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REVIEW

Mechanisms of pluripotency maintenance in mouse embryonic stem cells

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Abstract Mouse embryonic stem cells (mESCs), characterized by their pluripotency and capacity for self-renewal, are driven by a complex gene expression program composed of several regulatory mechanisms. These mechanisms collaborate to maintain the delicate balance of pluripotency gene expression and their disruption leads to loss of pluripotency. In this review, we provide an extensive overview of the key pillars of mESC pluripotency by elaborating on the various essential transcription factor networks and signaling pathways that directly or indirectly support this state. Furthermore, we consider the latest developments in the role of epigenetic regulation, such as noncoding RNA signaling or histone modifications.

Keywords Pluripotency · Mouse embryonic stem cells · Transcriptional regulation · Epigenetic regulation

Introduction

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass (ICM) of the blastocyst during development [1]. The ability to capture this otherwise transitional state in vitro comes as a result of culture techniques which maintain mESCs in a state of unlimited proliferation, i.e., self-renewal, whilst retaining their pluripotency [1]. Potency refers to the differentiation potential of a cell, ranging from totipotency to unipotency. Totipotent cells are able to give rise to any cell type, whereas unipotent cells are restricted to one linage [2, 3]. Pluripotent cells are capable of differentiating into any of the three germ layers (mesoderm, endoderm, and ectoderm), but are unable to differentiate into extra-embryonic (placental) tissue [4]. Accordingly, chimera formation using mESCs is the ultimate demonstration of their pluripotency, reflecting the developmental end-point of the ICM during embryogenesis [4]. Loss of pluripotency can be regarded as occurring not only upon directed or spontaneous differentiation, but also upon loss of range of potency or ability to differentiate. As such, the regulatory mechanisms that maintain mESCs in their pluripotent state must balance stability, to maintain pluripotency, with plasticity to allow entry into specific programs of differentiation. In this review, we integrate the different mechanisms supporting the maintenance of pluripotency in mESCs, from regulatory transcription factors and signaling pathways to small RNA signaling and epigenetic regulation, to thoroughly understand how this balance is maintained.

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Transcription factors for pluripotency maintenance

There are many reported transcription factors acting as a regulatory network that directly or indirectly drives the mESC identity. In particular, the transcription factors Oct4, Sox2, and Nanog form a core regulatory circuit that is controlled by an auto-regulatory feedback loop [5, 6]. The members of this core circuit are not necessarily restricted to complexing with each other. Several auxiliary transcription factors have also recently been found to be essential. Oct4 itself can also form its own network centered around secondary transcription factors that also play a role in pluripotency maintenance and differentiation.

Core regulatory circuit: Oct4

Oct4 (officially denoted as Pou5f1) belongs to the POU family, as defined by their bipartite DNA-binding POU domain, and can regulate gene expression either positively or negatively to maintain mESC pluripotency. For example, Oct4 synergizes with Sox2 to maintain mESC pluripotency [7–9] or acts as a repressor of ESC differentiation by interacting with lineage-specific transcription factors, such as FoxD3 [10]. The balance of Oct4 expression level itself is also critical for maintenance of pluripotency, and its disruption cripples the ability to derive mESCs from the ICM [11]. Moderate expression of Oct4 enables derivation and maintenance of mESCs, whereas high expression promotes differentiation into mesoderm or endoderm lineages and low expression leads to trophectoderm formation [12, 13]. This balance is fine-tuned by the interaction of Oct-4 with secondary transcription factors through three cis-elements, a distal enhancer, a proximal enhancer, and a proximal promoter [14]. As such, chromatin structure becomes important for Oct4 transcription; methylation in both enhancer regions has been shown to inhibit Oct4 expression [15]. Indeed, such chromatin modifications play an important role in pluripotency maintenance and will be discussed later in this review.

Core regulatory circuit: Sox2

Sox2, which belongs to the Sox family as characterized by their conserved high-mobility-group (HMG) DNA-binding domain, notably synergizes with Oct4 to support the maintenance of ESC pluripotency [16, 17]. This cooperation is established by structural interaction between their DNA-binding domains [19]. Depletion of Sox2 results in loss of pluripotency, although this phenomenon can be rescued by forced expression of Oct4, suggesting that Sox2 plays a secondary role to Oct4 in pluripotency [9, 18]. During embryogenesis, Sox2 expression persists during the development of the central nervous system, whilst the expressions of other pluripotency factors are lost [18]. Therefore, stringent spatiotemporal regulation of Sox2 expression is essential for pluripotency maintenance. As such, for ESC pluripotency, Sox2 not only plays a synergistic role with Oct4 but also maintains a certain expression level to avoid inducing differentiation.

