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Molecular control of pluripotency

Laurie A Boyer¹, Divya Mathur^{1,2} and Rudolf Jaenisch^{1,2}

Transcriptional regulators and epigenetic modifiers play crucial roles throughout development to ensure that proper gene expression patterns are established and maintained in any given cell type. Recent genome-wide studies have begun to unravel how genetic and epigenetic factors maintain the undifferentiated state of embryonic stem cells while allowing these cells to remain poised to differentiate into somatic cells in response to developmental cues. These studies provide a conceptual framework for understanding pluripotency and lineage-specification at the molecular level.

Addresses

¹Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA

²Massachusetts Institute of Technology, Cambridge, MA 02142, USA

Corresponding author: Jaenisch, Rudolf (jaenisch@wi.mit.edu)

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Introduction

Cells of the embryonic inner cell mass and their *in vitro* derivatives, embryonic stem (ES) cells, possess the remarkable property of pluripotency, the ability to give rise to all cells of the organism [1]. For this reason, ES cells are thought to hold great promise for regenerative medicine. Therefore, a detailed understanding of the mechanisms that enable propagation of ES cells in a pluripotent state, poised to execute a broad range of developmental programs, is essential to realizing their therapeutic potential. In metazoans, the establishment and maintenance of lineage-specific gene expression programs determines cell identity. Many regulators of these processes are highly conserved throughout evolution and are vital for development [2,3]. External environmental factors can also influence gene regulation [4–6] but are not discussed in this review. Here, we focus on studies over the past two years that reveal how genetic and epigenetic factors control ES cell identity and influence the balance between pluripotency and differentiation in mammals.

Genetic control of pluripotency

Oct4, Sox2 and Nanog are key regulators of pluripotency

The homeodomain transcription factors Oct4 (also known as Pou5f1) and Nanog have been identified as crucial regulators of pluripotency and are predominantly expressed in pluripotent cell types (Table 1) (for detailed review, see [5]). Loss of Oct4 causes inappropriate differentiation of the inner cell mass and ES cells into trophectoderm, whereas overexpression of Oct4 results in differentiation into primitive endoderm and mesoderm, suggesting that precise Oct4 levels are necessary for pluripotency [7,8]. Oct4 can regulate gene expression by interacting with other factors within the nucleus, including the high mobility group (HMG)-box transcription factor Sox2 [6]. Although Sox2 plays an important role in the maintenance of pluripotency and lineage specification, its expression is not restricted to pluripotent cells, because Sox2 is also found in early neural lineages [9]. Cells lacking Nanog spontaneously differentiate into primitive endoderm [10,11]. Conversely, overexpression of Nanog promotes self-renewal independent of the cytokine leukemia inhibitory factor (LIF), which functions by activating the transcription factor Stat3 [12]. Although the LIF–Stat3 pathway is dispensable in human ES cells, recent functional analyses indicate an analogous role for Oct4 and Nanog in these cells [13,14]. Thus, *Oct4*, *Sox2* and *Nanog* are the earliest-expressed set of genes known to maintain pluripotency. Together, these studies suggest that Oct4, Sox2 and Nanog function in distinct pathways that might converge to regulate certain common genomic targets. It is likely that the interplay among these factors is critical for early cell fate decisions.

The balance between a minimal set of lineage-specific transcription factors might drive early cell-fate decisions

The simplest model for how Oct4, Sox2 and Nanog function is that they collaborate with other transcription factors to specify a pluripotent state and thus form the basis of a transcription factor hierarchy. Consistent with this, the balance between the levels of Oct4 and the caudal-type homeodomain transcription factor Cdx2 has recently been shown to influence the first overt lineage differentiation in the embryo [15^{*}]. Oct4 and Cdx2 expression patterns become mutually exclusive during embryogenesis, owing, in part, to their ability to reciprocally repress each other's expression. Oct4 is associated with the establishment of the ICM, whereas Cdx2 is necessary for trophectoderm development [16]. Oct4 is lost from the outer cells of the morula that become fated for trophectoderm, whereas Cdx2 expression is restricted to these cells. Oct4 and Cdx2 also regulate the T-box transcription factor *eomesodermin* (*eomes*), which, like

