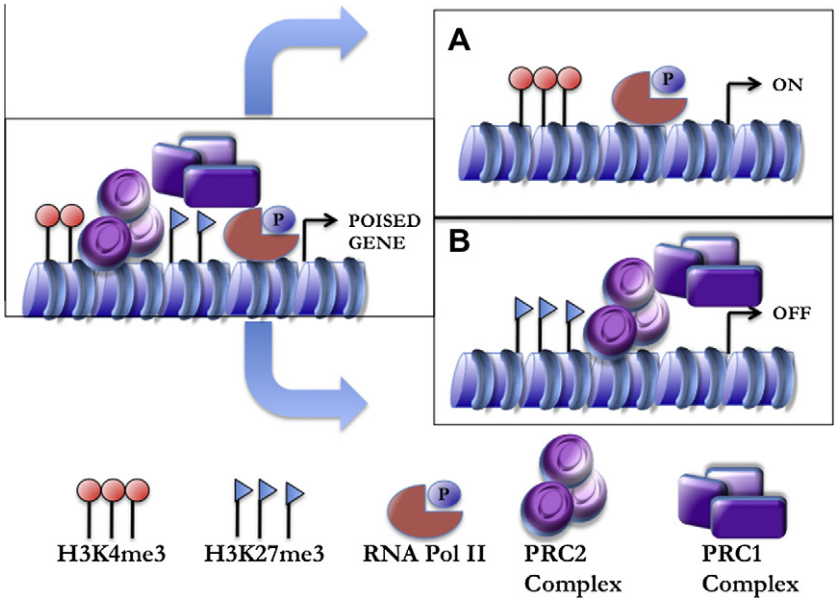

Fig. 1. PcG and chromatin bivalent domains. In ES and other lineage committed precursor cells, promoters of a wide range of non-transcribed developmental genes are characterized by a particular chromatin pattern that harbors both the "repressive" H3K27me3 and the "activating" H3K4me3 modifications. Such "bivalent promoters", contain a specific, non-elongating RNA Pol II isoform phosphorylated at Serine 5. This state fixes genes in transcription competent, "poised state", and constitutes an important regulatory step for genes that have to accommodate dynamic transcriptional responses to developmental cues. Bivalent domains are generally resolved during differentiation into either H3K4me3 (A) or H3K27me3 (B) regions depending on the expression state of the gene in a particular cell type. Considering the PcG targets, the bivalent domains can be divided in two classes-the first occupied by both PRC2 and PRC1 and the second specifically bound by PRC2 (B).

that control of developmental gene expression patterns is highly coordinated by the concerted activities of TrxG and PcG proteins.

Despite the concept that H3K4me3 is associated with transcriptional activation, bivalent genes display low expression levels. Such bivalent promoters contain a specific RNA Pol II isoform phosphorylated at Serine 5 (RNA Pol II S5) [82,83]. Also, PcG repressed promoters do not exclude RNA Pol II complex [84]. Indeed, recent genome-wide studies in *Drosophila* show that RNA Pol II binds transcription start sites of a large number of silent, developmentally regulated genes [83,85], suggesting that stalling RNA Pol II is an important regulatory step for genes that have to accommodate dynamic transcriptional responses to developmental cues. A similar picture is also characteristic of human cells [86]. In this context, it is highly relevant that Ring1 proteins play a decisive role in maintaining stalled RNA Pol II at promoters of bivalent domains of ES cells [82]. Thus the interplay between PcG and basal transcription machinery may be an integral part of cell memory.