Core regulatory circuit: Nanog

Nanog was first defined by the early embryo-specific NK (ENK) gene, by virtue of its homeodomain bearing similarities to the NK family [19]. However, due to low conservation of DNA sequence with the other members of the NK family, Nanog is regarded as a unique homeodomain transcription factor [20]. Nanog plays a role in maintaining mESC pluripotency and during mouse embryo development. Its expression starts in the morulae and gradually concentrates in the ICM before ultimately halting in the trophectoderm [21]. Some of the downstream targets of Nanog include inhibition of Trp53, a negative regulator of pluripotency [22]. However, the role of Trp53 in maintaining mESC pluripotency is not absolute, since Trp53-null ES cells fail to differentiate in vitro but retain pluripotency of contributing to chimeric embryos [23]. The Oct4-Sox2 complex, in addition to secondary transcription factors, such as FoxD3, binds to the proximal promoter of *Nanog* to modulate its high expression [24]. As the expression of *FoxD3* is also regulated by Oct4, this is a robust means by which Oct4 can regulate *Nanog* [25]. On the other hand, Tcf3 is reported to negatively regulate *Nanog* expression; its depletion would support high expression of Nanog for pluripotency maintenance [26]. Interestingly, it has also been observed that Nanog can regulate its own expression through auto-repression independently from Oct4–Sox2 [27]. Reflecting this, it has been shown that Nanog is dispensable for mESC pluripotency; *Nanog* deficient mESCs and iPSCs retain several hallmarks of pluripotency, such as self-renewal and potency, including the ability to form chimeric mice [21, 28].

Core Klf circuitry

Several Krüppel-like factors are also important for pluripotency maintenance [29]. Klf4 in combination with Oct4/Sox2/cMyc can transform terminally differentiated somatic cells to a pluripotent state thus giving rise to induced pluripotent stem cells (iPSCs). The core Klf circuitry connects to the core regulatory circuit of Oct4/Sox2/Nanog to prevent ESC differentiation and support pluripotency maintenance [30]. As members of the core KLF circuitry, Klf2, Klf4, and Klf5 together increase *Oct4*/

Sox2/*Nanog* expression by binding to their distal enhancer. Conversely, *Klf2* expression is then activated by Oct4, whilst *Klf4* and *Klf5* are activated by Nanog to form a feedback loop [31]. Klfs share a functional relationship with Nanog and both regulate similar targets [29].

Other transcription factors

To establish a clear picture of the molecular mechanisms regulating pluripotency, computational approaches have been utilized to elucidate the essential components and interactions sufficient maintaining ESC pluripotency [32]. Currently, 12 components and 16 interactions have been established and constitute the known regulatory network [32]. For example, Stat3 signaling increases the activity of the Klf circuitry and supports the expression of Tfcp2l1 with either Esrrb or Sall4 to facilitate core or Klf regulatory circuits [32]. Inhibition of two transcription factors, Tcf3 and Erk, is necessary to prevent differentiation and maintain the core regulatory circuit Oct4/Sox2/Nanog [32]. Together, these additional components form the known regulatory network which interacts with the core circuitry to maintain mESC pluripotency (Fig. 1).

Signaling pathways for pluripotency maintenance

Several signaling pathways are involved in the integration of external cues and induction of the mESC identity through modulation of the key transcription factors driving pluripotency and self-renewal. These pathways may furthermore crosstalk to maintain pluripotency.

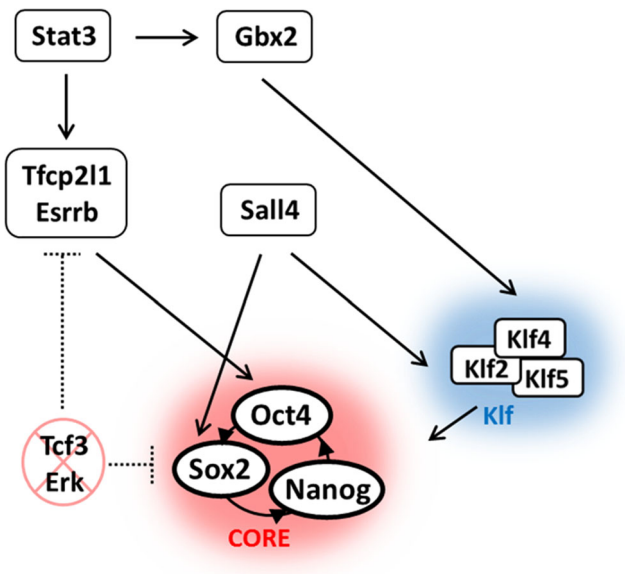

Fig. 1 Regulation of key transcription factors for pluripotency maintenance of mouse embryonic stem cells

LIF signaling

Leukemia Inhibitory factor (LIF), which belongs to the IL6 family, is secreted from murine embryonic fibroblasts and plays a crucial role in the maintenance of the mESC state [33]. LIF binds to the heterodimeric gp130 and LIF receptor beta, resulting in the activation of a broad range of downstream intracellular signaling pathways which regulate different aspects of the core transcriptional circuitry. Some of these are pro-pluripotency, such as Jak-Stat3, PI3K-Akt, and YES-YAP pathways, whereas others are actually pro-differentiation, such as MAPK-Erk [34, 35].

