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Role of Enhancer of Zeste Homolog 2 Polycomb Protein and Its Significance in Tumor Progression and Cell Differentiation

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1. Introduction

Epigenetics is a branch of genetics that focuses on the heritable changes of DNA or associated proteins, other than DNA sequence variations, which carry information content during cell division [1,2]. These heritable changes are ascribed to chromatin, which constitutes the ultrastructure of DNA and whose modifications affect the genetic material functionality. Differences in chromatin structure have been associated to transcription regulation [3-5] and chromosome stability [6,7], affecting both gene's information, expression and heritability. Noteworthy, these epigenetic modifications are involved in both transcriptional activation and repression, indicating their widespread role as modulators of gene expression in numerous biological processes [8,9].

Chromatin is subjected to numerous modifications roughly classified in two groups: DNA and histone post-translational modifications (histone-PTMs).

DNA methylation is the most studied epigenetic modification of DNA and corresponds to the covalent addition of a methyl (CH_3) group to the nucleotide cytosine within CG dinucleotides or CNG trinucleotides where N can be C, A, G or T. Usually, DNA methylation induces decreased protein-DNA binding of transcription factors and leads to the repression of gene expression [10].

DNA "methylable" sequences are not uniform across the human genome but restricted in CpG rich DNA regions termed CpG islands (CGI). CGI are localized at repetitive sequences, heavy methylated, to prevent the reactivation of endoparasitic sequences such as transposons, and at gene promoter sequences, which are normally refractory to methylation in normal somatic cells [8,11].

DNA methylation is specifically established by DNA methyltransferases proteins (DNMTs), which can be recruited by numerous DNA-binding molecular complexes. These enzymes were classically classified in two categories: de novo DNMTs, as mammalian DNMT3a and DNMT3b, in charge of the addition of the methyl group on a previously unmethylated DNA, and maintenance DNMTs, whose only known member is DNMT1, responsible for methylation renewal in the newly synthesized DNA copy. However, this classification does not entirely explain methylation establishment and maintenance in various molecular processes [10]. For instance, a number of studies demonstrated that all DNMTs are important in the maintenance of methylation during DNA replication, therefore indicating that it is not possible to distinguish classes of DNMTs based on their functional role [12-15]. Another important functional role in DNA methylation dynamics is constituted by the removal of methyl group, which is required to activate methylated genes. However, demethylation is a process not fully understood, in fact, until recently, it was current opinion that only a passive demethylation could occur, as a consequence of a lack of methylation maintenance during DNA replication. The discovery of several putative demethylases, as thymine DNA glycosylase (TDG), methyl-binding domain 2 (MBD2), and GADD45 [16-18], strongly suggested that an active mechanism of demethylation can occur in specific contexts, such as germ line reprogramming [19-22].

The second group of epigenetic changes is represented by histone post-translational modifications (PTM), which consist in the addition of chemical groups to amino acid residues of both canonical histones (H2A, H2B, H3 and H4) and variant histones (such as H3.1, H3.3 and HTZ.1).

Differently from DNA modifications, there are at least eight distinct types of histone post-translational modifications: acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization. Each chemical group can be established at multiple amino acid residues of nucleosomes in multiple levels of substrate modification by specific classes of enzymes. For example, lysine methylation can be established at numerous aminoacidic residues of N-terminus tails of histones H3 and H4, such as K4, K9, K20 and K27, in mono-, di- or tri-methylated forms. This variety of histone PTMs and its timing of appearance depends on the particular cell conditions giving the cells different functional responses [23]. Differently from DNA methylation, histone PTMs feature numerous functional roles. For instance, histone acetylation regulates DNA replication, repair and condensation; methylation, phosphorylation and ubiquitylation are involved in DNA repair or condensation. Moreover, all PTMs regulate transcription processes; acetylation is generally a marker of transcriptionally active genes, and methylation can be a marker of repressed or active genes depending on the amino-acid residues involved. For example, methylation of histone H3 lysine 4 (H3K4me) is considered a mark of transcriptionally active genes, while methylation of histone H3 lysine 9 and 27 (H3K9me; H3K27) are considered a mark of transcriptionally repressed genes [8,11,23].

Almost all cellular processes that require transcription dynamics and genetic stability can be considered epigenetic processes. Cellular differentiation is a good example of biological process, which is strictly connected to epigenetics. The genome sequence is static and it is the same for each cell of an organism (with some exceptions); however, cells are able to differentiate into many different types, with different morphology and physiological functions. During organism

development, the zygote, derived form a single fertilized egg cell, originates totipotent cells, able to potentially differentiate in all cell types of adult organisms. After several divisions, totipotent cells originate pluripotent cells, which are partially differentiated and able to differentiate in several cell types. Finally, pluripotent cells complete differentiation becoming adult somatic cells. The differentiation processes are characterized by transcriptional activation and repression of specific genes and, once completed, the cells maintain their characteristic gene-expression pattern, strictly dependent on the epigenetic modifications previously established [24]. Therefore, cell differentiation is rigorously related to the establishment of the correct epigenetic status and to the proper epigenetic maintenance. Epigenetic abnormalities alter gene expression, counteracting regular differentiation and cell physiology [25]. In support of this theory, cancer cells feature an aberrant epigenetic landscape, indicating the causal relationship between epigenome dynamics and cellular processes as proliferation, cellular identity maintenance and genomic instability. [26-28]. Frequently, CGIs in the proximity of tumor suppressor genes (TSG) are methylated in various cancers, inducing TSGs transcriptional repression and promoting cancer progression [29]. Furthermore, specific patterns of histones H3 and H4 acetylation and methylation are associated with numerous cancer types, and it has been shown that several epigenetic patterns enable to distinguish disease subtypes [30,31].

This chapter explores the role of the Enhancer of Zeste Homolog 2 (EZH2). EZH2, the catalytic subunit of Polycomb repressive complex 2, catalyzes the addition of methyl groups to lysine 27 of the N-tail of histone H3 (H3K27me). The importance to specifically focus on EZH2 raises from the evidence that it is involved in several differentiation processes and is often over-expressed in a wide variety of cancer types [32].

