The Epigenetic Paradox of Pluripotent ES Cells

Nicola Festuccia, Inma González, Pablo Navarro Gil

Review

The Epigenetic Paradox of Pluripotent ES Cells

Nicola Festuccia[†], **Inma Gonzalez**[†] **and Pablo Navarro**

Epigenetics of Stem Cells, *Department of Stem Cell and Developmental Biology, Institut Pasteur, CNRS UMR3738, 25 rue du Docteur Roux, 75015 Paris, France*

Correspondence to Pablo Navarro: *pnavarro@pasteur.fr*
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Abstract

The propagation and maintenance of gene expression programs are at the foundation of the preservation of cell identity. A large and complex set of epigenetic mechanisms enables the long-term stability and inheritance of transcription states. A key property of authentic epigenetic regulation is being independent from the instructive signals used for its establishment. This makes epigenetic regulation, particularly epigenetic silencing, extremely robust and powerful to lock regulatory states and stabilise cell identity. In line with this, the establishment of epigenetic silencing during development restricts cell potency and maintains the cell fate choices made by transcription factors (TFs). However, how more immature cells that have not yet established their definitive fate maintain their transitory identity without compromising their responsiveness to signalling cues remains unclear. A paradigmatic example is provided by pluripotent embryonic stem (ES) cells derived from a transient population of cells of the blastocyst. Here, we argue that ES cells represent an interesting "*epigenetic paradox*": even though they are captured in a self-renewing state characterised by extremely efficient maintenance of their identity, which is a typical manifestation of robust epigenetic regulation, they seem not to heavily rely on classical epigenetic mechanisms. Indeed, self-renewal strictly depends on the TFs that previously instructed their undifferentiated identity and relies on a particular signalling-dependent chromatin state where repressive chromatin marks play minor roles. Although this "*epigenetic paradox*" may underlie their exquisite responsiveness to developmental cues, it suggests that alternative mechanisms to faithfully propagate gene regulatory states might be prevalent in ES cells.

Introduction

During the development of a complex organism, distinct cell identities are generated by establishing different gene expression profiles. To instruct each identity multiple signalling pathways activate particular sets of sequence-specific transcription factors (TFs) that ultimately activate and repress the appropriate gene expression programs. This sequential activation of tissue-specific TFs is accompanied by the reshaping of the landscape of chromatin modifications in a mutually-dependent process that progressively generates new regulatory architectures [1–3]. TFs are able to dictate the appropriate pattern of gene activity by binding to the promoters and enhancers of specific groups of genes to drive and/or enhance their transcriptional activity. In eukaryotes, transcriptional activation is constrained and regulated by the physical accessibility of regulatory elements, which may or may not be permissive to TF binding. While a handful of master TFs, named pioneer TFs [4], are capable of engaging in stable interactions with DNA even when packed in nucleosomes, the binding of other sequence-specific TFs, general TFs, and the transcriptional machinery requires nucleosome-free regions. Hence, the control of TF binding is frequently associated with the recruitment of chromatin remodelling complexes that establish competent or refractory nucleosomal arrays [5]. Moreover, additional mechanisms based on post-translational histone modifications, the incorporation of specific histone

variants, or the direct methylation and hydroxymethylation of CpG dinucleotides of DNA also promote or restrict chromatin accessibility [6]. This is achieved either by directly affecting nucleosome stability or by serving as a scaffold for the recruitment of additional proteins, including chromatin modifiers and remodellers [5]. Therefore, the functional and biochemical interactions existing among TFs, nucleosome remodellersm and chromatin modifiers represent a key aspect of chromatin biology and gene regulation.

The landscape of chromatin modifications and the differential accessibility of regulatory regions are therefore instated by the local recruitment of chromatin modifiers and remodellers and orchestrated by TFs and other regulators such as non-coding RNAs (Fig. 1). In this model, TFs control over the transcriptional identity of a cell is reinforced and stabilised, but generally not determined, by the establishment of chromatin modifications. In turn, chromatin modifications ensure the preservation of cell identity over time, particular across cell division. In this regard, histone and DNA modifications are ideal candidates to establish an epigenetic memory; provided that they are maintained after replication and during mitosis (Fig. 1), they represent a simple mean for the inheritance of gene regulatory information [7,8]. The study of several epigenetic paradigms has shown that although reversible, epigenetic regulation is often extremely stable and made of several layers of information that ensure the propagation of regulatory states across generations, even in the absence of the initial molecular instructors of such states [9,10]. Therefore, while these mechanisms provide a powerful explanation to the long-term stability of cell and lineage identity in somatic, terminally differentiating cells, they are perhaps less suitable for more immature cells. Indeed, undifferentiated precursors and their immediate immature progeny must display the seemingly opposing faculties of rapidly adapting their transcriptional program to change cell fate in response to developmental cues while concomitantly maintaining their uncommitted state through multiple cell divisions. How this is achieved remains poorly understood.