Jak-Stat3 signaling begins with the phosphorylation of Jak upon binding with the SH2 domain of the gp130 receptor. pJak then phosphorylates and activates Stat3, resulting in its homodimerization and translocation into the nucleus [36]. Both pStat3 itself and its downstream intracellular targets, such as *Bcl3*, are reported to play important roles in regulating gene expression for the maintenance of pluripotency [37, 38]. pStat3 regulates the core pluripotency regulatory circuit Oct4/Sox2/Nanog through activation of Klf4 [39], whereas we have shown that Bcl3 interacts with Oct4 protein to regulate *Oct4* and *Nanog* promoter activity. Forced expression of Bcl3 partially maintains alkaline phosphatase activity, an indicator of stemness, after induction of differentiation [38].

PI3K-Akt contributes to the maintenance of pluripotency through two contrasting mechanisms. First, PI3K-Akt blocks MAPK-Erk signaling, which is a driver of endoderm differentiation [40, 41]. Second, Akt significantly increases Tbx3 activity and Nanog expression for ESC pluripotency and proliferation [39].

MAPK/Erk signaling negatively affects Nanog activity through antagonizing the nuclear localization of Tbx3 [39], whilst MEK activates the downstream Erk signaling to repress *Nanog* expression for primitive endoderm differentiation [42].

The YES-YAP pathway begins with activation of YES through binding with gp130 receptor [43]. Upon phosphorylation of gb130, YAP then translocates into the nucleus and binds to TEAD2, enabling it to bind the *Oct4* promoter and induce its expression [43].

BMP signaling

BMP, a member of the TGFβ family, acts through binding to and activation of heteromeric type I and II BMP receptors [44]. The BMP signaling pathway mainly acts through the Smad complex, which consists of three categories: receptor-regulated Smads (R-Smads), cooperating Smad (Co-Smad), and inhibitory Smads (I-Smads). BMP activation leads to phosphorylation of the R-Smads, two of which will then complex with one Co-Smad. This complex then translocates to nucleus to directly regulate pluripotent gene expression.

I-Smad competes with Co-Smad for R-Smad binding to drive ubiquitin degradation of R-Smads, thereby blocking signaling [45]. BMP4 signaling also cooperates with LIF to support pluripotency maintenance of ESCs during in vitro culture, since LIF alone promotes neural differentiation of ESCs under serum-free condition—this differentiation can be halted by induction of BMP4 signaling [46].

Wnt signaling

Similar to the inhibition of MAPK/Erk signaling by LIF, Wnt signaling contributes to pluripotency maintenance by acting as a repressor of ESC differentiation [47]. More specifically, it has been shown that mutations in Wnt3a lead to ectopic neural tube formation in the gastrulating embryo, suggesting that Wnt signaling mainly inhibits neural differentiation [48]. Wnt binds and activates the heterodimeric receptor Frizzled and LRP, resulting in the phosphorylation of GSK3β. This leads to the release of β-catenin, thereby preventing its degradation. Upon translocation into the nucleus, β-catenin binds directly to activate Oct4 or repress Tcf3 to regulate their transcriptional activity [49, 50]. Cytosolic β-catenin can also associate with the membrane and complexes with Oct4 and E-cadherin. This complex is destroyed upon differentiation [51].

Crosstalk between signaling pathways

Crosstalk between these diverse pathways is also essential for the regulation of pluripotency maintenance in mESCs. Within the LIF pathway, Akt (PI3K-Akt signaling) can inhibit both Erk (MAPK/Erk signaling) and GSK3β (thereby activating Wnt signaling) to prevent ESC differentiation [52–54]. The Co-Smad/R-Smad complex from BMP signaling also inhibits Erk (LIF signaling) to keep ESCs in undifferentiated state [55]. Therefore, each signaling pathway not only possesses its own function but also connects with others in an integrated system for the maintenance of pluripotency (Fig. 2).

Reflecting the importance of this integration to stabilize the pluripotent state is the landmark development of 2i culture conditions, which target two separate pathways: PD03 (PD0325901) to inhibit MEK (upstream of Erk signaling) and CHIRON (CHIR99021) to inhibit GSK3 [56]. Crucially, the transition from using serum to 2i culture conditions enabled the derivation of mESCs capable of forming chimeric mice regardless of genetic background [57]. Therefore, the development of 2i culture conditions demonstrates that pluripotency could be maintained solely by inhibition of intrinsic signalings.