Table 1

Gene expression analyses of transcription factors in ES cell pluripotency and embryonic development

Transcription factor	Protein family	Expression pattern	Loss-of-function phenotype		Gain-of-function phenotype in ES cells
			Embryonic development	ES cells	
Oct4	Pit-Oct-Unc protein family	Oocytes, fertilized embryo, ICM, epiblast, ES cells, embryonic carcinoma cells, germ cells	Embryonic lethal (blastocyst stage), differentiation of epiblast into trophectoderm lineage	Loss of pluripotency, differentiation into trophectoderm lineage	Differentiation into primitive endoderm and mesoderm
Nanog	Novel homeodomain protein	Morula, ICM, epiblast, ES cells, embryonic carcinoma cells, germ cells	Embryonic lethal (E5.5), lack of epiblast, differentiation of ICM into primitive endoderm	Loss of pluripotency, differentiation into primitive endoderm	LIF-Stat3-independent self-renewal, resistance to retinoic acid-induced differentiation
Sox2	SRY-related HMG box protein	Oocytes, ICM, epiblast, germ cells, multipotent cells of extraembryonic ectoderm, cells of neural lineage, brachial arches, gut endoderm	Embryonic lethal (E6.5), failure to maintain epiblast	Unknown	Unknown
Stat3	Signal transducer and activator of transcription family protein	Wide ranges of cell types	Embryonic lethal (E6.5–7.5)	Differentiation into primitive endoderm and mesoderm (Stat3 signaling is dispensable in human ES cells)	LIF-independent self renewal
Cdx2	Caudal-type homeodomain protein	Outer morula cells, trophectoderm cell lineages	Embryonic lethal due to implantation failure (lack of functional trophectoderm)	Normal contribution to all cell lineages except trophectoderm and intestinal cells	Differentiation into trophoblast
Gata6	GATA-binding protein	Extraembryonic endoderm lineages	Embryonic lethal (E5.5–7.5), defects in visceral endoderm formation	Unknown	Differentiation into primitive endoderm
Gata4	GATA-binding protein	Extraembryonic endoderm lineages	Embryonic lethal (E8–9), defects in heart morphogenesis	Can generate cardiac myocytes, inability to generate visceral endoderm and definitive endoderm of foregut	Differentiation into primitive endoderm

Cdx2, is necessary for trophectoderm maintenance [15^{*}]. These studies suggest that the interaction between these factors is essential for segregation of the inner cell mass and trophectoderm lineages during early development.

A similar balance between Nanog levels and the transcription factors Gata4 and Gata6 might be necessary for differentiation into primitive endoderm, a derivative of the inner cell mass of the developing blastocyst. Forced expression of Gata4 or Gata6 in ES cells leads to differentiation into primitive endoderm, an effect similar to that caused by the loss of Nanog function [11,17,18]. Moreover, Gata4 and Gata6 expression was upregulated in the absence of Nanog [11]. Together, these studies suggest that a minimal set of lineage-specific transcription factors can drive early cell fate decisions (Table 1). However, it is likely that other