Bivalent domains are generally resolved during differentiation into either H3K27me3 or H3K4me3 regions depending on the expression state of the gene in a particular cell type (Fig. 1). Ku et al. [29] found that bivalent domains can be divided in two classes-the first occupied by both PRC2 and PRC1 and the second specifically bound by PRC2. PRC1/PRC2 positive bivalent domains appear functionally distinct as they more efficiently retain H3K27me3 upon differentiation, show stringent conservation of chromatin state and associate with an overwhelming number of developmental regulator gene promoters (Fig. 1) [29]. Thus, the binding of PcG proteins in ESCs may facilitate repression at a particular set of genes during differentiation by using different stable silencing mechanisms, such as DNA methylation [23,87]. Indeed, promoters associated with H3K27me3 in ESCs are more likely to become DNA methylated during differentiation [80,88]. The resolution of bivalent domains is also probably facilitated by a class of histone demethylases that selectively remove H3K4me3, consistent with their essential roles in development and differentiation [89,90]. Conversely, loss of PRCs or H3K27me3 may facilitate the activation of genes necessary for lineage commitment. Two histone demethylases, Jmjd3 and UTX are necessary for proper development and differentiation in a variety of mammalian systems and are targeted to developmental regulators such as Hox genes during ESCs differentiation [91,92]. Therefore, demethylation of H3K27me3 may be one way to disrupt PcG-mediated gene repression, although there are probably other mechanisms that work in concert such as those mediated by signalling pathways in response to developmental cues (Fig. 2) [93–95]. Thus, the potential discovery of mechanisms that govern the dynamics and the recruitment of PcG proteins to target sites in the genome may facilitate efforts to direct differentiation of stem cells in vitro and to control disease progression in vivo.

6. PcG-mediated epigenetic control of skeletal muscle differentiation

Vertebrate skeletal muscle formation constitutes an excellent system to study the signals and the molecular mechanism that govern cellular differentiation. Myogenesis is a multistep process, which begins with the commitment of multipotent mesodermal precursor cells to pursue muscle fate. These committed cells, the myoblasts, then differentiate and fuse into multinucleated myotubes to finally give rise to the syncytium of multinucleated cells to organize myofibers. The specification, proliferation and terminal differentiation of skeletal muscle cells is controlled by several transcription factors that interact with enzymes modifying the nucleosomes structure to control the regulated formation and recruitment of specific protein complexes to muscle gene loci [33,96,97]. First evidence of the involvement of PcG proteins in the control of this differentiation system derives from the work of Laible et al. who reported the identification and phenotypic analysis of mammalian E(z) SET domain homologue [98]. In ES cells, the silent master gene *MyoD* locus is occupied by PcG proteins although *bivalently* marked by H3K27me3 and the trxG H3K4me3 modifications [8]. Subsequently, in skeletal muscle cell precursor cells, the *MyoD* locus is no longer occupied by PcG