During mammalian development, the radical, structural, transcriptional, and epigenetic changes that follow fertilisation culminate in the formation of a population of pluripotent cells localised in the inner

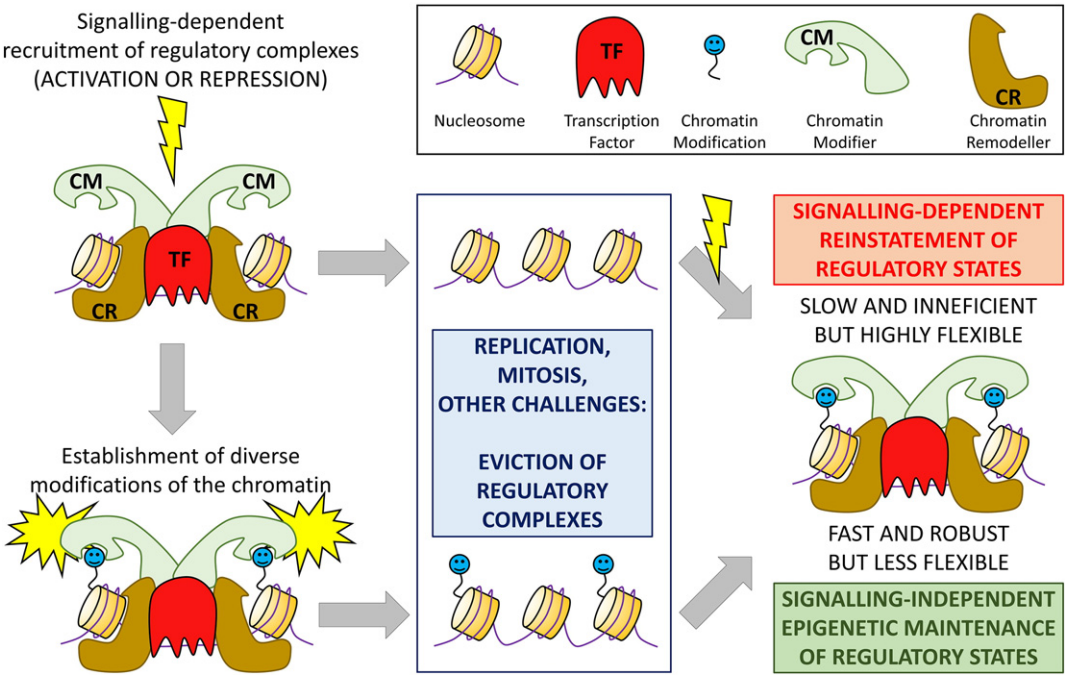

Fig. 1. Facing replication and mitosis is the role of epigenetic memory of chromatin states. Gene regulatory processes are often initiated by a signal that culminates in the establishment of a functional complex at a particular genomic location. This process involves TFs that bind the DNA at specific sequences, chromatin remodelling factors that modify the nucleosomal array, and chromatin modifiers. When the complex disassembles, for example, during replication and mitosis, two outcomes are possible depending on whether or not the regulation has established a form of epigenetic information (denoted by a blue circle). In the absence of such information (upper part), the initial signal is strictly required to re-instruct the regulatory process. In contrast, when an epigenetic mark is established (generally a histone or DNA modification or a histone variant), the signal is not required any longer to enable the reassembly of a functional complex (lower part).

cell mass of the developing blastocyst [11,12]. Whereas *in vivo* pluripotency is rapidly extinguished between implantation and the onset of somitogenesis [13], pluripotent embryonic stem (ES) cells can be derived from pre-implantation embryos [14,15]. While ES cells maintain their identity extremely efficient throughout virtually infinite cell divisions, a process known as self-renewal, they are also strongly responsive to signalling cues *in vitro* and *in vivo*. Crucially, they are capable of effectively recapitulating normal development upon reintroduction into host blastocysts [16,17]. Therefore, ES cells constitute a precious model to understand the molecular mechanisms underlying the unrestricted developmental potential of pluripotent precursors along with the maintenance of these mechanisms across cell division. In this manuscript, we describe three major aspects of ES cells that may underlie their robust yet plastic identity. First, we describe their atypical cell cycle structure and highlight why epigenetic gene regulation should be of particular importance in this cell type. Second, we discuss their overall independence from systems responsible for the deposition of repressive chromatin modifications, which are considered at the foundation of epigenetic memory. Third, we review their globally accessible chromatin configuration and the apparent importance of TFs and chromatin remodelling in preserving their transcriptional identity. Finally, we suggest that based on these three different aspects, alternative mechanisms of mitotic inheritance must be operational in ES cells, as suggested by the recent discovery of mitotic bookmarking by a pluripotency TF, Esrrb [18].