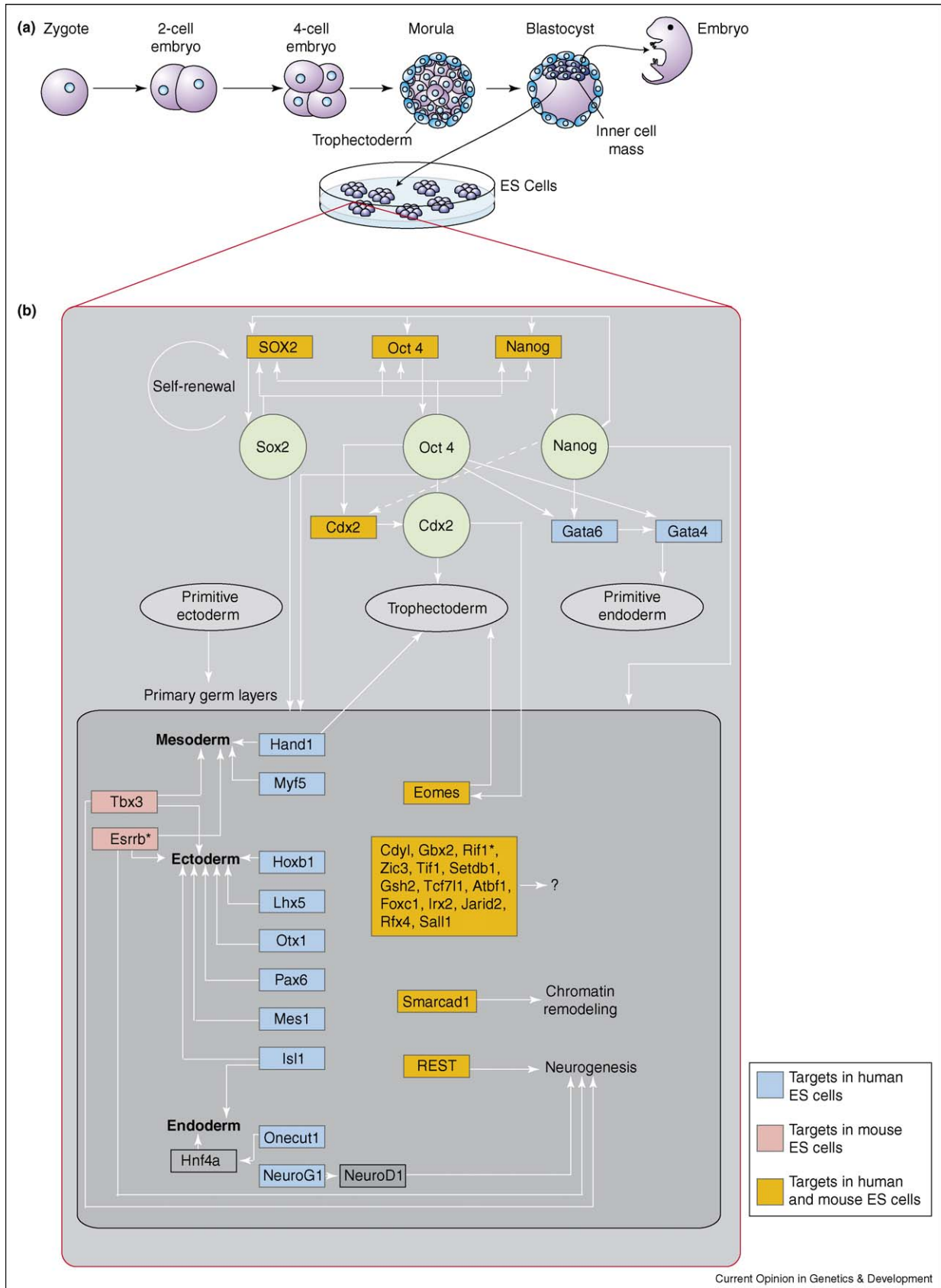
genetic, epigenetic and environmental factors play an important role in this process.