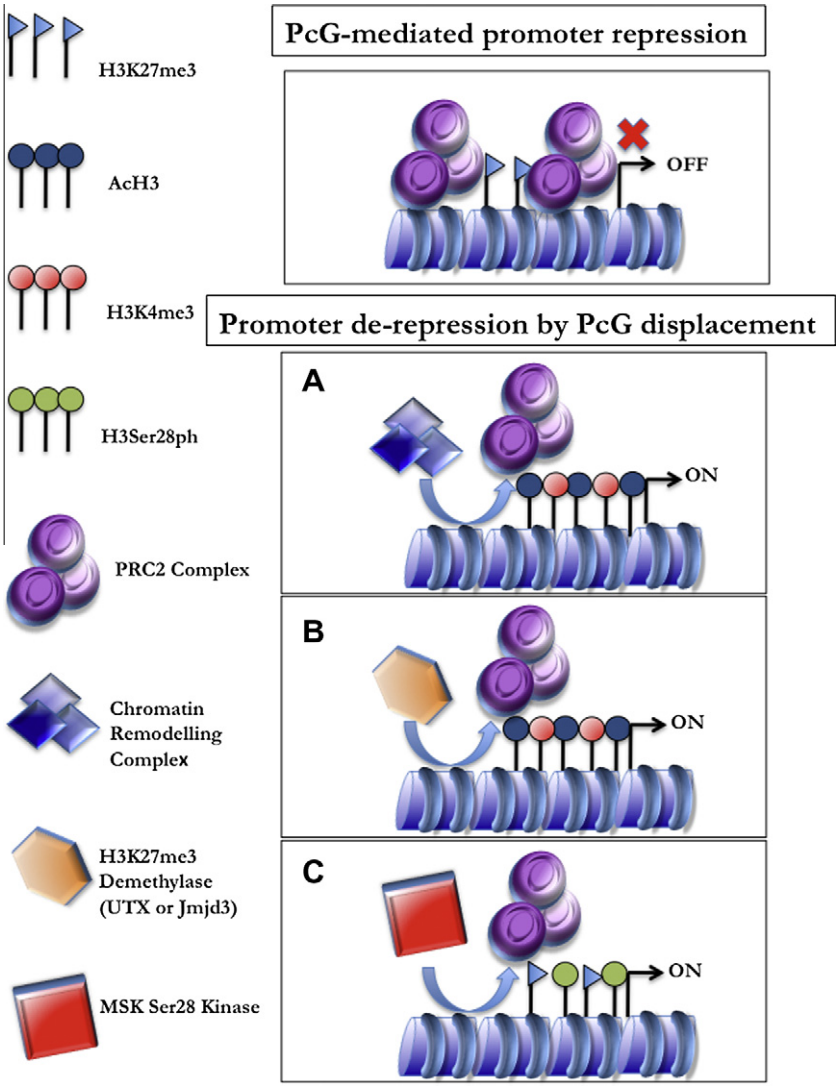

Fig. 2. Different mechanisms of promoter de-repression by PcG displacement. To permit gene activation, PcG complexes and the repressive mark H3K27me3 have to be displaced from their target genes. Different epigenetic pathways have been proposed, in various differentiation systems, that would contribute to switching transcriptional program. One way is the recruitment of chromatin remodelling complexes on target genes (A). For example, during muscle differentiation, the p38 MAPK pathway is essential for the recruitment of SWI/SNF chromatin remodelling complex to the *Myog* and *CKm* promoters. Another mechanism by which lineage specific genes repressed by PcG-mediated H3K27me3 become derepressed in differentiated cells postulates the existence of specific H3K27me3 demethylases (B). Alternatively, a phospho/methyl switch mechanism has been reported to explain PcG removal from the chromatin. Phosphorylation of H3Serine28, via mitogen and stress activated kinases Msk1/2, is able to counteract the docking site H3K27me3 determining the PcG chromatin displacement and gene activation, in response to stress signalling, mitogenic signalling and retinoic acid (RA)-induced neuronal differentiation (C).

proteins and H3K27me3, allowing for its transcriptional activation. However, while *MyoD* is expressed in proliferating myoblasts, certain *MyoD* target genes although occupied by PcG protein are marked by H3K27me3 and kept in standby position for subsequent activation. After additional signals initiate the myogenic program PcG binding and H3K27me3 are lost at *MyoD* target loci, resulting in appropriate muscle gene expression and skeletal muscle cell differentiation. Caretti et al. [99] proposed a two-step activation model to define PcG dependent muscle gene expression and cell differentiation. In undifferentiated myoblasts, a PcG complex formed by Ezh2, YY1 DNA binding protein and the deacetylase HDAC1 is bound at muscle specific gene regulatory regions to maintain chromatin silent and preventing premature transcription. Their presence correlates with trimethylation of K27 of H3 histone. Upon gene activation, Ezh2, HDAC1 and YY1 dissociate from muscle loci, H3K27 becomes hypomethylated and MyoD and SRF (Serum Response Factor) are recruited to chromatin, whereas Ezh2 knock down causes premature expression of skeletal muscle genes and acceleration of myoblasts to myotubes switch program [99,100]. As Ezh2 levels drop in myotubes, the question remains opens about who is maintaining H3K27m3 and silencing in terminally differentiated myotubes.