2. PcG proteins and PRC-mediated silencing

Polycomb group proteins (PcG) were discovered in *Drosophila melanogaster* as responsible of homeotic gene silencing, also referred as Hox clusters. Hox proteins are a group of transcription factors that determine cell identity along the anteroposterior axis of the body plan by the transcriptional regulation of hundreds of genes [33-42]. After the initial discovery in fruit flies, PcG proteins were detected in plants and in mammals, where they are involved in development, stem cell biology and cancer [43-48]. Polycomb-mediated gene silencing is required in many processes like mammalian X-chromosome inactivation and imprinting [49,50]. Furthermore, PcG proteins are required to maintain stem cell identity [51]. Indeed, their numerous target genes encode for transcription factors and signaling components involved in cell fate decision, therefore in differentiation processes [32].

Principal PcG proteins are conserved from Drosophila to human indicating that PcG-mediated gene silencing is conserved among eukaryotes [42,52,53]. In mammals, each of the fly proteins has two or more homologs [54]. PcG proteins form two main complexes: Polycomb-repressive complex 1 and 2 (PRC1, PRC2) [55-59].

In mammals PRC1 is formed by BMI1, RING1A/B, CBX, and PHC subunits [60]. RING1A/B are ubiquitin E3 ligases that catalyze the monoubiquitylation of histone H2A at lysine 119 (H2AK119ub1), a histone PTM associated with transcriptional silencing [48,53].

As previously explained, PRC2 has a histone methyltransferase activity on lysine-27 of histone H3 [56-59]. EZH2 is the catalytic subunit of the complex and is activated by other PRC2 subunits like EED, SUZ1 and RbAp46 [61,62]. Recent studies have identified an EZH2 homolog, EZH1 that originates an alternative PRC2 complex and, as EZH2, is able to methylate H3K27. EZH1 and EZH2 can occupy similar target genes, and in some cases have been proposed to play redundant roles. However, during development, it has been demonstrated that the two proteins, can also have distinct and context-dependent roles [63-65].

PRC1 and PRC2 are able to induce gene silencing independently by each other [66,67] or by a synergistic mechanism. In fact, establishment of H3K27me3 by PRC2 complex can induce the recruitment of PRC1 by binding the chromodomain of the PHC subunits [39,58]. Once recruited, PRC1 induces transcriptional repression of target gene by catalyzing the ubiquitilation of lysine 119 of histone H2 or by an H2Aub-independent mechanism [68-70]. Therefore, in gene promoters of PRC1 and PRC2 common target genes, H3K27me3, can be reckoned as the hallmark of PcG mediated repression, whereas PRC1 carries out the gene silencing (Figure 1) [48,71].
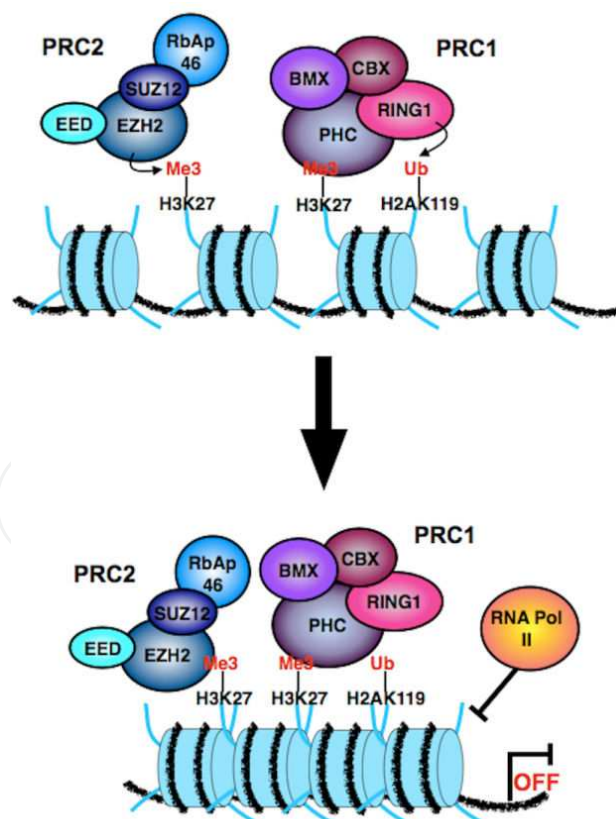

Figure 1. Epigenetic gene silencing PcG-mediated. PRC2 induces EZH2-mediated H3K27me3. H3K27me3 recruits PRC1 that ubiquitylates H2AK119 promoting chromatin compaction and gene silencing.

For what concerns PRC1-independent target genes, it has been shown that PRC2 is able to catalyze *in vitro* the methylation of lysine 26 of histone H1, which in turn recruits heterochromatin binding protein 1 (HP1) to chromatin, influencing its structure [72,73].

PRC2 is also able to cooperate with other epigenetic silencing enzymes. Recent studies demonstrated that it acts upstream of DNMTs in order to silence target genes [74]. The mechanism is not yet clear, but a hypothesis is that target genes are initially repressed through histone H3K27 methylation. Afterwards, PRC2 induces a more stable transcriptional silencing by recruiting DNMTs and establishing CGI methylation [75-77]. Moreover, PRC2 associates with histone deacetylases, reinforcing transcriptional repression and providing functional synergy to stable silencing of target genes (Figure 2) [32,56-59,61,78,79].