Transcriptional regulatory networks in pluripotent ES cells

Given that factors orchestrating early cell fate decisions also regulate ES cell pluripotency, Oct4, Sox2 and Nanog are thought to establish the initial genomic state from which all other gene expression patterns are derived during development. Recent genomics studies have enabled the construction of transcriptional regulatory networks in ES cells that provide a foundation for understanding how Oct4, Sox2 and Nanog control pluripotency and influence subsequent differentiation events. Two groups have used chromatin immunoprecipitation (ChIP) combined with genome-wide methodologies to map the

(Figure 1 Legend) Core transcriptional regulatory circuitry in pluripotent mouse and human ES cells. **(a)** Embryonic stem (ES) cells are derived from the pluripotent cells of the inner cell mass (ICM), which normally gives rise to the embryo. **(b)** Genomics studies have enabled the construction of a core transcriptional regulatory network in ES cells, initiated by Oct4, Sox2 and Nanog. This network reveals an integrated circuitry comprising genes that specify the development of both the extraembryonic and the embryonic lineages. Shown are a few examples of the circuitry components identified in the mouse and human studies. Boxes and circles indicate genes and proteins, respectively. Arrows represent interactions only, and not positive or negative effects. Genes for which binding information with mouse Sox2 is also available are marked with an asterisk.

Figure 1



binding sites for OCT4 and NANOG throughout the human and mouse ES cell genomes [19^{••},20^{••}]. These studies identified a large number of target genes and revealed that OCT4, NANOG and, in the case of human ES cells, SOX2 share a substantial portion of their targets. These studies have begun to reveal the circuitry that is responsible for the combined biological output of these ES cell regulators.

Similarities and differences between mouse and human ES cell genomic targets

Oct4, Sox2 and Nanog occupied both transcriptionally active and inactive genes in mouse and human ES cells (Figure 1). Active genes include the transcription factors *Oct4*, *Sox2* and *Nanog* themselves, as well as others that are highly expressed in ES cells, such as *Rif1*, *Jarid2* and *SmarcaD1*. *Rif1* has been implicated in regulating telomere length and might be important for self-renewal [21]. Although *Jarid2* and *SmarcaD1* have important roles in development [22,23], their contribution to pluripotency is unknown. Interestingly, a large portion of the inactive targets identified in mouse and human ES cells include transcription factors involved in lineage-specification (Figure 1) [19^{••},20^{••}]. The developmental importance of these genes suggests that Oct4, Sox2 and Nanog act in concert to maintain pluripotency by directly controlling a transcriptional regulatory hierarchy that specifies differentiation into extraembryonic lineages in addition to derivatives of the primary germ layers.

A comparison of Oct4- and Nanog-bound regions identified in these studies, however, revealed only modest similarity between the target genes in the two species. For instance, certain genes such as *Heart and neural crest derivatives expressed 1 (HAND1)* and *MYST3* were identified as targets of OCT4 and NANOG exclusively in human ES cells, whereas others such as *Estrogen-related receptor b (Esrrb)* were observed only in mouse cells. It is interesting to note that although *Hand1* was not identified as a target in mouse ES cells, its expression was upregulated upon RNAi-mediated silencing of both *Esrrb* and *Rif1* in mouse ES cells [20^{••}]. The lack of orthologous genomic targets could be due to genuine differences between the gene regulatory networks or a result of the dissimilarities in genomic platforms used in these studies. Detailed comparisons of Oct4, Sox2 and Nanog target genes between the two species will be imperative for determining the extent to which genetic regulatory information can be extrapolated from one species to the other.

Although these studies provide an initial framework for deciphering the mechanisms by which these key regulators elicit their effects, genetic manipulation of *Oct4*, *Sox2* and *Nanog* combined with gene expression analyses are necessary to elucidate which of their targets are important for the maintenance of pluripotency or downstream differentiation events. Such analyses, reported in the same

study that identified mouse Oct4 and Nanog targets [20^{••}], as well as in another recent study [24[•]] in which mouse ES cell gene expression patterns were profiled under a wide range of conditions, are critical steps in this direction [24[•]]. In addition to confirming a role for *Esrrb* in mouse, Ivanova and colleagues [24[•]] recognized *T-cell leukemia/lymphoma 1 (Tcl1)* and *T-box protein 3 (Tbx3)* as being important factors for sustaining an undifferentiated state. Interestingly, *Esrrb* has been shown to be important for placental development and germ cell proliferation [25], and *Tcl1*, which is highly expressed in ES cells [11], enhances cell proliferation and survival through augmentation of phosphoinositide-3 kinase PI3K–Akt signaling [26,27]. Thus, how these factors contribute to ES self-renewal and pluripotency is of particular interest. Together, these genome-wide studies suggest that Oct4, Sox2 and Nanog form the basis for specialized transcriptional regulatory circuitry that allows for consistent gene expression patterning during ES cell propagation.