Thus, PcG-repressed system controls precocious induction of genes during differentiation, allowing for integration of multiple signals in a temporally ordered manner. To overcome PcG repression as muscle differentiation proceeds, it's important to analyze the role and coordination of signalling pathways operating with Polycomb proteins during skeletal muscle cell differentiation that by changing epigenetic codes control correct activation of developmentally regulated genes. Among the cofactors feeding into the myogenic process, the p38 mitogen-activated protein kinase (MAPK) is crucial in establishing the muscle specific gene expression program [101,102], whereas the role of the NF-kB pathway acts both positive and negative [103–105]. The observation that muscle specific genes are targeted for repression by PcG proteins suggests that activation of these genes should require the antirepressive

function of TrxG proteins. Indeed, the p38 MAPK pathway has been shown to be essential for the recruitment of SWI/SNF chromatin remodelling complex to the *Myog* and *CKm* promoters (Fig. 2) [106]. Further, Rampalli et al. [107] demonstrated that *Myog* and *CKm* genes became epigenetically marked for gene expression (H3K4me3) during muscle differentiation by recruiting TrxG Ash2L–HMT complex. Targeting of Ash2L is mediated by the transcriptional regulator Mef2d and this interaction is modulated through activation of the p38 MAPK signalling pathway, via phosphorylation of Mef2d. Furthermore, it has been shown that Mef2d cooperates with myogenin to recruit SWI/SNF to the promoter region of the *CKm* gene [108]. Thus SWI/SNF chromatin remodelling complex appears to be recruited and to counteract PcG at *Myog* and *CKm* promoters through different mechanisms. In contrast to the activating role of p38-alpha in muscle gene activation, p38-gamma represses MyoD transcriptional activity by direct phosphorylation, via association with H3K9 methyltransferase KMT1A [109]. Thus, p38 kinases can either activate or repress gene expression, depending on the engagement with specific p38 isoforms. Notably, recent data show that in satellite cells p38 signalling to PRC2 complex specifically directs the repression of genes that are typically down-regulated during muscle differentiation, via phosphorylation of Ezh2 (e.g. *Pax7*), but it has no direct impact on PRC2-mediated repression of muscle specific genes [110], suggesting that other pathways control PcG function in myoblast-myotube transition.

To permit muscle gene activation, chromatin remodelling complexes have to be recruited on muscle specific loci. At the same time, also PcG complexes and repressive mark H3K27me3, have to be displaced from their targets (Fig. 2). One way of doing it would be to reduce HMT PcG intracellular levels. Juan et al. [100] provided evidences that miR-214 can affect PcG complex gene expression by regulating Ezh2 protein levels in skeletal muscle and embryonic stem cell. Mice with experimental ablation of the miR-199/214 locus display developmental defects, suggesting a relevant role of these microRNAs in skeletal myogenesis [111]. Several microRNA influence myogenesis [112]. In particular, miR26a regulates Ezh2 in skeletal muscle cells and promote their differentiation [113]. It is likely that miR-214 and miR-26a affect Ezh2 at distinct developmental steps. Indeed, miR-214 accumulation is evident at the very initial stages of cell differentiation-coinciding with the initial and most evident Ezh2 protein reduction [100]-whereas miR-26a could not be detected until SMC had completed their terminal differentiation [113]. Recent studies open interesting questions about the paradigm that PcG derepression has to be accompanied by the loss of H3K27me3 repressive mark. Seenundun et al. [114] showed that the histone demethylase UTX is targeted to muscle specific genes by the transcriptional activator Six4 to mediate removal of repressive H3K27me3 mark during myogenesis (Fig. 2). However, a novel mechanism inducing PcG chromatin displacement has recently been proposed. Phosphorylation of H3Serine28 (H3S28ph), via mitogen and stress activated kinases Msk1/2, is able to counteract the docking site H3K27me3 determining the PcG chromatin removal and gene activation, in response to stress signalling, mitogenic signalling and retinoic acid (RA)-induced neuronal differentiation (Fig. 2) [94,95]. Data from our lab show that a similar mechanism appears to occur in differentiating myoblasts in which Msk1 regulates a phosphomethyl H3S28ph/H3K27me3 switch to allow removal of PRC2-Ezh2 complex and muscle gene activation (Stojic, Prezioso, Jasencakova et al., unpublished data). Interestingly, our data show that another PRC2 complex that includes Ezh1 subunit substitutes for PRC2-Ezh2 and it is required in order to complete myogenic program. A similar dynamics of Ezh2 being replaced by Ezh1 has been reported in epidermis differentiation [51]. Notably, both in vivo and in vitro chromatin binding experiments show that this complex is insensitive to H3S28ph, suggesting that Msk-phosphomethyl switch pathway is specific for Ezh2 but not for PRC2 complex as such (Stojic, Prezioso, Jasencakova et al., submitted).