Epigenetic regulation of pluripotency maintenance

It has emerged that noncoding RNAs and the regulation of chromatin packing dynamics by histone modifications and DNA methylation play an important role in pluripotency maintenance. These factors provide epigenetic regulation of gene expression and cellular functions both positively and negatively.

miRNAs in transcriptional and post-transcriptional regulation

MicroRNAs (miRNAs) are small (22–24 nt) noncoding RNAs which modulate gene expression through negative post-transcriptional regulation. miRNAs first emerge as primary miRNAs (pri-miRNAs) generated by RNA polymerase II. These are processed into precursor miRNAs (pre-miRNAs), with their stem-loop hairpin structures, in the nucleus upon cleavage by the Drosha-DGCR8 endonuclease [58] and then exported to the cytoplasm to be further cleaved by another endonuclease, Dicer, to generate double-strand RNAs (dsRNAs) [59]. One of the RNA strands becomes the mature miRNA and binds to AGO proteins, and becomes integrated into the RNA-induced silencing complex (RISC) which targets the 3′UTR of mRNAs as directed by sequence complementarity with the

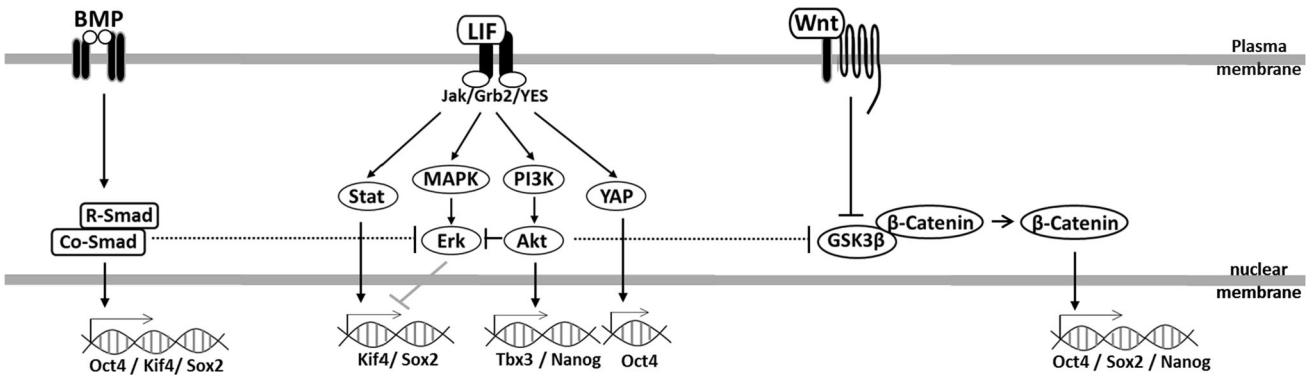

Fig. 2 Regulation of major signaling pathways for pluripotency maintenance of mouse embryonic stem cells

guide miRNA [60]. This targeting silences gene expression through mRNA degradation and deadenylation or inhibition of mRNA translation [61, 62].

Studies of *Dicer* and *Dgcr8* knockout mESCs have shown that miRNAs play an important role in both maintaining pluripotency and the ability to transition into differentiation. Whilst *Dicer* knockout mESCs remain viable, they constitutively express Oct4 and fail to properly differentiate in both in vitro and in vivo differentiation assays [63]. Similarly, DGCR8-deficient mESCs are also unable to fully downregulate pluripotency markers upon attempted differentiation and retain the ability to produce ESC colonies. This is again due to impaired silencing of mESC self-renewal that normally occurs with the induction of differentiation [64]. Interestingly, the phenotype of *Dicer* and *Dgcr8* knockout mESCs differs and it has been suggested that the processing machinery itself may play a miRNA-independent role in ESC function [64].

To identify the miRNAs involved in mESC self-renewal, Wang et al. transfected miRNA mimics in an attempt to rescue proliferation defects in *Dgcr8* knockout mESCs. A group of miRNAs involved in regulating G1-S transition that also shared similar seed sequences were identified: miR-20a, miR-20b, miR-93, miR-106a, miR-291a-3p, miR-291b-3p, miR-294, miR-295, miR-302b, miR-302c, and miR-302d [65].

Of these, the miR-290 family (miR-291a-3p, miR-291b-3p, miR-294, and miR-295) was singled out for investigation due to being an embryonic-specific group of microRNAs found to be enriched in undifferentiated mESCs and rapidly decrease upon differentiation [66–68]. Their transcription is regulated by key pluripotent transcription factors [69]. *Rbl2* and *Lats2* are considered the potential targets by which this miRNA family promotes G1-S transition given that they are the inhibitors of the cyclinE-Cdk2 pathway [65] (Table 1).