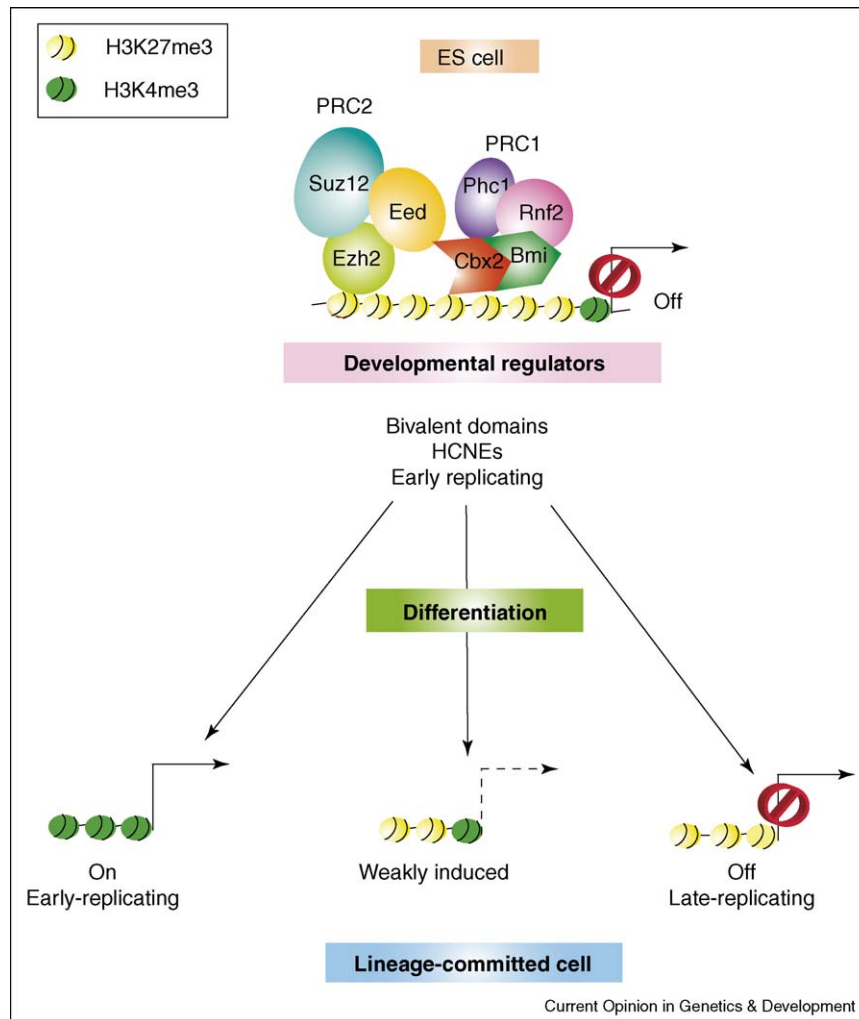
Epigenetic control of pluripotency Chromatin dynamics and epigenetic profile of pluripotent ES cells

Chromatin reorganization is essential for the establishment of new heritable gene expression programs that accompany lineage specification (Figure 2) [27]. For example, ES cell chromatin displays characteristics of transcriptionally permissive euchromatin, such as an abundance of acetylated histone modifications and increased accessibility to nucleases. Conversely, lineage specification is typified by a decrease in acetylation and concomitant increase in heterochromatin formation, indicating that restriction of developmental potential is associated with a marked decrease in genome plasticity. Recent studies have revealed additional unique properties of pluripotent chromatin that distinguish these cells from their differentiated progeny.