The role of PcG epigenetic memory in terminally differentiated myofibers remains to be elucidated. To this PcG comprehensive dynamics and genome-wide mapping analysis will be required to decipher the link between signalling and PcG epigenetic regulation during muscle differentiation.

7. PcG-mediated epigenetic control of epidermal differentiation

The mammalian epidermis has turned out to be a highly informative model to explore the functional significance and physiological relevance of PcG-mediated chromatin repression during embryonic tissue development and homeostasis. Epidermal lineage originates from a single layer of multi-potent progenitors, basal cells, which adhere to an underlying basement membrane separating epidermis from dermis [115]. Basal cells continually fuel the production of $\approx$ 10 suprabasal layers. Once cells exit the basal layer, they down-regulate proliferation-associated genes and execute a terminal differentiation program that is marked by a stepwise transcriptional transition from early differentiation spinous layers to late differentiation granular layers. In the last step, all metabolic activity ceases as dead squames of the protective stratum corneum are formed and subsequently sloughed from the skin surface. Maintaining the relative size of these compartments requires that the cell proliferation rate in the basal layer, the tendency of cells to survive versus differentiate in the suprabasal layers and terminal cell death in the cornified layer be co-ordinately controlled and balanced [116].

Recent studies suggest that PcG gene products regulate these processes at different levels, with a particular emphasis on Bmi-1, a PRC1 subunit that acts as a cofactor in H2A ubiquitylation by Ring1A/Ring1B [117]. Although Bmi-1 was characterized as a stem cell maintenance protein that is required for efficient renewal of hematopoietic, leukemic and neuronal stem cells [118], in the epidermis it does not appear to function exclusively as a stem cell regulator. Indeed, if this were the case, one would expect expression to be restricted to epidermal stem cell compartment. However, the available evidence suggests that Bmi-1 is localized in the nucleus of proliferating keratinocytes and in the basal and suprabasal layers [119,120] and is not strictly confined to stem cells [121–124]. These findings are consistent with a previous report indicating a nuclear localization of this protein in fibroblasts [125] and this localization appears necessary for function, as Bmi-1 mutants that cannot localize in the nucleus are inactive [125]. Several studies suggest that the pro-survival, pro-proliferation action of Bmi-1 may be due to its ability to suppress expression of proteins that regulate cell cycle progression for example, the *Ink4a/Arf* locus genes [118,126]. Senescence, the process whereby aging cells gradually lose proliferation potential [127], is controlled by the expression of products of *Ink4a/Arf* locus, $p16^{INK4a}$ and $p14^{ARF}$ and Bmi-1 seems to play a key role in this context. Silva et al. [119] showed that cells that have high proliferative potential have high Bmi-1 and low $p16^{INK4a}$ [119,128]. This relationship is also observed in aging epidermis, as Bmi-1 levels are high in keratinocytes isolated from young individuals and lower in cells isolated from older individuals [121,128]. In addition, Cordisco et al. [128] found that the Bmi-1 levels are also reduced in keratinocytes isolated from young xeroderma pigmentous group C, trichothlodystrophy, and progeria patients as compared with keratinocytes isolated from healthy young individuals and lower levels are present in keratinocytes derived from photo-aged epidermis than those collected from non-exposed epidermis of the same patients. Altogether, we can conclude that Bmi-1 has a role in controlling