Rbl2 is also a means by which the miR-290 family regulates pluripotency through control of DNA methylation. Transcriptome analysis of *Dicer* null mESCs indicates that this may be due to the downregulation of DNA de novo methyltransferase (Dmnt) genes *Dnmt3a*, *Dnmt3b,* and *Dnmt3l*, likely by Rbl2, which leads to decreased methylation of the *Oct4* promoter during differentiation. The silencing of *Oct4* in differentiating *Dicer* null mESCs relies on repressive histone marks [70]. Accordingly, this can be rescued by ectopic expression of DNA de novo methyltransferases or miR-290 family microRNAs [70]. As can be seen, DNA methylation, therefore, plays an important role in the regulation of pluripotency in mESCs and will be discussed later in this review.

Interestingly, the miR-290 family can also enhance the generation of mouse iPSCs through somatic reprogramming using by Oct4, Sox2, and Klf4 [71]. As such, these embryonic-specific miRNAs function in maintaining the pluripotency of mESCs, somatic reprogramming, and sustain expression of DNA de novo methyltransferases for *Oct4* promoter methylation during differentiation.

lncRNAs in transcriptional and post-transcriptional regulation

Long noncoding RNAs (lncRNAs) are arbitrarily defined as RNAs longer than 200 nt. Similar to mRNAs, they are transcribed by RNA polymerase II. Nascent transcripts are processed with 5′-capping, splicing and 3′ polyadenylation. Several functions of lncRNAs have been reported, such as modulation of chromatin structure, regulation of transcription, and post-transcriptional regulation. This is through acting as signals to recruit transcription factors, as molecular decoys titrating proteins away from chromatin, as scaffolds to stabilize protein complex, as RNA guides to recruit chromatin modifiers or targeting miRNAs for degradation [72–75].

Xist is a well-known lncRNA which mediates X chromosome inactivation in female mammals during embryogenesis to balance the dosage of X-linked gene expression [76–79]. In female embryo, the paternal X chromosome is inactivated during cleavage and then transiently reactivated in the inner cell mass of pre-implanting embryo. After implantation, one of the X chromosomes becomes randomly inactivated again [80]. Reflecting this cycle of inactivation, X chromosome inactivation status of mESCs is one of the characteristics defining naïve and primed states of pluripotency. mESCs, which are defined as displaying naïve ground pluripotency, are characteristics of the ICM and possess two activated X chromosomes. In contrast, in vitro

Table 1 Noncoding RNAs regulate pluripotency maintenance in mESCs

	Name	Function	References
miRNA	miR-290 family	To maintain self-renewal property, especially G1-S transition To regulate DNA methylation in the pluripotency state	[65, 70]
lncRNA	Xist RepA	Negatively regulate naïve pluripotency status by recruitment of PRC2 complex and X chromosome inactivation	[82, 83]
lncRNA	AK028326	Oct4 co-transcriptional factor and activate Oct4 expression	[87]

epiblast stem cells (EpiSCs), which are derived from primed epiblasts and defined as displaying primed pluripotency, carry one inactivated X chromosome [81]. *RepA* is an lncRNA that is also transcribed from the *Xist* locus and recruits polycomb repressive complex 2 (PRC2) to form heterochromatin [82]. In mESCs, Oct4, Sox2, and Nanog repress *Xist* expression [83] (Table 1). Notably, during somatic reprogramming, it has been reported that reactivation of X chromosome is necessary and occurs through several mechanisms, such as DNA demethylation and induction of endogenous pluripotency transcription factors [84].

Many other lncRNAs have now also been identified in mESCs using chromatin IP sequencing. Through identification of potential transcriptionally active domains, conserved large noncoding genes, and pluripotency transcription factor binding potential, more than 100 lncRNAs have been discovered in mESCs [85]. Guttman et al. identified 30 lncRNAs related to repression of lineage-specific differentiation through a loss of function screening study knocking down mESC enriched lncRNAs. Using a *Nanog* promoter driven luciferase as a pluripotency reporter, they found 26 lncRNAs involved in regulating mESC pluripotency. Subsequently, through mapping of transcription factors in the genome, they also found that most of these lncRNAs are, in turn, regulated by pluripotency associated transcription factors and bind with several chromatin protein complexes, such as polycomb repression complexes, histone modifiers, and DNA-binding proteins [86]. The function of several other pluripotency related lncRNAs has also been studied. LncRNA *AK028326* transcription is activated by and collaborates with Oct4. LncRNA *AK141205* overexpression positively regulates Oct4 expression [87]. Inhibition of *AK028326* or *AK141205* results in downregulation of Oct4 expression, with abrogation of *AK141205* reducing cell proliferation, and promotes differentiation [87] (Table 1).