A Short G1 Phase, AHallmark of Pluripotent Cells

The relatively fast ES cell cycle (12 h in average; Fig. 2) is characterised by the absence of a G1/S checkpoint leading to a very short G1 and the consequent large prevalence of actively replicating cells in regular ES cultures [19]. In somatic cells, the G1/S transition is highly regulated, particularly by the Extracellular signal-regulated kinase-2 (MEK/ERK) pathway that activates the Cyclin/Cdk complexes leading to cell cycle progression upon stimulation by growth factors. Notably, the lack of a G1/S checkpoint in ES cells is in line with their large and similarly atypical independence from MEK/ERK signalling: ES cells can proliferate upon the chemical inhibition or the genetic inactivation of MEK/ERK, with relatively unchanged cell cycle dynamics [20,21]. Three related features have been proposed to explain the unusual cell cycle of ES cells (Fig. 2): Retinoblastoma (Rb) hyperphosphorylation [19], low expression of cell cycle inhibitors [22–25], and strongly attenuated fluctuations of cyclins and their associated kinase activities [24,26,27]. Briefly, the association of Cyclin D3 with Cdk6 forms a complex that is not affected by the low levels of p16ink4a inhibitor [22,25], leading to

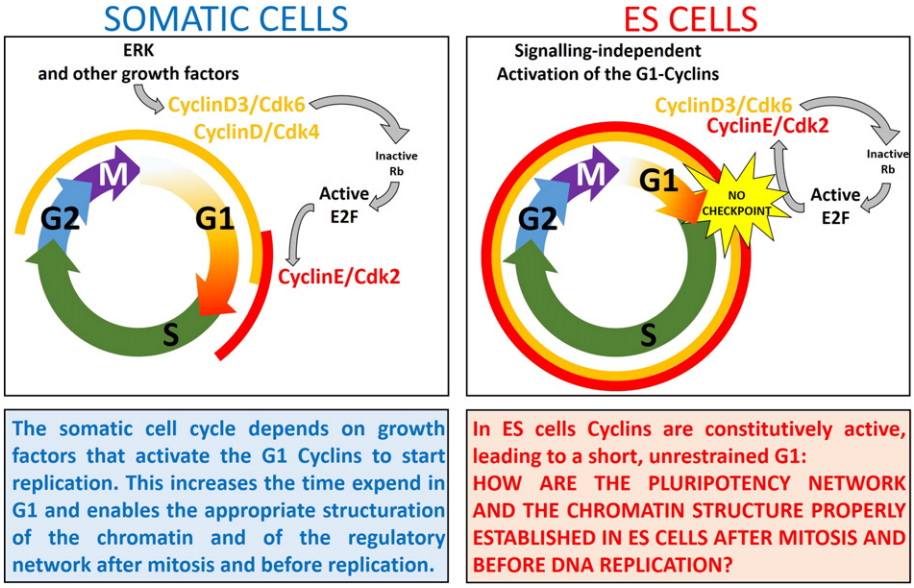

Fig. 2. The particular ES cell cycle suggests that epigenetic regulation should be of paramount importance. The figure depicts the structure of the cell cycle in somatic (left) and pluripotent (right) cells. The different phases are shown in line with the expression profile of the Cyclins controlling the G1/S transition (Cyclin D in yellow and E in red). Note the lack of growth factor stimuli in ES cells and the shortened G1 phase due to a lack of G1/S checkpoint. A minimal network explaining the underlying molecular basis of both cell cycle states is shown (see text for details).

the constitutive phosphorylation of retinoblastoma (Rb) [19] that cannot therefore sequester E2F as normally occurring during G1 in somatic cells. As a consequence, E2F activates its targets and leads to elevated levels of Cyclin E [23,24,26]. Together with the complete absence of expression of the Cdk inhibitiors p27Kip1 and p21Cip1, this leads to higher CyclinE/Cdk2 activity in ES than in differentiated cells [22–24]. The hyperactivity of CyclinD/Cdk6 and CyclinE/Cdk2 largely explains the unrestrained, ERK-independent transition from G1 to S phases in ES cells [28].

The unusual cell cycle structure of ES cells, particularly the presence of a shortened G1 phase, has profound consequences in terms of gene regulatory processes and seems to be mechanistically linked to the maintenance of pluripotency. Indeed, seminal work in embryonic carcinoma cells demonstrated that G1 represents a phase of the cell cycle in which pluripotent cells are more susceptible to respond to differentiation cues [29]. This observation was recently extended to human [30,31] and mouse [32] ES cells. Differentiation by withdrawal of Leukaemia Inhibitory Factor (LIF), a cytokine that strongly stimulates self-renewal, coincides with lengthening of G1 [32,33]. Moreover, the experimental extension of G1 by knockdown of combinations of Cyclins D and E increases ES cell propensity to differentiate [30,32]. More directly supportive of the existence of a connection between rapid G1 progression and pluripotency, the transient knockdown of all three forms of Cyclin D results in overt human ES cell differentiation [30]. Conversely, the upregulation of Cyclin E shields ES cells from differentiation [32]. Therefore, it is usually thought that the shortened G1 phase of ES cells reduces their window of opportunity to initiate differentiation. Although the molecular mechanisms underlying the facilitated differentiation in G1 are not yet fully elucidated, it has been proposed that both Cyclin E and D may be directly involved in the post-translational regulation of pluripotency regulators [30] or in the activation of differentiation-associated genes [30,34,35]. Therefore, it seems that there is a direct connection between key components of the cell cycle machinery and some regulators of pluripotency and differentiation. More generally, the lack of a proper G1 phase results in ES cells effectively existing in two alternating states of replication and mitosis. At the molecular level, both replication and mitosis have major consequences for gene regulatory processes, particularly regarding their long-term maintenance.

Major Regulatory Consequences of the Rapid Cell Cycle in ES Cells: In Need of Epigenetics?