A recent analysis of global chromatin dynamics revealed a highly dynamic association of structural chromatin proteins (e.g. core and variant histones, the linker histone H1, and the heterochromatin associated protein HP1 α) with the chromatin of pluripotent cells compared with that of differentiated cell types [28^{••}]. This study also showed that replacement of histone H1 with a version that binds more tightly to chromatin inhibited ES cell differentiation, whereas genetic manipulation of the association of histone H3 and its variant H3.3, a marker of active transcription, with chromatin caused accelerated differentiation. These data posit that structural proteins remain loosely associated with chromatin in pluripotent cells, thereby enabling the reorganization of chromatin structure during differentiation.

Consistent with the observation that the chromatin of pluripotent nuclei is in an 'open' conformation, recent studies have shown that tissue-specific genes that are

Figure 2



Epigenetic characteristics of pluripotent and lineage committed cells. PcG proteins have recently been shown to reversibly silence developmental regulators in ES cells, a process that might be necessary for the propagation of an undifferentiated state [33*,34*]. These regulators, which are early-replicating, contain highly conserved non-coding elements (HCNEs), which are rich in bivalent domains that consist of both H3K27me3 and H3K4me3 modifications [31*,32*]. These domains might provide an epigenetic indexing system to mark genes for expression at later developmental stages. During differentiation of ES cells, the bivalent marks resolve, because early-replicating genes that are expressed in the lineage-committed cells maintain or acquire activating H3K4me3 marks, and late-replicating genes that are turned off in these cells possess repressive H3K27me3 modifications. Notably, genes that are weakly induced still possess bivalent domains.

expected to be silent in undifferentiated cells might be in a semi-permissive transcriptional state in ES cells [29,30]. For example, active epigenetic marks were noted in ES cells at discrete sites within the B cell-specific *λ5-VpreB1* locus prior to gene activation during B-cell commitment [30]. Two recent reports [31*,32*] support such an epigenetic indexing mechanism by revealing the existence of dual marks or 'bivalent' domains, consisting of repressive histone H3K27me3 and activating histone H3K4me3 modifications at a large set of developmentally important genes that are silent in ES cells but activated upon differentiation. These studies suggest that lineage-specific genes are cued in ES cells for subsequent activation

during differentiation. Furthermore, bivalent domains coincide with the most highly conserved non-coding elements in the mammalian genome, suggesting an evolutionarily conserved role for these chromatin domains [32*]. The additional observation that Oct4, Sox2 and Nanog occupied a significant subset of genes that harbor bivalent domains supports a link between the repression of developmental regulators and stem cell pluripotency [19**,32*-34*]. It is important to note that not all tissue-specific genes appear to contain these bivalent marks, and the underlying chromatin structure at these genes and their contributions to pluripotency await further characterization.

A role for Polycomb group proteins in maintaining ES cell identity?

Gene expression is influenced by enzymatic activities that can induce both global and local changes in chromatin structure. Polycomb group (PcG) proteins were first identified in *Drosophila* as transcriptional repressors of homeotic gene expression during embryogenesis [35]. PcG proteins comprise at least two distinct repressor complexes (PRC1 and PRC2–PRC3), the core components of which are highly conserved between fly and human [36]. A role for PcG proteins in pluripotency in mammals was suggested on the basis that PcG components are required for early developmental gene expression patterning [37–40], the establishment of pluripotent ES cell lines [39], and for adult stem cell maintenance [41,42].