One kind of lncRNA named *pRNA*, mediating heterochromatin formation especially in ribosomal DNA (rDNA) region. To exit the pluripotent state, *pRNA* maturation is required. In undifferentiated mESCs, chromatin structure, including rDNA, mostly remains open. The proceeding of *pRNA* maturation from *IGS-rRNA* (intergenic spacer rRNA) is restricted. Upon differentiation, mature *pRNA* interacts with a transcription terminator factor, TTF1, and a TTF1 interacting protein TIP5, on rDNA region, and this recruitment of TIP50 initiates rDNA heterochromatin formation. rDNA heterochromatin triggers genome-wild heterochromatinization. Ectopic expression of mature *pRNA* to induce heterochromatin formation leads to loss of pluripotency [88], suggesting that chromatin remodeling by lncRNA is able to drive the exit of pluripotency state.

Chromatin remodeling and histone modification complexes in pluripotent stem cells

Chromatin modifiers function through mediation of the post-translational modification (PTM) of histone proteins or via ATP-dependent chromatin modifier release of DNA from histone binding for transcription, DNA repair, and replication [89].

Two important complexes mediating PTM modifications of histone proteins are polycomb repressive complex 1/2 (PRC1/2) and the MLL complex, which are responsible for epigenetic repression and activation of gene transcription, respectively (Fig. 3). Study of these PRC complexes originates from work carried out on Drosophila *polycomb* (*Pc*) mutants, which displays abnormal body segmentation due to aberrant *Homeotic* (*Hox*) gene silencing, [90, 91] and several other mutants displaying the same phenotype. Collectively, the proteins involved in this patterning are referred to as the polycomb group (PcG) proteins.

PRC1 and PRC2 were defined based on the nature of their post-transcriptional modification. PRC1, whose core components include Cbx proteins, Ring1A/B, Phc proteins and Pcgf proteins (Fig. 3), and monoubiquitylates lysine 119 of Histone 2A (H2AK119ub) via its Ring1A and Ring1B ubiquitin ligase subunits [91]. A double knockout of *Ring1A/B* impairs mESC proliferation and self-renewal [92]. Notably, PRC1 can also mediate gene silencing with or without its enzymatic activity [93]. Meanwhile, PRC2, the key components of which are Ezh1/2, Suz12, Eed, and RbAp46/48 (Fig. 3), mediates lysine 27 di- or tri-methylation of histone 3 (H3K27me2/3) via its Ezh1 and Ezh2 methyltransferase subunits [94]. Other components, such as Aebp2, Pcl, and Jarid2, are important in the positive regulation or negative regulation of this enzymatic activity [95–99]. Genome-wide analysis of protein binding shows that PRC1 and PRC2 complex proteins co-occupy on the promoters of several transcription factors related to development, such as the Gata family [100]. Although the PRC2 function is dispensable in pluripotency maintenance [101], *Eed*-deficient cells de-repress developmental gene expression [100]. This suggests a role of PRC2 in gene silencing in mESCs [100]. Inactivation of PRC2 components delays the reduction of Oct4 and Nanog expression during differentiation [97, 102, 103]. PRC2 complexes are, therefore, crucial for the repression of developmental regulators during maintenance of pluripotency and then silencing pluripotency upon mESC differentiation through its histone modification activity.

In mESCs, around half of H3K27 chains are dimethylated, 20% of H3K27 are monomethylated, and 10–20% of H3K27 are trimethylated [104]. Trimethylated H3K27 is enriched in repressive chromatin regions or bivalent domains in mESCs [105–107]. PRC1 can be considered