The passage of the replication fork, a large multiprotein complex, at a speed of around 3 kb per minute [36], has tremendous consequences at the level of the structure of the chromatin, at least transiently. Indeed, around 10–15 nucleosomes are disrupted every minute during active replication. Therefore, a major challenge for the replicating cell is to reconstitute the appropriate chromatin environment just after DNA duplication such that TFs can re-engage specific interactions with selected regulatory regions (Fig. 1). Several chromatin proteins can be directly transferred from the parental to the newly synthesised DNA, including H3/H4 tetramers via the reloading possibly mediated by Mcm2, Asf1, or Fact [37]. Although this enables the maintenance of the local chromatin environment, it is also associated with a twofold dilution of nucleosome density. However, new nucleosomes are rapidly incorporated to the new chromatin fibres, particularly by Caf1, whose involvement in cell potency is starting to be revealed [38]. Subsequently, newly deposited nucleosomes are modified by several proteins that directly interact with components of the replication machinery. Notably, the systems that reproduce the modification landscape of the parental chromatin on the new chromatin fibres are particularly efficient at reproducing chromatin marks associated with gene repression: Proliferating cell nuclear antigen (PCNA), a replication processivity factor, interacts directly or indirectly with (i) Dnmt1/Uhrf1 complexes to reestablish CpG methylation on DNA [39], (ii) several Histone deacetylases (HDACS) to deacetylate newly incorporated histones [40], (iii) and G9a to trigger H3K9 methylation [41]; additionally, Caf1 recruits the H3K9 methyltransferase Setdb1 [42]. Moreover, the segregation of old histones between the daughter chromatin fibres also allows the reproduction of repressive histone marks by template-binding principles. In these mechanisms, the marks of a methylated nucleosome are reproduced on its neighbours, as shown for the Eed–Ezh2 tandem in Polycomb-mediated spreading of H3K27me3 and for HP1–Suv39H-mediated regulation of H3K9me3 maintenance [37]. In contrast, the reproduction of the active chromatin modifications after replication, if any, is less understood. Nevertheless, it has been proposed that p300, a key histone acetyltransferase enriched at active promoters and enhancers, interacts with proliferating cell nuclear antigen (PCNA) [43]. Furthermore, evidence is accumulating that suggests that more precise regulation is needed. For instance, since newly incorporated histones tend to be acetylated [44], a transient environment promoting inappropriate transcription may also be created. In addition, the deposition of new nucleosomes has been recently proven to compete for TF binding [45]. Similarly, the exact position of nucleosomes, in particular at and around gene bodies, requires active transcription to be appropriately reorganised following replication [46]. Therefore, since ES cells are essentially permanently replicating their genome during interphase, this suggests that efficient epigenetic mechanisms may have

evolved to instruct the rapid reconstitution of silent chromatin around differentiation-associated genes and the fast reassembly of active transcription complexes at the right regulatory elements.

Mitosis represents a second period during the cell cycle that is a major challenge to gene expression control. Here, molecular and structural changes alter the function of not only the chromatin, as in the case of replication, but more generally the whole nucleus [47]. Briefly, the phosphorylation cascades initiated by the CyclinB–Cdk1 complex lead to dramatic consequences: (1) the chromosomes condense by several orders of magnitude, losing their micro- (promoter–enhancer looping) and macromolecular organisation (topologically associated domains (TADs), lamina-associated domains (LADs), and chromosome territories); (2) the structure of the interphase nucleus is abolished (the nuclear envelope, the nucleoli, and other nuclear bodies, such as the speckles, are disassembled); and (3) a large number of gene regulators, including several members of the transcriptional machinery, ubiquitous and cell-type-specific TFs, and multiproteic complexes involved in chromatin organisation and structure are post-translationally modified, resulting in their targeting for proteosomal degradation or the interference with the ability to interact with their DNA or protein targets, leading to a general loss of TF binding. For instance, the pluripotency TF Oct4 is specifically phosphorylated by Aurora Kinase b during mitosis, leading to a severely impaired ability to bind DNA [48]. Another interesting example is provided by the SWI/SNF complex, a key regulator of chromatin dynamics, which is phosphorylated by Erk1 during mitosis, resulting in its disassembly and the degradation of several of its subunits, such as hBrm [49,50]. Therefore, essentially all aspects of gene regulation, from *cis–trans* interactions to the 3D organisation of the nucleus, are targeted by the mitotic machinery to induce the silencing of transcription and dismantling of several regulatory processes. (Fig. 1). Moreover, and in contrast to replication, these dramatic changes occur simultaneously over the entire genome. As such, mitosis must be perceived as a major obstacle for the perpetuation of cell identity [51]—how a functional nucleus is reconstituted early in G1, and how this is driven or enhanced by specific mechanisms operating in mitosis, remains a crucial question to be fully addressed.