The functional link between PcG proteins, HDACs and DMTs demonstrated a synergic control of gene silencing involved in both physiological and pathological processes.
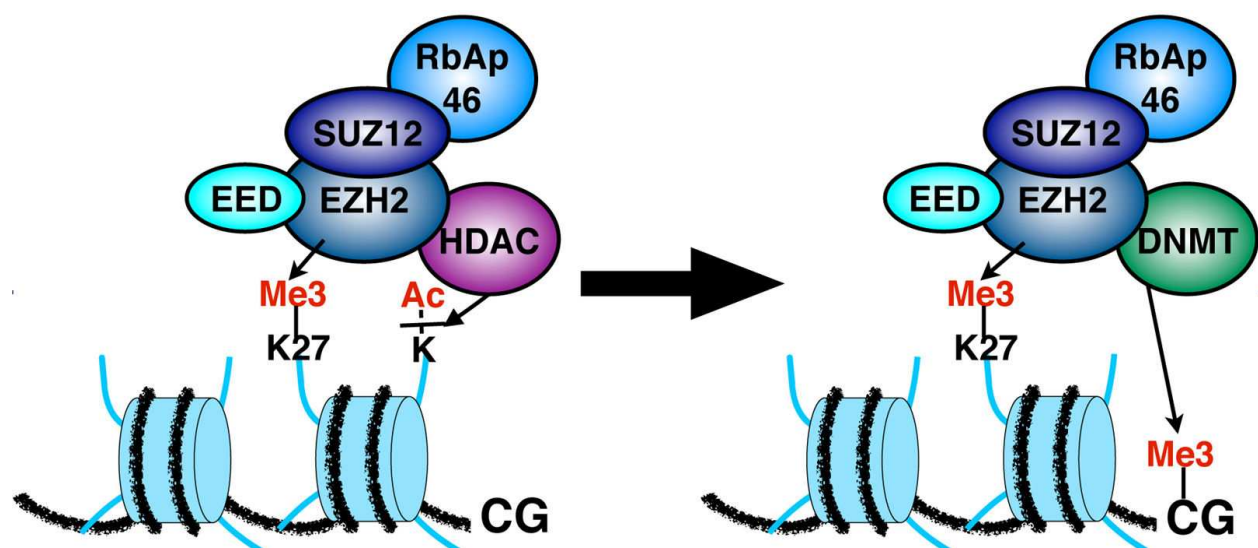

Figure 2. Functional link between PRC2 HDACs and DNMTs. Target genes are initially deacetylated by a histone deacetylase. PRC2 silences target genes by H3K27me3. PRC2 may also recruit DNMTs that methylate DNA promoting a more strongly silenced chromatin state.

3. Regulation of PRC2 activity

Polycomb group proteins are epigenetic regulators of embryonic development and stem cell maintenance [48,51,80] and their deregulation contributes to cancer [28,81]. The crucial role of Polycomb-repressive complexes in the regulation of these biological processes strongly supports the presence of multiple molecular mechanisms involved in PRC activity modulation, such as regulation of expression, post-translational modification and recruitment of other molecular complexes to target genes.

3.1. Regulation of PRC2 components expression

As already explained, PRC2 functions are strictly tissue-specific. Therefore, it should not surprise that the expression of PRC2 subunits has been reported to be context- and tissue-specific, despite the activity of PRC2 promoters has not yet fully understood. Recently, it has been proposed a general rule by which PRC2 expression is maintained by molecular factors that control cell proliferation and self-renewal, such as E2F factors and c-myc, whereas its transcriptional repression is induced by differentiation-promoting factors, such as pRb and p16INK4b [65,82-85].

For what concerns the transcriptional regulation by the pRb/E2F pathway, it has been demonstrated that E2F factors are required for the transcriptional activity of EZH2 and EED in mouse embryonic fibroblasts (MEF). Ectopic expression of pRb and p16INK4b, both involved in E2F target gene repression, induces PRC2 subunits transcriptional repression, whereas pRb silencing increases their transcript levels [82-84].

Furthermore, it has been recently reported that c-myc, a key regulator of ES cells pluripotency maintenance, is directly involved in transcriptional upregulation of all components of PRC2; c-myc binds PRC2 subunits promoters and induces the acetylation of histones H3 and H4, an epigenetic modification involved in transcriptional activation [85].

Finally, it has been demonstrated that EZH2 is post-transcriptionally regulated by a micro-RNAs-mediated translation-inhibition mechanism. MicroRNAs (miRNAs) are small non-coding RNA ~22 nt long (ncRNA), involved in various biological processes, which exert gene expression regulation. Several studies showed a role of miRNA in chromatin structure, they are indeed able to regulate transcriptional levels of epigenetic enzymes as for example PcG proteins [86,87]. Initially, it has been shown that miRNA-101 and miRNA-26a negatively regulate EZH2 expression by binding to its 3'-UTR. However, recent studies have reported an increasing number of miRNAs, able to inhibit the translation of PRC2 subunits (reviewed in [87]). For example, miR-214 regulates EZH2 expression during muscle differentiation [88]. Furthermore, downregulation of several miRNAs promotes EZH2 overexpression in cancer; for instance, miR-25 and miR30d in thyroid carcinoma [89], let-7 in prostate cancer [90], miR-98 and miR-214 in esophageal squamous cell carcinoma.

3.2. Post-translational modification of EZH2

Several studies demonstrated that post-translational modifications of PRC2 subunits can regulate their recruitment to target genes and molecular activity [91-93].

The first post-translational modification that will be analyzed is EZH2 phosphorylation, which has been extensively studied. In order to bind to PRC2 complex and exert its molecular function, EZH2 must be phosphorylated in several specific sites. EZH2 phosphorylation can be classified in two groups: dependent by cell-cycle-dependent signals and dependent by extracellular-regulated kinases [94]. In the first mechanism, EZH2 is phosphorylated by Cdk1 and Cdk2 during cell cycle progression [92,93,95]. In murine model, phosphorylation of threonine 345 (Thr345) increases the binding of EZH2 to specific regulatory ncRNAs as HOTAIR that induces the recruitment of PRC2 to HOX gene promoters, and Xist RepA that

induces the inactivation of X chromosome [93]. In humans, phosphorylation of threonine 350 (Thr350) corresponds to murine Thr345. Recently, Chen *and collaborators* demonstrated a crucial role for the phosphorylation of Thr350 by Cdk1 and Cdk2 in both EZH2-dependent gene silencing and EZH2-mediated cell proliferation and migration [92].