keratinocyte senescence via regulation of the *Ink4a/Arf* locus. The role of Bmi-1 in keratinocytes survival and proliferation was also confirmed by treatment with chemiopreventive agent or keratynocyte differentiating/apoptosis-inducing agent, that reduces the expression of Bmi-1 and other PcG proteins, such as Ezh2 and Suz12 and also H3K27me3 levels [120,129]. An interesting finding is that overexpression of Bmi-1 is associated with increased expression of Ezh2, suggesting that Bmi-1 somehow functions to maintain expression of the enzyme that prepares its chromatin binding site, H3K27me3 [129]. While much is known about the Bmi-1 role in controlling cell survival, little is known about its involvement in apoptosis. Recent works show that Bmi-1 expression maintains survival of cells following challenge with stress agent, such as OA, TPA, UVB [120,129]. Only one report has addressed the possible role for Bmi-1 in regulating apoptosis, via inhibition of Myc-dependent increase of $p19^{ARF}$ [130,131]. Other PcG proteins have also been studied in epidermis, including the PRC2 complex components, Ezh2, Suz12 and Ezh1. Interesting evidence was reported in the work of Ezhkova et al. [51]. They showed that Ezh2 is expressed in epidermal progenitors but diminishes concomitant with embryonic differentiation and with post-natal decline in proliferative activity. Loss of PcG function in the developing skin alters epithelial stem cells proliferation and accelerates the timing of skin development. Indeed, Ezh2, like Bmi-1, controls proliferative potential of basal progenitors by repressing *Ink4a/Arf* locus and tempers the developmental rate of differentiation by preventing premature recruitment of Ap1 transcriptional activator to the structural genes that are required for epidermal differentiation. In agreement with recent data [50], also in epidermis while Ezh2 declines, Ezh1 levels increase in upper and terminal differentiated layers [51].

Further analysis on the role of Ezh2 and Ezh1 in epidermis [52] revealed new insight into tissue specific PcG-dependent control and provided a new twist to how different progenitors within the same tissue respond to loss of H3K27me3. Sen et al. [132] showed that epigenetic derepression is controlled by Jmjd3 histone demethylase during mammalian epidermal differentiation (Fig. 2). Perhaps the most surprising finding of these studies was that the global loss of H3K27me3 did not cause wide-spread differentiation defects in epidermis. Thus, even though many PcG-repressed differentiation genes [6] showed signs of transcriptional activation in the absence of Ezh2/1, the levels of non-epidermal differentiation programs were still low to reroute already established skin fates [52]. These observations underscore the importance of additional epigenetic modifiers and backup mechanisms (other repressive histone marks, DNA methylation, non-coding RNA) that appear to have evolved in complex organism to ensure the establishment and maintenance of lineage programs in adult tissues. Mechanisms of tissue repair like wound healing are closely related to processes that control development and cell proliferation. It is interesting that three major components of the PRC2 complex, Eed, Suz12 and Ezh2 are reduced in epidermis during wound healing [124]. In agreement with this observation, it has been reported that in *D. melanogaster* the expression of the TrxG and PcG family genes is modulated during transdetermination events [133]. Indeed, in fragmented imaginal discs-the determined structures responsible for the development of the adult cuticular parts-some PcG genes are downregulated in the proliferating cells at the wound site upon activation of the Jun N-terminal kinase (JNK) pathway [134]. Downregulation of PcG genes reduces the silencing of the target genes and increases the plasticity by facilitating the accessibility of the new transcription factors to developmental regulator and pro-stem cell genes [135] as well as easing the switch to new epigenetic imprints. These results appear to conflict with the idea that PcG protein expression correlated with enhanced cell survival and increased expression of survival genes. However these findings highlight the potential complexity of the epidermis regulation and suggest that the role of the PcG proteins may be context dependent.

8. PcG cell memory function in neuronal differentiation

Although for long decades the adult brain had been thought to represent an exception to the general concept that most adult tissues retain a reservoir of self-renewing multipotent stem cells generating differentiated tissue components, it's now clear that this organ also retains stem cells that produce neuronal cells throughout life [136]. Neuronal stem cells (NSCs) are defined as cells that possess the ability to self-renew and to generate the three major cell types in the central nervous system (CNS): neurons, astrocytes and oligodendrocytes. NSCs and neurogenesis persist throughout life in the subventricular zone (SVZ) and dentate gyrus of the hippocampus. These differentiation processes are defined by the dynamic interplay between extracellular cues including cytokine signalling and intracellular programmes such as epigenetic modifications.