Hence, the mitotic stability of chromatin modifications is a key aspect in determining their epigenetic role. DNA methylation does not seem to be specially challenged by mitosis, and most of the histone marks are, to some extent, maintained during mitosis, at least as established by imaging approaches [52]. However, whether these marks are enriched at the same promoters and enhancers as in interphase, and with similar profiles, has only been sparingly assessed [53–55]. Moreover, histone methylation marks invariably occur adjacent to threonine or serine residues that are phosphorylated during mitosis (H3T3K4, H3K9S10, and H3K27S28), and several reports have shown that this alters the interaction of specific proteins with H3K4, K9, or K27 methylation [7]. For example, H3T3 phosphorylation interferes with the interactions of TFIID, a key general TF, with H3K4me3; H3S10 phosphorylation alters HP1 binding to H3K9me3, influencing the stability of heterochromatin; H3S28 phosphorylation can displace Eed from H3K27me3 and, hence, interfere with Polycomb-mediated silencing. Therefore, even though methyl marks can be passed on during mitosis, the dephosphorylation occurring at the end of mitosis is also a key event ensuring that such marks are immediately functional. In addition, the modification of TFs and other gene regulatory proteins such as Brahma-related gene 1 (Brg1), together with the strong condensation of the chromatin fibre—significant enough to trigger DNA stress as measured by the generation of single-stranded DNA [56]—leads to a global disassociation of protein complexes from DNA [51]. How these factors are efficiently recruited to the right targets in G1 is far from understood. Nevetheless, it has been shown that several chromatin regulators such as Brd4 [57] or Mll1 [58], general TFs such as TBP [59], and tissue-specific TFs such as Gata1 [60] or the pluripotency factor Esrrb [18], among others, can interact with mitotic chromatin. This process, known as mitotic bookmarking, has been proposed to operate through two non-mutually exclusive modes of regulation: the retention of binding at a subset of the promoters and enhancers bound in interphase, ensuring a specific and almost immediate resumption of transcription of the target genes in G1, or a more global binding that may increase the local concentration of the factors nearby their target sites upon chromatin decondensation.

Overall, both replication and mitosis represent dramatic periods of the cell cycle during which most of the regulatory mechanisms of gene expression are challenged, particularly those regarding the key interactions established between *cis* and *trans* regulators of transcription. In this context, it is noteworthy that ES cells rely on the permanent activity of pluripotency TFs, for which deviation from steady-state expression levels leads to variable alterations of self-renewal [16]. While Oct4 and Sox2 expression needs to be maintained within strict limits to enable self-renewal [61,62], the individual perturbation of other pluripotency TFs such as Klf2, Klf4, Klf5, Esrrb, and Nanog is associated with poor self-renewal efficiency unless differentiation pathways are inhibited [17,63–66]. Hence, efficient mechanisms must be in place that ensure the expression and activity of pluripotency TFs is rapidly reinstated following replication and mitosis to avoid excessive spontaneous differentiation. Conversely, ectopic expression of several lineage-specific TFs leads to the loss of self-renewal and differentiation. This is the case, for instance, for Cdx2 [67] and Gata4 and 6 [68], which drive trophectoderm and endoderm

differentiation, respectively. Therefore, specific mechanisms may have evolved to rapidly reconstitute the refractory chromatin state associated with repression of these genes after replication and mitosis. Consequently, one would expect that the highly proliferative ES cells would strongly rely on the deposition of classical epigenetic marks. Intriguingly, as we will highlight subsequently, ES cells are largely insensitive to the inactivation of systems responsible for establishing key DNA and histone modifications, a particularity not shared by other pluripotent cell types such as epiblast stem cells [69–71].

Polycomb Group Proteins, Bivalent Chromatin and Control of Cell Differentiation Programs

Polycomb Group (PcG) proteins have been widely associated with gene repression in all metazoans so far analysed. They organise mainly into two classes of large multimeric polycomb repressive complexes (PRCs): PRC1 and PRC2. The general but not unique model for gene repression by PcG proteins is based on the deposition of H3K27me3 by PRC2. This methyl mark is then recognised either by PRC2 itself to modify neighbouring nucleosomes and allow spreadingor by PRC1 complexes to induce chromatin compaction and monoubiquitination of H2AK119, possibly inhibiting RNAPII elongation. In mammals, canonical PRC1 complexes include Chromobox (CBX) chromodomain proteins, responsible for the recognition of H3K27me3, Bmi1, and Mel18, with a role in DNA binding and chromatin compaction, the Sterile alpha motif (SAM) domain scaffold PH, and the catalytically active Ring1a or Ring1b subunits, responsible for ubiquitination of H2AK119. The PRC2 complex includes the SET domain lysine methyltransferases Ezh1 or Ezh2, responsible for H3K27 mono-, di-, or trimethylation; Suz12, Eed which can bind to H3K27me3; the histone binding proteins RbAp46 and RbAp48, and in ES cells the catalytically inactive histone demethylase Jarid2 [72]. Seminal experiments using *Drosophila* as a model system established the main function of PcG proteins as key regulators of development and as mediators of the inheritance of gene silencing. At the molecular level, PcG proteins have been shown to associate with replication forks, perhaps contributing to the maintenance of silent states. In addition, the Eed subunit of PRC2 can bind H3K27me3 at the recycled histone H3 post-replication, ensuring the trimethylation of newly incorporated histones [73]. It has been proposed that PRC2 increases its methylation activity during G1 in order to ensure a sufficient amount of recyclable H3K27me3 during replication [74]. Moreover, although there is contradictory data in the literature, several PcG proteins have been proposed to remain bound to mitotic chromatin [75,76], suggesting that they may directly participate into the mitotic transfer of regulatory information beyond the establishment of potentially mitotically stable H3K27me3 and H2AK119ub1.