Moreover, Cdk1 is able to phosphorylate EZH2 at Thr487. This modification is associated with the disruption of EZH2 binding with other PRC2 components with subsequent methyltransferase activity inhibition [95]. Surprisingly, these data are in contrast with studies of Kaneco *and coworkers*, which demonstrated that EZH2 phosphorylated at Thr487 is able to bind other subunits of PRC2 complex and to maintain its activity. In addition, another recent work showed that inhibition of Cdk1 suppresses hoxA gene expression in contrast to the findings of Chen *and colleagues*. [92,93,95]. Bearing in mind that these three findings use different models to analyze EZH2 phosphorylation, becomes noticeable that the apparent discrepancies could be explained with different mechanisms of tissue-specific regulation. Further studies are needed to resolve these specific incongruities. Mechanisms of EZH2 regulation during cell cycle are summarized in figure 3.
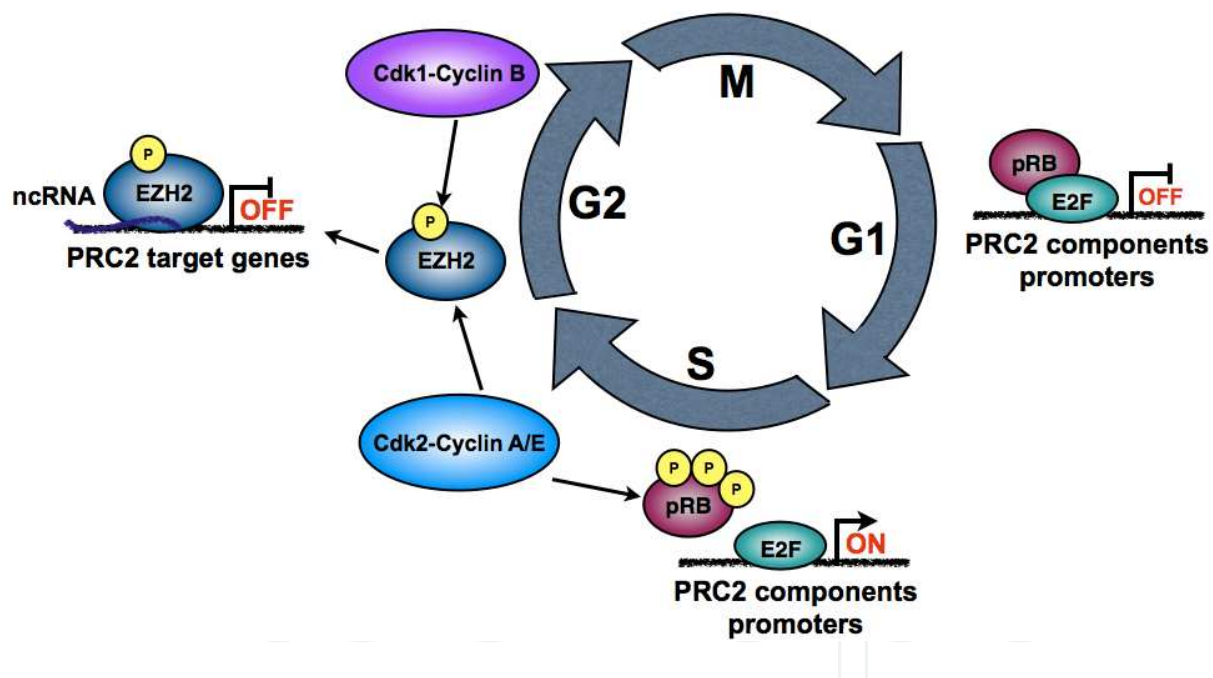

Figure 3. Model for regulation of EZH2 activity during cell cycle. PRC2 subunits are E2Fs target genes. E2Fs activity is inhibited by hypo-phosphorylated pRb during G1 phase of cell cycle. Activity of Cdk/cyclin complexes triggers the transition from G1 to S phase through phoshorylation of pRb and consequent activation of E2F target genes. Moreover Cdk2 and Cdk1 are able to phosphorylate EZH2 promoting the binding of ncRNA, a crucial step for the recruitment of PRC2 to its target genes.

Phosphorylation of EZH2 is also modulated by environmental signals. Extracellular signals induce Akt activation that in turn is able to phosphorylate EZH2 at Serine 21 (Ser21), which results in a suppression of the PRC2 activity. Differently by previous phosphorylation mechanisms, Akt-dependent phosphorylation does not affect the binding with other PRC2

components but it reduces the affinity of EZH2 with histone H3, which results in a decrease of the H3K27 methylation and consequent de-repression of the EZH2-silenced genes [96].

Furthermore, recent studies reported that EZH2 can be phosphorylated at threonine 372 (Thr372) by p38α kinase in muscle stem (satellite) cells in response to tumor necrosis factor (TNF), an inflammatory cytokine highly expressed in muscle regeneration process [97]. The phosphorylation of EZH2 at Thr372 promotes the repression of Pax7, a marker of stem cells, by inducing the interaction between PRC2, YY1 and PRC1. This leads to the transcriptional activation of genes involved in muscle regeneration and to transcriptional repression of genes involved in cell proliferation. This data are in apparent conflict with the fully studied role of EZH2 in the cell proliferation promotion, but it is possible that in response to specific signals and in particular cell types, PRC2 can silence genes involved in cell cycle regulation, resulting in an antiproliferative activity [97]. Other studies are needed to confirm this hypothesis.

Finally EZH2 and SUZ12 can be sumoylated *in vitro* and *in vivo* but the role of this modification is still not yet clear [91].

3.3. PRC2 recruitment to target genes

PRC2 core subunits bind to the DNA sequences with low affinity, this, therefore, suggests the existence of recruiting mechanisms that direct PRC2 to target genes [98].

In *Drosophila melanogaster*, PcGs are recruited by Polycomb response elements (PREs), DNA sequences of several hundred base pairs [42,48,99] located both in proximal region of gene promoters and in long-range enhancer elements. PREs contain consensus sequences for various transcription factors [100]. For instance, Drosophila's pleiohomeotic (PHO) and pleiohomeotic-like (PHO-like) are PcG proteins conserved in mammalian cells and involved in the recruitment of PRC complexes.

It is important to stress that PREs are element and not short stretches of nucleotides and contain numerous TF binding sites, therefore, although several Drosophila transcription factors are essential for the recruitment of PRC complexes to specific promoters, a single TF is not sufficient alone. Moreover, "universal" factors able to bind all PcG target genes have not been found yet, and it is strongly suggested that the PcG protein recruitment is a cell type-specific mechanism dependent by various combinations of TFs [52,53]. Mammalian PREs have been just recently discovered [101]. The mammalian orthologue of *Drosophila* DNA-binding protein PHO is YY1, however, studies in mouse stem cells showed a little overlap between sequences bound by YY1 and PRC2 suggesting a cell-type specific role rather than a general one [47].