There is increasing evidence that the PcG group proteins are closely associated with fate specification of NSCs. These epigenetic modifiers could represent a coordinated system for regulating gene expression at each step of neuronal cell differentiation. The dynamic gain and loss of PcG and TrxG-mediated histone modifications occur during in vitro developmental progression from embryonic stem cells (ESCs) to fully differentiated neurons [88,137,138]. Using a murine system that progresses from stem cells to lineage-committed progenitors to terminally differentiated neurons, Mohn et al. [88] suggested a model how de novo DNA methylation and dynamic switches in Polycomb targets restrict pluripotency and define the developmental potential of progenitor cells. These observations are compatible with a role for Polycomb in targeting DNA methyltransferase activity [139,140]. It's important to consider that both proneuronal bHLH and SoxC transcription factors (TFs)-together involved in neuronal fate determination and differentiation-seem to be regulated by a distinct temporal pattern of PcG-mediated repression. The PcG-mediated repression of the *Sox2* promoter occurs during differentiation of neuronal progenitors cells (NPCs) [88]. Unlike the pluripotency and multipotency factor Sox2, developmental TFs, such as Ngn2, NeuroD1, NeuroD2, Sox4 and Sox11 are repressed by a PcG-mediated mechanism already in pluripotent ESCs. They all have bivalent promoters, suggesting they are poised to be activated [80]. In multipotent NPCs, Ngn1, Ngn2 and NeuroD2 retain the "closed" promoter conformation, with H3K27me3 repressive mark, whereas promoters of Sox4 and Sox11 become "open", losing H3K27me3 (Fig. 1) [80,88,141]. Thus, these data suggest that PRC2-dependent marker is related to changes in transcriptional programs associated with transition from ES cell-derived NSCs to neuronal progenitors and their differentiated progeny. The impact of PcG regulation on NSC self-renewal is more limited and it is derived mostly from studies in loss-of-function models of Bmi1. Knockout of Bmi1 has little effect on progenitor cell self-renewal during development but it is essential for neuronal stem cell maintenance in the adult CNS [118]. Moreover, Bmi1 is essential for cerebellar development and it is overexpressed in human medulloblastoma [142]. Therefore, Ring1B promotes embryonic NSC self renewal by sustaining their proliferative activity and maintaining their undifferentiated state and developmental potential [143]. Interestingly, Pereira et al. [144] reported that Ezh2 is essential for controlling the rate at which development progresses within cortical progenitor cells lineages. Loss of function of Ezh2 removes H3K27me3 repressive mark in cortical progenitor cells and also prevents its establishment in post-mitotic neurons. This dynamics correlates with marked up-regulation in gene expression, the consequence of which is a

shift in the balance between self-renewal and differentiation toward differentiation, both directly to neurons and indirectly via basal progenitor cell genesis. Although the temporal order of neurogenesis and gliogenesis are broadly conserved under these conditions, the timing of neurogenesis, the relative numbers of different cell types and the switch to gliogenesis are altered, narrowing the neurogenic period for progenitor cells and reducing their neuronal output. The findings reported here contrast with a recent report about the consequence of Ring1B and of Ezh2 depletion in the E12 cortex during neurogenic period [145]. Removal of Ring1B from the developing cortex during neurogenesis lengthens the period of neurogenesis and delays the onset of oligogenesis [145]. Deletion of Ezh2 at the same developmental stage produces the same phenotype [145]. This difference points to a potential role for Polycomb in regulating major developmental transition in cortical progenitor cells: changing the competence of cortical progenitors cell to generate neurons of different laminar fates; switching from neurogenesis to gliogenesis. The requirement of Ezh2 in regulating neurogenesis and differentiation appears to be in line with its proposed role in maintaining stem cell populations, such as hematopoietic stem cells [146,147]. However, it is becoming clear that the role of PRC2 is more complex in this cell type. PRC2 not only acts to promote self-renewal but also controls fate choices within this multipotent lineage, so that in its absence neural progenitors cells alter their fate decisions. The work by Sher et al. [148] has been very instrumental to better clarify the role of Ezh2 in the control of neuronal differentiation programs. They reported that Ezh2 is highly expressed in proliferating NSCs and its expression decreases when the NSCs differentiates into neurons and it is completely suppressed during differentiation into astrocytes. Surprisingly, Ezh2 levels remain high in NSCs that differentiate into an oligodendrocytic cell lineage. Depletion of Ezh2 in NPCs leads to a delay in the differentiation capability to form oligodendrocytes. Thus, identifying Ezh2 targets responsible for these altered progenitor cell behaviour will provide crucial insights for neuronal progenitor cell multipotency and other fate choices.