Recently, the location of PcG components throughout the genome was mapped in *Drosophila* [43–45] and mammals [33*,34*,46]. Studies in human and mouse ES cells revealed that PRC1 and PRC2 bind to a large set of genes composed of transcriptional regulators and signaling factors with known roles in development. Genes occupied by PcG proteins also contained H3K27me3 in their promoter regions, a repressive histone modification catalyzed by PRC2. Many of the target genes were de-repressed in the absence of the PRC2 components *Eed* or *Suz12*, indicating a direct functional link between PRC2 and gene silencing in ES cells [32*,33*]. ES cells lacking *Eed* can contribute to most cell lineages, suggesting that PcG proteins are not necessary for maintaining pluripotency [47]. However, the observations that *Eed* mutant ES cells spontaneously differentiate [33*] and that ES cells cannot be derived from blastocysts deficient for the PRC2 component *Ezh2* [39] suggest that PcG proteins are necessary for ES cell identity, leaving this an open question.

These studies revealed a dynamic role for PcG proteins in gene silencing in ES cells. The finding that PcG target genes were preferentially activated upon ES cell differentiation implied that these genes were poised for activation [33*,34*]. In flies, the maintenance of heritable epigenetic states requires the interplay between repression mediated by PcG proteins and activation mediated by Trithorax group (Trx) proteins [35]. Trx proteins catalyze lysine 4 trimethylation on histone H3 (H3K4me3) [48]. Interestingly, many of the PcG target genes contained bivalent chromatin domains in their promoter regions [31*–34*], which were resolved during ES cell differentiation into either H3K27me3 or H3K4me3 domains, consistent with the idea that gene expression is governed by the balance between positively and negatively acting factors [49].

Many of the PcG target genes that harbour both H3K27me3 and H3K4me3 marks replicated early in

S phase in ES cells, a property associated euchromatin [31*,50]. However, replication timing was not significantly altered in *Eed* mutant ES cells [30], suggesting that the presence of H3K4me3 or additional factors is required to maintain these genes in a semi-permissive transcriptional state. Notably, a significant subset of PcG target genes were also bound by Oct4, Sox2 and Nanog, suggesting that these ES cell regulators play a role in recruiting PcG complexes [33*,34*]. Identifying the components that catalyze the addition of the activating mark at these genes in ES cells, as well as identifying the factors that recruit PcG and Trx proteins, will be important to better understand how these genes are regulated in pluripotent cells.

A recent study also revealed a role for methyl-CpG binding domain protein 3 (Mbd3), an essential component of the nucleosome remodeling and histone deacetylation (NuRD) complex, in ES cell differentiation [51*]. In *Caenorhabditis elegans*, germline-specific chromatin states specified through PcG-like activities are reorganized in somatic cells by a NuRD-like activity [52]. Thus, it is likely that the balance between pluripotency and lineage commitment is orchestrated by both genetic and epigenetic factors whose roles are to establish and maintain correct gene expression programs during development.

Conclusions and prospects

The recent studies highlighted in this review have made important contributions toward elucidating the complexity of cell fate determination and suggest mechanisms for the stable propagation of a pluripotent state. Despite these efforts, Oct4 and Nanog remain the only two transcriptional regulators identified to date whose role is specific to pluripotent cells. Therefore, it will continue to be of interest how Oct4 and Nanog themselves are regulated and whether there are any other similar pluripotency factors.

The limited success with cloning embryos by nuclear transfer, and the failure to re-establish the gene expression patterns of an early embryo has established an obligatory role for epigenetic processes during development and for nuclear reprogramming [53]. Thus, an understanding of how chromatin states are organized in the embryo and in ES cells is essential for our understanding of pluripotency. The changes in gene expression that lead to differentiation are generally initiated through a response to external cues, where transcription factors are most often the ultimate targets of such signals. Studies have indicated that mouse and human ES cells differ in their requirements for various exogenous factors, such as LIF and bone morphogenic proteins [4–6]. Thus, investigating how extrinsic signals specify intrinsic gene expression programs and cell identity is an important next step. Ultimately, it will be essential to determine

which of these genetic and epigenetic mechanisms are conserved in all embryo-derived pluripotent cell types.

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