Similar to YY1, the embryonic stem (ES) cell-related transcription factors OCT4, SOX2 and NANOG co-occupy a subset of PcG target genes in human and mouse ES cells [45,46]. Interestingly, recent studies demonstrated that the serine/threonine protein phosphatase-1 (PP1), together with its regulatory partner NPP1, is capable of complexing PRC2 at its target genes, modulating the DNA occupancy of EZH2 and therefore its activity [102].

Current data suggest that, similarly to flies, various transcription factors may be involved in the recruitment of mammalian PRC2, varying in different cell types and context. Recent studies

showed that Twist-1 recruits EZH2 at ARF-INK4a locus in Mesenchymal Stem Cells (MSCs), inducing transcriptional repression of both p14ARF and p16INK4a, and suppression of senescence initiation [103].

Moreover, PRC2 can also be associated with another PcG protein, called PHF1. PHF1 is not a core subunit of PRC2 but its association with the complex influences the recruitment of PRC2 to target genes and stimulates the enzymatic activity [104,105].

Furthermore, several reports have identified in mouse and human ES cells, a novel DNA-binding component of PRC2 complex, Jarid2, which is a member of the Jumonji C (JmjC) family that binds GC and GA-rich motifs [106-109]. Despite this, it has been demonstrated that Jarid2 promotes PRC2 recruitment to target genes, but its precise role in PRC2 activity has not yet been defined. Knockdown of Jarid2 causes an increase of H3K27me3 levels on some PRC2-target genes [106,107] and a decrease on others [108,109]. Different effects of Jarid2 on PRC2 activity could depend from additional factors, and it has been suggested that it acts as a ''molecular rheostat'' that finely calibrates PRC2 functions at developmental genes [106].

Finally, long ncRNAs have been implicated in the recruitment of PRC2 [48,53,80,81,110]. For example, in primary human fibroblasts the ncRNA HOTAIR recruits PRC2 complex to HOXD locus for regulating gene silencing in *trans* [111]. Several long ncRNA have been discovered and its tissue-specific expression allows assuming PRC2-dependent roles in organogenesis [112].

4. Role of PRC2 in differentiation and cell fate commitment

In past decades, several studies demonstrated that PcG proteins play a key role during invertebrate differentiation but, only recently, the involvement of these proteins during vertebrate organogenesis as regulators of developmental gene expression has been confirmed. Various tissues are regulated by PRC2 during development (Table 1).

Embryonic stem cells (ESC) are able to differentiate into all derivatives of the three primary germ layers and their pluripotency is preserved by the inhibition of differentiation and the promotion of proliferation [71]. Therefore, ESC can be an extremely valuable model to study cell fate transition mechanisms involved in mammalian development. As previously explained, during development, epigenetic changes regulate the activation determining cell fate.

Genome wide analysis revealed that epigenetic changes regulate the activation or the inhibition of lineage-specific transcription factors in cell fate transition, suggesting their role in the maintenance of ESC pluripotency. For what concerns the polycomb repressive complexes, they occupy gene promoter sequences of the main developmental genes, impeding their transcriptional activation through repressive marks [44-46].

Major targets of PRC2 are tumor suppressor genes, such as Ink4b/Arf/Ink4a locus and their inhibition promotes cell proliferation [44,132,136-141].

In ESC, numerous differentiation-related genes feature a bivalent epigenetic regulation in preparation of lineage commitment [65]. This bivalent epigenetic regulation consists in the

Tissue	Specie	Role/Stage	References
Brain	human	Neuronal stem cell maintenance; oligodendrocytes differentiation	[113-118]
	mouse		
Spinal Cord	chicken	Dorsoventral patterning	[119]
Skin	human	Epidermal stem cell maintenance; hair follicle homeostasis	[120-122]
	mouse		
Cardiac Muscle	mouse	Endocardial cushion formation and cardiomyocyte proliferation	[123-125]
Skeletal Muscle	mouse	Skeletal muscle stem cells maintenance; somites developing and myoblasts proliferation	[88,126-129]
Liver	mouse	Hepatic stem/progenitor cells proliferation and self-renewal	[130,131]
Pancreas	mouse	β-cell proliferation	[132,133]
Adipose Tissue	mouse	Adipogenesis promotion	[134]
Mammary Gland	mouse	Alveolar progenitors maintenance	[135]

Table 1. Tissues under PRC2 regulation

presence of both H3K27me3 and H3K4me3, which respectively are a repressing and an activating mark of transcription [142]. Upon differentiation, PRC2 complex dissociates from these gene promoters, inducing H3K27me3 removal and gene expression [45,46].

Similarly to ESC, PRC2 is involved in organ development through tissue-dependent mechanisms. As a general rule, EZH2 prevents differentiation by inhibiting genes involved in its completion. For instance, EZH2 negatively regulates skin development by repressing premature differentiation of skin progenitors. Specifically, it has been shown that in this specific differentiation model, EZH2 prevents epidermal differentiation by inhibiting the recruitment of AP1, a transcriptional activator, to Ink4/ARF locus, thus maintaining proliferative potential of epidermal progenitors [120]. Likewise, it has been reported that silencing of EZH2 in hepatic stem/progenitor cells promotes the differentiation into hepatocytes and further enhances the maturation of hepatocytes through Ink4a-Ink4b dependent and independent mechanisms [130].

EZH2 also contributes to pancreatic regeneration, by the suppression of Arf/Ink4a locus and the promotion of pancreatic β-cells proliferation, [132] and to terminal differentiation inhibition of mammary gland alveolar cells during pregnancy, in order to prevent milk production and secretion until parturition [135].