The observations reported until now that lineage specific genes repressed by PcG-mediated H3K27me3 have to be derepressed in differentiated cells postulated the existence of a specific H3K27me3 demethylase. First evidence was reported in the work of Burgold et al. [149]. They found that Jmjd3, recently identified as a H3K27me3 specific demethylase which is recruited by H3K4 methyltransferase Mll1 [138,149,150], controls the expression of key regulators and markers of neurogenesis and is required for commitment to the neural lineage (Fig. 2). Thus, regulation of H3K27me3 is highly gene- and context-specific, suggesting that the interplay of methyltransferases and demethylases enables the fine-tuning more than the on/off alternation of methylated states. A recent study added novel aspects to signal-dependent release of PcG mediated silencing. Gehani et al. [94] proposed a model for PcG protein displacement on gene promoters in response to Msk-mediated H3K27me3S28 phosphorylation during retinoic acid (RA)-induced neuronal differentiation (Fig. 2). As already commented before (see Section 6), these data suggest that we have to reconsider demethylation of H3K27me3 as the only mechanism for gene derepression of PcG group protein-bound genes during differentiation. Related to this, we need to understand if H3S28 phosphorylation affects H3K27 demethylation by JmjC family members. If demethylation is blocked by S28 phosphorylation, H3K27me3S28 phosphorylation mechanism for PcG displacement speaks for a more transient nature of derepression as compared to regions where H3K27me3 is removed by JmjC proteins. Furthermore, a recent report demonstrated that the Dnmt3a-dependent non-proximal methylation promotes expression of neurogenic genes by functionally antagonizing Polycomb repression [151]. Given that both DNA methylation and Polycomb pathways are indispensable for normal development and are implicated in diseases, including neurological disorders and cancer [152,153], it will be of interest to fully elucidate mechanisms by which these two epigenetic machineries are targeted to specific genomic loci and are cross-regulated.

9. Concluding remarks

The studies reported in this review reinforce the concept that cell differentiation is a dynamic and also reversible process, involving the concerted action of signalling pathways, transcription and epigenetic factors. A long-standing notion in developmental biology has been that organ/tissue specific stem cells are restricted to differentiating into cell types of tissue in which they reside. In other words, they have irreversibly lost the capacity to generate other cell types in the body. However, recent evidences suggest that tissue-specific stem cells may overcome their intrinsic lineage-restriction upon exposure to a specific set of signals in vitro and in vivo [154–161]. The transition from somatic cell to induced pluripotent stem cell (iPS cell) involves radical alterations in the epigenetic landscape of the cell. The epigenetic signature of iPS cells resembles but is not identical to the epigenetic signature of embryonic stem cells [162–164]. For this reason, iPS cells retain an epigenetic memory of their somatic progenitors. The importance of PcG proteins in this context is strongly represented in the work of Pereira et al. [165] in which they showed that ESCs require PRC2 components to direct the successful reprogramming of differentiated cells toward pluripotency. Thus, the differentiation of progenitor cells into terminally differentiated cell types and the de-differentiation of somatic cells into progenitor cells are governed by epigenetic functions. The loss of cellular memory is a crucial step not only for reprogramming events but also in the cancer, in which cells appear to forget their lineage features and re-acquire aggressive proliferative potential. PRC2 components are upregulated in various cancers such as melanoma, lymphoma, breast and prostate cancer. In particular Ezh2 has been reported to be not just a marker but also a major player in the aggressive stages of a large number of tumors [166–169]. Thus, unravelling the molecular basis of cell memory will be crucial for the comprehension of fundamental aspects of the biology cell differentiation as well as complex diseases like cancer, in which loss of cell memory can be eventually fatal.

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