From the considerations above, PcG proteins appear as ideal candidates to maintain gene silencing in self-renewing ES cells, particularly at developmental genes that must be kept in check. Pioneering studies showed that even though the vast majority of CpG-rich RNAPII promoters are associated with the

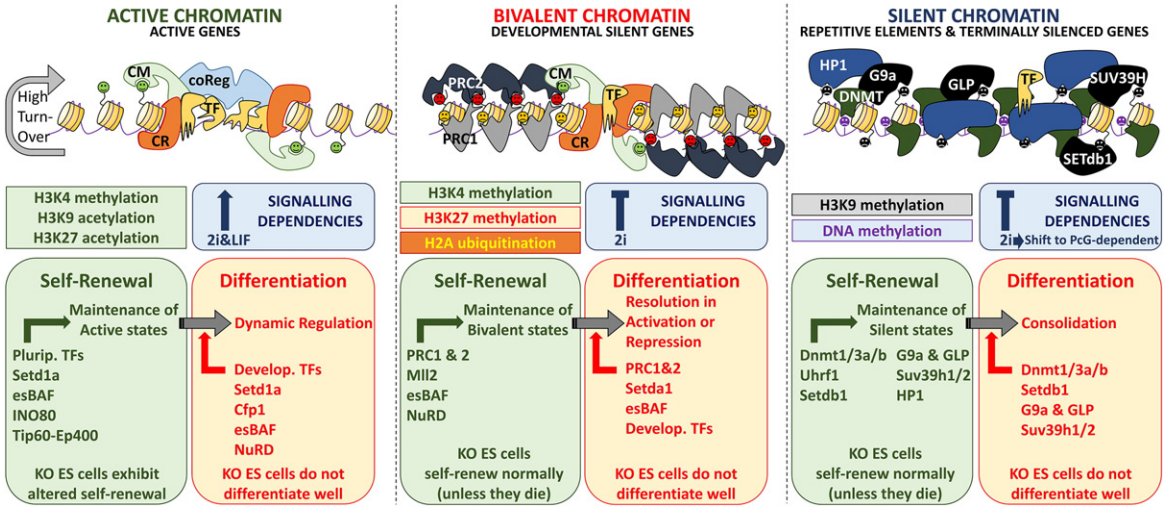

Fig. 3. The three main chromatin states of ES cells and their relationships with self-renewal and differentiation. The three most prominent chromatin states encountered in ES cells are schematised. Below each diagram, key features are outlined, such as characteristic chromatin modifications of each state, the influence of the signalling pathways generally associated with ES cells identity, and the chromatin regulators controlling their maintenance in self-renewing or differentiation conditions. The main phenotypical outcome of their invalidation is also depicted.

activating H3K4me3 mark in ES cells [77], around 20% are also targeted by PRC2 and are embedded within wide domains of H3K27me3 [78–80]. Of those, one-third are also occupied by PRC1, and a further third shows ubiquitination of H2AK119 [81,82]. The simultaneous presence of H3K4me3 and K27me3, a signature defined as "bivalent" (Fig. 3), has been observed in the Inner Cell Mass (ICM) of mouse blastocysts [83] and in several other cell types displaying developmental potential [77]. Therefore, it was proposed that bivalency allows the maintenance of gene silencing while preparing future activation processes. Accordingly, the presence of H3K4me3, but also of the p300 acetyltransferase and H3 acetylation, does not correlate with active transcription at bivalent genes. Instead, bivalent promoters are enriched for transcriptionally paused Ser5-phosphorylated RNAPII [84]. Ring1 activity seems to be responsible for restraining productive RNAPII elongation at these genes, both directly through the deposition of H2AK119ub1 [84] and by promoting chromatin condensation [82]. Despite the presence of Ser5-phosphorylated RNAPII at bivalent promoters, the general TF mediating such modification, TFIIH, is depleted. The phosphorylation of RNAPII on Ser5 is mediated by ERK, which is directly recruited at bivalent promoters [85]. This suggests a direct link between MEK/ERK signalling, a major driver of ES cell differentiation [21], and the preparation of developmental promoters to be rapidly activated via pause–release mechanisms. More generally, upon differentiation, bivalent domains typically resolve in monovalent H3K4me3 or H3K27me3 (Fig. 3) at genes that are activated or silenced, respectively, in a given cell type [77].