In opposition to PRC2-dependent mechanisms mentioned above, there are some tissues and organs, which require PRC2 activity for differentiation completion. For instance, recent studies showed a promoting role of EZH2 methyltransferase activity in adipogenesis. EZH2 is required, indeed, for silencing of Wnt1, -6, -10a, and -10b genes, which are inhibitors of adipogenesis [134]. Moreover, EZH2 contributes to the correct development by preventing the inappropriate gene expression, typical of different cell types. The cardiac differentiation is an example of this PRC2-dependent regulatory function; indeed, EZH2 is involved in transcrip-

tional repression of genes as Six1, responsible of skeletal muscle genes activation in cardio-myocytes. EZH2-knockout mice feature postnatal myocardial pathologies and altered cardiac gene expression [123,125]. EZH2 promotes evenly, by indirect mechanism, liver differentiation by the inhibition of Pdx1 gene, which is involved in pancreatic differentiation promotion [133].

The complexity of PRC2-dependent molecular pathways in organogenesis has been specifically demonstrated by extensive studies in neurogenesis and myogenesis.

4.1. Role of EZH2 in neurogenesis

Neurons and astrocytes derive from common neural precursors (neuronal stem cells: NSC), which sequentially pass through phases of expansion, neurogenesis and astrogenesis. The timing of the switch from neurogenic to astrocyte differentiation is crucial for the determination of neuron numbers.

Analysis of EZH2 expression in neurogenesis showed that EZH2 decreases when NSCs differentiate into neurons and is completely suppressed in astrocyte differentiation. In contrast, EZH2 expression remains high in oligodendrocyte differentiation, from precursor cells to the immature stage [113]. EZH2 silencing and overexpression in NSCs confirmed these results, indeed forced expression of EZH2 increases the number of oligodendrocytes and reduces the number of astrocytes [113]. Furthermore, forced expression of EZH2 in astrocytes induces a partially dedifferentiation to NSCs [117], supporting a key role for EZH2 towards oligodendrocyte commitment. For what concerns EZH2 silencing, it has been reported that inhibition of EZH2 or EED in neural precursor cells extends neurogenic phase, inducing an increased production of neurons and a delay in gliogenesis [114]. However, Pereira and colleagues found that loss of EZH2 results in a shift from self-renewal towards differentiation, accelerating the timing for both cortical neurogenesis and gliogenesis [115]. These differences could be accounted to differential EZH2 inhibition timing before or after neurogenesis onset; further studies are required to clarify this pathway, but all data confirm an essential role for PRC2 in the regulation of developmental transitions timing.

4.2. Role of EZH2 in skeletal myogenesis

Proliferation and differentiation of skeletal muscle cells are controlled by a family of myogenic transcription factors, known as bHLH proteins. MyoD is one of the most important bHLH factors, which is crucial for complete muscle differentiation [143]. In ESC, PRC2 binds and represses numerous MyoD target genes [46]. In skeletal myoblasts, despite MyoD expression, PRC2 is recruited by YY1 to muscle-specific genes, inhibiting their expression and preventing premature differentiation. After the commitment of myogenesis, EZH2 expression decreases and H3K27me3 at MyoD-target loci is removed. Consequently, muscle-specific genes are transcriptionally active [126]. This process is finely regulated by miR-214, a miRNA expressed after myogenic commitment of MyoD. In myoblasts, PRC complexes occupy and repress transcription of the intronic region containing miR-214. During myogenesis decreased levels of EZH2 allow derepression of

the miR-214 locus. miR-214, on the other hand, targets EZH2 3'UTR reducing its mRNA translation, thus inhibiting EZH2 mRNA translation [88].

It has been shown that UTX, a specific demethylase that accomplish the muscle specific genes activation, is specifically involved in removal of H3K27me3 and in establishment of H3K4me3, an epigenetic marker of active genes [127].

Interestingly, EZH1 expression increases during myogenesis and its levels remain elevated in differentiated myoblasts [144]. It has been demonstrated that PRC2-EZH1 complex has a crucial role in the correct timing of transcriptional activation of muscle specific genes, as myogenin, allowing proper recruitment of MyoD on its target promoters. This mechanism involves another epigenetic modification, the phosphorylation of serine 28 of the histone H3 (H3S28ph), which is fundamental for the displacement of the PRC2-EZH2 complex [129].

This example proves the complexity of PRC2 dependent mechanism during development and demonstrates how distinctive complexes can regulates various stages of differentiation.

Despite the numerous roles of PRC2 in differentiation and organogenesis are attributable to a tissue-specific behavior, further studies are required to clarify each time its role in any process of differentiation.

It is certainly clear that both PRC2 and its catalytic subunit EZH2 can be defined as key factors in the regulation of development and in preserving cell identity.

5. EZH2 and cancer

Epigenetic abnormalities lead to altered gene expression and cellular physiology and occur in several pathologies such as cancer [145,146]. Cancer epigenetics is a branch of cancer biology that focuses on the epigenetic malfunctions involved in cancer initiation and progression [11]. EZH2 is differentially expressed in many tumors with abnormally elevated levels in cancer tissues versus the corresponding normal ones. Of interest is that EZH2 expression is generally correlated with metastatic cancer cells and poor prognosis [32].

Microarray studies in breast and prostate cancers were the first reports addressing the implication of EZH2 in tumor progression [79,147]. Currently, a wide number of human cancers associated with the deregulation of EZH2 have been discovered (Table 2).

The role of PcG proteins in cancer epigenetics is partially attributed to their contribution in transcriptional repression of INK4b-ARF-INK4a locus, which encode p15INK4b, p16INK4a and p14ARF proteins. These proteins constitute a homeostatic mechanism that protect organism from inappropriate growth signals, which would eventually lead to uncontrolled proliferation, promoting in contrast senescence or apoptosis [139]. Various tumors are characterized by mutations or transcriptional repression at INF4b-ARF-INKa locus, which is frequently a consequence of an aberrant epigenetic landscape established by factors as EZH2.

p15INK4b and p16INK4a are cyclin-dependent kinase inhibitors (CdkI) that function up-stream in the retinoblastoma protein (pRb) pathway. pRb can be found in two isoforms: hypo-

phosphorylated pRb is the biologically active form, while hyper-phosphorylated pRb is inactive. Hypo-phosphorylated pRb binds and inhibits E2F transcriptional factor activity. Cdks, through phosphorylation of pRb, render E2F an active transcriptional activator on the E2F target genes. INK4 proteins bind Cdk4 and Cdk6, blocking the assembly of catalytically active Cyclin–Cdk complexes.