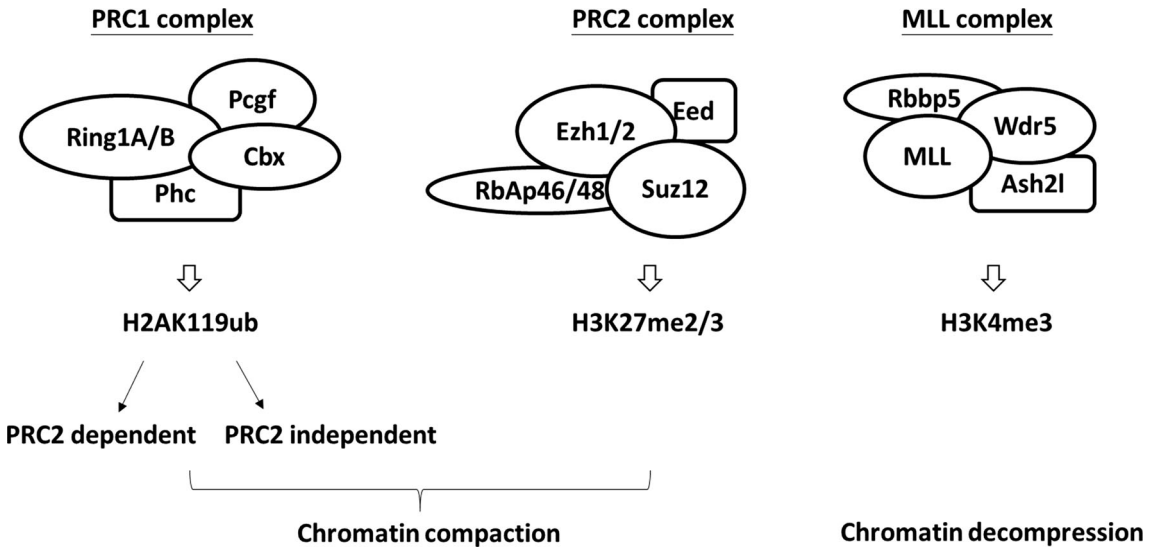

Fig. 3 Complexes-mediating histone modifications for pluripotency maintenance in mouse embryonic stem cells. Key subunits of PRC1 complexes are Cbx (Cbx2/4/6/7/8), Ring1A/B, Phc (Phc1/2/3), and Pcgf1/6. Ring1A/B are ubiquitin ligases responsible for ubiquitylization of lysine 119 of Histone 2A. Key subunits of PRC2 complexes are Ezh1/2, Suz12, Eed, and RbAp46/48. Ezh1/2 are methyltransferases responsible for di- or tri-methylation of lysine 27 of Histone 3. MLL is composed of Wdr5, Ash2l, and Rbbp5. MLL is a histone methyltransferase responsible for tri-methylation of lysine 4 of Histone 3

downstream of PRC2 given that the PRC1 component, Cbx proteins, targets to H3K27me3, a catalytic product of PRC2 [108]. As such, both PRC2 and PRC1 co-occupy H3K27me3-modified gene domains encoding developmental regulatory factors. However, PRC1 can act independently from PRC2 and, furthermore, can take over PRC2′s targets in its absence [94, 109]. The binding of PRC1 to target genes is mediated by Oct4 in this case [92]. L3mbtl2, a PcG protein, is an atypical member of the PRC1 complex and plays a crucial role in regulating mESC self-renewal during the G1-S transition through a noncanonical PRC1-mediated repression mechanism—its knockout results in embryonic lethality [110]. L3mbtl2 targets gene loci characterized by lysine 9 dimethylation of histone 3 (H3K9me2), low histone acetylation, and lysine 199 monoubiquitination of histone 2A. These target genes are not bound by canonical PRC1 and PRC2 complexes, so their modifications are dependent upon the recruitment of PRC1-related components, such as G9a methyltransferase, Hdac1 histone deacetylase, and Ring1B ubiquitin ligase.

In contrast to PcG proteins, trithorax group (TrxG) proteins play a role in epigenetic gene activation through histone 3 lysine 4 tri-methylation. During development, TrxG and PcG proteins have opposite functions but often target similar chromatin regions in mESCs [107, 111, 112]. The mammalian TrxG proteins form the mixed-lineage leukemia (MLL) complex, whose key components include Mll1/2/3, Wdr5, Ash2l, and Rbbp5 [113] (Fig. 3). Wdr5 is a downstream target of Oct4 and Nanog, and its reduction is correlated with a decrease in H3K4me3 status during differentiation. Knockdown of Wdr5 causes mESCs to lose stemness properties, including cell morphology, alkaline phosphatase activity, and self-renewal ability [114]. Using genome-wide mapping of Wdr5, Rbbp5, H3K4me3, and Oct4 binding, Ang et al. found that pluripotency factors cooperate with the MLL complex to activate the transcription of regulators of self-renewal [114], suggesting a crucial role in mESC pluripotency.

General ATP-dependent chromatin-remodeling protein complexes include SWI/SNF, CHD, and INO80. esBAF is a unique ESC-specific SWI/SNF complex which regulates mESC self-renewal and pluripotency. It has been found to colocalize with Oct4, Sox2, Nanog, Stat3, and Smad1, indicating that esBAF has a wide ranging involvement in the core transcription circuitry and the LIF and BMP signaling pathways [115, 116].

Chd1 is an ATP-dependent DNA helicase maintaining euchromatin for gene activation. Although *Chd1* knockdown cells remain in an undifferentiated state, these cells show the defect to differentiate into primitive ectoderm and show a bias in differentiation towards the ectoderm lineage [117]. This suggests that chromatin compaction can lead to a reduction in potency.

Ino80 has been shown to target pluripotency gene promoters through interaction with Oct4 and Wdr5, and its knockdown reduces pluripotency gene expression and promotes cell differentiation [118]. Tip60-400, a complex belonging to the INO80 family, was implicated in the maintenance of mESC pluripotency in an RNAi screening study [119]. More specifically, the Tip60-400 complex binds to H3K4me3 marked chromatin and enables access to Nanog targeted promoters [119]. Accordingly,

knockdown of its subunits alters cell morphology and cell cycle profile, suggesting a loss of pluripotency. As such, the INO80 family as a whole can be considered to facilitate DNA binding of the core circuitry.