Given the bivalent configuration observed at the promoters of genes encoding developmental regulators, one should expect PcG mutants to exhibit drastic phenotypes in ES cells, particularly a strong spontaneous induction of differentiation. However, this is largely not the case, particularly for PRC2. For example, Ezh2 –/– ES cells can be generated in culture or derived from blastocysts [86]. Even though H3K27me3 is not completely abolished in these cells, because the low levels of Ezh1 expression partially compensate for the loss of Ezh2, this strongly argues against H3K27me3 providing an essential function. In fact, Ezh2 –/– ES cells remain undifferentiated and only show deregulation of a fraction of bivalent genes upon additional knockdown of Ezh1. Similar observations were made in Suz12 –/– lines derived from mutant blastocysts, which show marginal levels of H3K27me3 and display only a partial de-repression of differentiation-specific genes [87]. Finally, Eed –/– ES cells gradually lose all three forms of H3K27 methylation upon passaging [88,89], arguing in favour of a key role for Eed in the long-term maintenance of PRC2 activity. However, no overt differentiation is observed in Eed –/– cells, which only show reduced expression of pluripotency regulators and a concomitant upregulation of bivalent genes. Therefore, the genetic invalidation of three key constituents of the PRC2 complex indicates that its activity is not strictly required for maintaining the undifferentiated state (Fig. 3). In agreement, culture of wild-type ES cells in the presence of inhibitors of MEK/ERK and GSK3b (thereafter 2i), which leads to systematic self-renewal, is accompanied by a loss of H3K27me3 at many bivalent genes without inducing their upregulation [90], perhaps because, as mentioned above, ERK is also required to trigger RNAPII phosphorylation at these loci.

The phenotype of PRC1 inactivation in ES cells appears more severe. After 4 days of inducing Ring1b deletion evident cell death, morphological changes and upregulation of around 10% of Ring1b bound genes have been reported [91,92]. Furthermore, the inducible deletion of Ring1b in Ring1a –/– cells results in stronger phenotypes, with a relatively fast decrease of H2A119Ub1 at bivalent promoters accompanied by the concomitant loss of other PRC1 subunits, PRC2 and H3K27me3 [84,93]. Moreover, 8 days after inducing the loss of Ring1b, double Ring1a/b knockout cultures display clear morphological signs of differentiation, but Oct4 expression is nevertheless maintained in a significant fraction of the cells [93], as also observed 4 days after single Ring1b deletion [92]. This heterogeneous phenotype suggests that the loss of both Ring1 proteins is not fully compatible with ES cell self-renewal, at least in cultures containing functionally active ERK signalling. While no detailed characterisation is available of stable lines lacking expression of both Ring1a and Ring1b, these results indicate that Ring1 proteins display important functions in the control of self-renewal. However, whether this is exclusively mediated through the ubiquitination activity of Ring1 or through other mechanisms independent from chromatin regulation remains unclear.

Self-renewing ES cells lines lacking both Eed and Ring1b can be derived despite the near absence of H3K27me3 and H2A119Ub1, and the upregulation of a third of PRC1/2 targets [94]. Moreover, catalytically inactive Ring1b is capable of rescuing some of the defects observed in Ring1a/b double knockout ES cells [82]. More strikingly, enzymatically inactive Ring1b proteins are compatible with early embryogenesis [95,96], suggesting that the effects observed in double knockout of Ring1 proteins may be attributable to the role of these proteins in other complexes. In line with this observation, various components of the PRC1 and PRC2 complexes have been shown to be required for embryonic development in mouse, but only after implantation, when the first signs of epiblast differentiation appear. Mutations in Ring1b [97], Ezh2 [98], Eed [99,100], and Suz12 [87] all show defects arising post-implantation and are associated with altered gastrulation and differentiation. Similarly, all the

mutant ES cells described above with altered PRC1 and/or PRC2 activity display strong phenotypes only during cell differentiation. For instance, Eed –/– cells reintroduced into mouse blastocysts contribute to derivatives of all three germ layers until E9.5 but are then completely lost by E12.5 [88]. More dramatically, Eed –/–;Ring1b –/– ES cells cannot differentiate into all three germ layers *in-vitro*, as also shown for Ezh2 –/– [86] and Suz12 –/– [101] cells, and are unable to form teratomas or survive in the embryo after E10.5 [94].

Taken together, the current knowledge on PRC1 and PRC2 (Table 1 and Fig. 3) indicates that they are not required for the maintenance of pluripotent cell identity but become essential for the survival of differentiated cells and the maintenance of stable lineages by consolidating transcriptionally inactive states [102]. This view is further supported by the observation that chemically blocking key signalling activities associated with differentiation of ES cells (MEK/ERK and GSK3b) leads to limited or no transcriptional changes in Suz12 –/–, Ezh2 –/– or Ring1A/B double knockout compared to wild-type ES cells [102].

H3K9 Methylation, Beyond Silencing of "Non-Genic" Transcription

In mammals, H3K9 methylation is triggered by enzymes containing a SET domain: H3K9me2 is principally deposited by two related lysine methyltransferases, G9a and GLP, mostly working as a heterodimer [103,104]; H3K9me3 is instead established by Suv39h1 and Suv39h2 [105,106] or Setdb1 (also called ESET) [107]. The principal targets of H3K9me2/3 are non-unique elements of the genome, including transposable elements and pericentric major satellites of the centromeres. Therefore, H3K9 methylation represents a key hallmark of constitutive heterochromatin, which is crucial to silence "non-genic" transcription (Fig. 3) and ensure genomic stability [108]. More specifically, H3K9me3 is strongly enriched at chromocenters and 4',6-diamidino-2-phenylindole (DAPI) dense foci where several centromeres cluster together in a wide range of cell types, including ES cells. Conversely, H3K9me2 is widely diffuse in the nucleus, including at regions occupied by euchromatin [104,109]. The involvement of histone methyltransferases in the establishment of H3K9 methylation post-replication has been well described, and these marks have been shown to be associated with mitotic chromatin [110]. Hence, H3K9 methylation is likely to be involved in the epigenetic maintenance of heterochromatin (Fig. 3).