The result of an elevated transcription of INK4 proteins is a pRb-dependent cell-cycle arrest in G1-phase [44,132,136-141,188,189]. Differently from INK4 proteins, p14ARF activates p53 pathway by inhibiting MDM2 functions. Indeed, MDM2 modulates p53 activity by inducing its transcriptional repression and by promoting its proteasome-mediated degradation. p14ARF induction generally causes cell-cycle arrest in G1 and G2 phases and apoptosis [139,190,191]. Interestingly, EZH2 activity on p16INK4a promoter is Rb family-dependent. Indeed, EZH2 is not able to bind INK4a locus in Rb proteins-deficient cells. A model has been proposed where pRb recruits PRC2 to the p16INK4a promoter, which in turn promotes its transcriptional repression [192].

EZH2 has shown a functional role evenly on pRb2/p130. pRb2/p130 is a member of Rb family that binds and recruits HDAC1 at Cyclin A promoter, inducing gene silencing. Cyclin A is a protein with a crucial role in cell cycle advancement. EZH2 competes with HDAC1 for its binding with pRb2/p130, disrupting both proteins occupancy on cyclin A promoter, inducing cyclin A activation and cell cycle progression [193,194].

As well as Rb family members, EZH2 inhibits tumor suppressor genes as p21 and phosphatase and tensin homolog (PTEN) [170,195]. For example, oncogenic stimuli in melanocytes provoke an oncogene-induced senescence, termed melanocytic nevus, which is a benign precursor of melanoma. EZH2 overexpressing cells escape senescence through the inhibition of p21. EZH2 depletion indeed, results in p21 activation and senescence induction in human melanoma cells [195]. A similar functional role has been reported in B-cell acute lymphoblastic leukemia (B-ALL) cells, where EZH2 overexpression induces p21, p53 and PTEN silencing whereas its knockdown induces cell cycle arrest and apoptosis [170].

As already stated, EZH2 is involved in apoptosis regulation [196,197]. High levels of EZH2 induce silencing of DAB2IP, a Ras GTPase-activating protein that promotes apoptosis through the tumor necrosis factor-mediated JNK signaling pathway [196], and Bim, a protein that promotes E2F1-dependent apoptosis [197].

DAB2IP is downregulated by epigenetic modifications in multiple aggressive cancers such as lung, breast and prostate. In medulloblastoma, EZH2-dependent-DAB2IP repression corre-lates significantly with a poor prognosis, independent by the metastatic stage [187].

Recent reports, using genome-wide technologies, reported that a large number of differentia-tion-related factors are PRC2-target genes [43-47]. Consequently, numerous differentiation-related factors as Gata, Sox, Fox, Pou, Pax, components of Wnt, TGF-β, Notch, FGF and retinoic acid pathways are silenced by EZH2 [32,44-46]. It has been proposed that similarly to ESC, the role of EZH2 in cancer is linked to its activity in self-renewal promotion and in the maintenance of undifferentiated state of cells; EZH2 deregulation indeed, strongly contributes to the transcriptional silencing of tumor suppressor and differentiation genes, promoting therefore

uncontrolled cell proliferation and cancer progression [32]. For instance, EZH2 is upregulated in Rhabdomyosarcoma (RMS) cell lines and primary tumors [180]. RMS is a tumor that arises from muscle precursor cells, characterized by a partial myogenic differentiation. RMS cells do not form functional muscle units and feature a strong proliferative ability. Specifically, as shown in a recent study, EZH2 binds and silences several muscle gene promoters evenly under differentiated conditions. The silencing of EZH2 promotes the reduction of H3K27me3 establishment, the recruitment of elongating RNA Polymerase II at these loci and the activation of muscle specific genes, with a partial recovery of skeletal muscle phenotype [182].

Finally, PRC2 complex inhibits the expression of several tumor suppressor miRNA. For instance, downregulation of miR-31, a common event of various melanomas, is caused by epigenetic silencing of EZH2-mediated histone methylation [177].

Moreover, in metastatic liver cancers, up-regulation of EZH2 inhibits miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b, promoting cell motility and metastasis-related pathways [163].

Cancer	Histological Origin	References
Breast	Epithelial	[84,147-151]
Prostate	Epithelial	[79,149,152-157]
Lung	Epithelial	[158-160]
Colon	Epithelial	[161]
Liver	Epithelial	[162,163]
Gastric	Epithelial	[164]
Lymphoma	Mesenchymal	[165-171]
Myeloma	Mesenchymal	[172-174]
Ovarian	Epithelial	[175]
Skin	Epithelial	[149,176,177]
Bladder	Epithelial	[178]
Endometrial	Epithelial	[149,179]
Sarcoma	Mesenchymal	[180-182]
Pancreas	Epithelial	[156,183,184]
Kidney	Epithelial	[185]
Brain	Nervous Tissue	[186,187]

Table 2. Human cancers associated with overexpression of EZH2

5.1. Extra-nuclear function of EZH2

The role of EZH2 as chromatin regulator has been extensively analyzed in a number of normal and pathological models. Recent studies demonstrated a localization of EZH2 and other PRC2

components in the cytoplasm of murine and human cells [198]. Cytoplasmic EZH2 maintains its methyltransferase activity and interacts with Vav1, a GDP-GTP exchange factor (GEF) for members of the Rho-family of GTPases. EZH2-Vav1 complex is necessary for actin reorganization and cellular proliferation in T-lymphocytes and fibroblasts, promoting cytoskeletal dynamics and cell migration as well as proliferation [198]. An example of this specific cytoplasmic function could be found in prostate cancer cells, characterized by increased levels of both nuclear and cytoplasmic EZH2. Cytoplasmic EZH2 might influence cell adhesion and migration, contributing to invasiveness and metastatic ability of tumors [199,200]. The nuclear and cytoplasmic functions of PRC2 thereby could co-operate to promote tumorigenesis.