Bivalent histone modifications

Chromatin packing into either 'active' euchromatin or 'inactive' heterochromatin is dependent on the type of post-translational modification present on histone tails, which affects their charge. Therefore, the pattern of modification is considered to 'code' the epigenetic regulation of gene expression. For example, H3K4me3, H3K9ac, and H3K14ac are associated with euchromatin, whereas H3K27me3 and H3K9me3 are associated with heterochromatin [120, 121]. Interestingly, H3K27me3 (an 'inactive' code) is often accompanied with H3K4me3 (an 'active' histone code) in the promoters of developmental genes in mESCs. This pairing is referred to as a bivalent histone modification and it is proposed that these promoters are "poised" for further activation [107]. Furthermore, bivalent modifications are established and maintained by a combination of PRC2 and MLL complexes as mentioned above in combination with DNA methylation, transcription factors, and noncoding RNAs [107, 122].

DNA methylation

The impact of DNA methylation on gene expression is widely known [123–125]. This form of modification typically occurs at cytosines in CpG dinucleotide, resulting in the formation of 5-methylcytosine (5mC) which then recruits methyl-DNA-binding (MBD) proteins and methyl-CpG binding protein 2 (MeCP2). These proteins further recruit histone modifiers and chromatin regulators for higher order chromatin organization to repress gene expression [126].

DNA methyltransferase 3A and 3B (Dnmt3A and 3B) are responsible for de novo methylation, whereas Dnmt1 maintains methylation during DNA replication; knockout of Dnmt in mESCs causes DNA hypo-methylation, which can have an impact on differentiation and lineage determination. *Dnmt1*-deficient mESCs die through apoptosis upon attempted induction of differentiation [127]. When cells are cultured in trophoblast stem cell medium, they are able to generate 25% of trophoblast giant cells [127]. DNA hypo-methylation de-represses *Elf5*, a trophoblast-specific transcription factor, which contributes to trophoblast cell lineage [128]. Those *Dnmt3a*- or *3b*-deficient ES cells with severe global DNA hypo-methylation, which possess just 0.6% CpG methylation, are not able to initiate differentiation but remain viable whilst retaining stemness characteristics [129]. Mesoderm cells derived from *Dnmt3a*- and *Dnmt3b*-deficient ES cells retain their ability to convert into endoderm lineage by Gata4 induction [130].

Tet proteins, which are responsible for DNA demethylation, convert 5mC to 5-hydroxymethylcytosine (5hmC), which then becomes 5-formylcytosine (5fC) and 5-carboxylcytosine (5-caC) [131, 132]. Tet1 and Tet2 are enriched in undifferentiated cells and become downregulated after differentiation. Although it has been shown that depletion of Tet1 and Tet2 reduces global 5hmC, no effect on mESC self-renewal ability was observed. On the other hand, differentiation capability was restricted [133]. *In vivo*, Tet1 and Tet2 double knockout mice showed partial perinatal lethality and abnormal DNA imprinting [134].

Others

Several other factors also contribute indirectly to the maintenance of the mESC state. For example, the histone protein variant H2A.Z interacts with and maintains Nanog protein levels through inhibition of protein degradation [135]. RNA polymerase-mediated transcription machinery assists core transcription factors in pluripotency maintenance and somatic cell reprogramming [136]. Cell cycle protein Geminin antagonizes chromatin-remodeling proteins during the S phase to maintain the expression of Oct4, Sox2, and Nanog [137]. Geminin also restrains mesodermal lineage commitment and is associated with antagonism of Wnt signaling and enhanced repressive polycomb-mediated repression [138]. The THO protein complex functions in coupling mRNA transcription and export to cytoplasm. Knockdown of Thoc2 and Thoc5, two subunits of THO complexes, inhibits export of pluripotency gene transcripts. This highlights the importance of mRNA export system in the regulation of mESC pluripotency [139].

Concluding remarks

In the past few decades, scientists have successfully managed to maintain pluripotency in vitro using LIF treatment and signaling inhibitors [4, 31, 54, 140–142] to enable the study into this remarkable state. What has emerged is a picture, whereby pluripotency in mESCs is an embodiment of several levels of balance, from the balance of pluripotency gene expression to that of different regulatory mechanisms that can both agonize and antagonize each other. Offset of just one element can result in a shift in the equilibrium towards differentiation, or, indeed, the inability to make this shift. Intriguing questions about these regulatory mechanisms still exist and the complexity of the involved regulation networks continues to be constructed. Indeed, epigenetics has emerged as a crucial player, with

elements such as chromatin modeling capable of overriding signaling induced gene expression programs. It is hoped that this review, which presents a thorough, up-to-date aggregation of the reported regulatory mechanisms in maintenance of mESC pluripotency, will represent a comprehensive aid to the study of pluripotency.

Compliance with ethical standards

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