5.2. Tumor suppressor roles of EZH2

Up to few years ago, EZH2 and PRC2 upregulation were assumed to hypermethylate H3K27, repressing the transcription of tumor suppressor genes. In 2010, Morin and colleagues identified a somatic mutation (Tyr641), which affects the EZH2 catalytic domain activity in diffuse large B-cell lymphoma but not in mantle cell or T-cell lymphoma. Specifically, mutations in lymphoma were heterozygous but haploinsufficient for the enzymatic activity, resulting in global deficit of H3K27 methylation and derepression of gene expression [168]. It has been supposed that the loss of EZH2 in lymphoma may lead to derepression of genes, promoting cell growth [201]. Other reports demonstrated that specific mutations in the EZH2 enzyme display limited capacity to carry out H3K27 monomethylation but have high efficiency for driving di- and tri-methylation. In B-cell lymphomas, mutant and wild type EZH2 co-operate increasing the trimethylated form [202].

Although the data analyzed as of now allow us to classify EZH2 as an oncogene, it must be stated that in particular cellular environments the picture becomes less clear, like for example in malignant myeloid diseases. Three different reports showed the inactivation of EZH2 in myelodysplastic syndromes (MDSs) and in myeloproliferative disorder (MPD) [203-205]. Point mutations of EZH2 gene in MDSs, MPD, and primary myelofibrosis (PMF) are predictors of poor overall survival, independently by risk factors [206,207]. Similarly, three studies conducted in T-acute lymphoblastic leukemia (T-ALL) demonstrated that PRC2 displays a tumor suppressor role in this pathology [171,208,209]. Particularly, Simon and colleagues demonstrated that in mouse, loss of EZH2 in hematopoietic stem cells induces aggressive T-ALL. Similar studies in human showed a comparable decrease of EZH2 levels in T-ALL [171]. Moreover, Ntziachristos and coworkers found that EZH2 and other PRC2 core components are frequently mutated in T-ALL samples [209]. Of interest is that the frequency of PRC2 mutations is higher in pediatric subtype of leukemia [208].

Despite mutations of EZH2 seem specific for a few type of cancers, latest reports suggest a fine balance of H3K27 methylation, necessary for normal cell growth. Recent studies showed an indirect EZH2-dependent mechanism involved in pancreatic cancer inhibition. Jon Mallen-St. and co-authors investigated the role of EZH2 in pancreatic regeneration and in cancer progression using a mouse model characterized by KRas activation, frequently mutated in pancreatic tumors. In particular, they show that KRas mutated mice developed preneoplastic lesions but rarely progressed into invasive adenocarcinoma. The loss

of EZH2 function in this experimental model increases by 6 times the development of pancreatic intraepithelial neoplasia, suggesting a protective role of EZH2 in pancreatic carcinogenesis. Since EZH2 is transiently upregulated after injury and returns to basal levels after tissue recovery, it has been proposed that, in injured tissue, surviving acinar cells de-differentiate into metaplastic epithelial intermediates are able to proliferate and restore pancreatic injury. Proliferation is induced by EZH2 activation through P16INK4a inhibition. Subsequently, acinar cell mass and function is finally restored through re-differentiation, which corresponds to restored basal levels of EZH2. EZH2 is involved therefore in homeostatic mechanisms that controls pancreatic regeneration, decreasing the risk of pancreatic cancer in patient with chronic pancreatic injury [156,184].

6. Conclusions

Epigenetic alterations in cancer cells represent an important aspect of tumor biology. Differently from genetic modifications, epigenetic alterations can be reversed by specific drugs inducing the restoration of "normal" cellular pathways, which in turn promote cellular senescence or apoptosis. Therefore, epigenetic changes are excellent target candidates for chemotherapeutic intervention in cancer.

Several HDAC and DNMT inhibitors are already available as putative anticancer drugs, and several clinical trials are underway [210,211].

A pharmacological therapy, which specifically targets EZH2, may constitute a novel approach to the treatment of cancer, assuming its role in inhibition of several tumor suppressor or differentiation genes.

Recently, an S-adenosyl-L-homocysteine (AdoHcy) hydrolase inhibitor, 3-Deazaneplanocin A (DZNep), has been demonstrated to deplete EZH2 and remove H3K27me3 at PRC2 target genes. The inhibition of AdoHcy hydrolase fosters accumulation of AdoHcy, which in turn stops S-adenosyl-L-methionine (SAH)-dependent methyltransferases.

DZNep promotes apoptosis in cancer cell lines as breast and colorectal cancer cells, but not in normal cells. DZNep reduces cellular levels of PRC2 subunits, inhibits H3K27 methylation and promotes the reactivation of PRC2 silenced genes and apoptosis [212]

Of interest is that, in several non-small cell lung cancer (NSCLC) cell lines, DZNep treatment results in p27 accumulation, G1 cell cycle arrest and apoptosis, whereas immortalized bronchial epithelial and fibroblast cell lines are less sensitive and show apoptosis with lesser extent, which render it a potential candidate in anti-cancer therapy [160].

Studies in various gastric cancer cell lines and in primary human gastric cancer cells showed that the DZNep responsiveness is attenuated in p53-depleted cells. p53 genomic status is therefore a potential predictive marker of DZNep response in this specific cell type [213].

Despite its potential usefulness in cancer therapy, further studies need to address its target specificity. Indeed, it has been reported that DZNep inhibits H4K20 methylation, another

epigenetic modification, which is involved with chromosome stability [212]. The effect of DZNep on several methyl transferases activity is a strong limiting factor for its use as anti-cancer drug.

New PRC2 targets have been recently developed. GSK126 is a small molecule, competitor of S-adenosyl-L-methionine. Unlike DZNep, that reduce levels of EZH2 indirectly, GSK126 specifically inhibits EZH2 methyltransferase activity with no alterations in EZH2 expression. In lymphoma cells, GSK126 treatment decreases global H3K27me3 levels and reactivates PRC2 target genes. [169]. Finally, it has been discovered a natural compound, 16-hydroxyclero-da-3,13-dien-15,16-olide (PL3), which is able to promote apoptosis in leukemia K562 cells by the modulation of various histone-modifying enzymes among which EZH2 and SUZ12 [214].

Other studies are needed to design inhibitors specific for PRC2 and to develop new strategies for epigenetic therapy in cancer.

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