Pericentric chromatin serves as a scaffold for centromere assembly and is essential for the correct partition of the genetic information during mitosis. It is composed of a long (several Mb) tandem array of short repetitive sequences, called major satellite repeats. It juxtaposes to a central core of minor satellite repeats where the kinetochore assembles during mitosis. Interestingly, although the chromocenters are clearly visible in ES cells, they appear partially decondensed and form fewer and larger spots, a feature promptly reversed upon differentiation [111,112]. As in other cell types, Suv39h1/2 play a key role in regulating pericentric heterochromatin in ES cells: in double knockout ES cells, H3K9me3 is lost, resulting in the displacement of HP1, impaired recruitment of Dnmt3b, and ultimately reduced levels of CpG methylation [106]. Interestingly, the loss of H3K9me3 at these regions is accompanied by the deposition of H3K27me3, indicating that compensatory mechanisms by different repressive systems must be in place [109]. Similarly, although loss of CpG methylation by knocking out all three DNA methyltransferases is associated only with a reduction in H3K9me3 [113,114], accumulation of PRC1 and PRC2 components and H3K27me3 is detected at pericentric heterochromatin in mutant cells [113]. Also, when the cells are grown in 2i and Vitamin C, DNA methylation is almost completely erased and the chromocenters lose H3K9me3 in favour of H3K27me3 [115]. While this highlights the atypical plasticity of pericentric heterochromatin in ES cells, with a certain correlation to the efficiency of self-renewal and pluripotency, it also argues for the importance of maintaining a certain level of condensation at these regions. Indeed, Suv39h1/2 double knockout leads to elevated transcription from major satellite repeats [106,115] and profound alterations in chromosome segregation during mitosis [105], highlighting the decisive role of Suv39h1/2-mediated H3K9me3 at pericentric heterochromatin. However, additional mechanisms have been proposed, such as the direct activity of Pax transcription factors [116]: Pax3 is reported to bind and repress major satellites in ES cells by interacting with KAP1, a well-known repressor that recruits Setdb1 [117]. More recently, a key pluripotency TF, Nanog, was also shown to be recruited to pericentric heterochromatin to favour the relatively less compacted structure of these regions in ES cells [118]. Although the mechanistic details of these interactions are not yet fully elucidated, these observations suggest that in ES cells, canonical epigenetic structures like pericentric heterochromatin are under the control of TFs, in a manner that correlates with cell potency: differentiation-associated genes such as Pax3 promote the compaction and heterochromatinisation of the chromocenters and pluripotency-associated TFs such as Nanog ensure a relative relaxation.

Transposable elements make up 40% of the mouse genome and, based on their evolutionary origin, can be divided in different families. Among the most prevalent are endogenous retroviruses (ERVs; around 12% of the mouse genome), which are characterised by the presence of retroviral long terminal repeats (LTR) in

Table 1. Major phenotypes associated with the loss of function of regulators of heterochromatin in ES cells and during early embryogenesis

Factor	Role in development and ESC	Complexes
Polycomb repressive complex (PRC) family		
Ezh2	• Ezh2−/− embryos die around 7.5/8.5 dpc. Severely growth retarded, defects in gastrulation, accumulation of mesoderm in extra-embryonic posterior regions [98] • Ezh2−/− ES cells can be generated in culture or derived from mutant blastocysts. Self-renewal is unaffected, but there are defects in mesoderm/endoderm differentiation and impaired silencing of pluripotency genes [86].	PRC2
Ezh1	• Ezh1 KD in Ezh2−/− ES cells results in deregulation of developmental genes [86].	PRC2
Eed	• Eed−/− embryos die around 8.5 dpc. Defects in gastrulation, morphogenetic movements and reduction of embryonic mesoderm [99,100,243] • Eed−/− ESC are able to self-renew but de-repress bivalent developmental genes and show defective contribution to chimeras after 9.5 dpc [88,89].	PRC2
Suz12	• Suz12−/− embryos are growth retarded and arrest around 8.5 dpc. Defects in embryonic and extra-embryonic tissues, no neural ectorderm nor signs of organogenesis [87] • Suz12−/− ES cells are viable and self-renew but de-repress differentiation genes and show defects during neural differentiation in monolayer or Embryoid Bodies (EB) [101].	PRC2
Rinb1b	• Ring1b−/− embryos do not progress beyond 9.5 dpc. Growth retarded, defects during gastrulation, accumulation of mesoderm in the posterior, and defective anterior mesoderm formation [97] • Ring1b−/− ES cells are able to self-renew, de-repress developmental genes, and show impaired ability to differentiate in EBs [91–93,244].	PRC1
Ring1a	• Ring1a−/− mice are viable and fertil but show skeletal abnormalities [245]. • Ring1a−/− ES cells are able to self-renew, but additional acute deletion of Ring1b in these cells results in significant upregulation of bivalent genes and widespread differentiation after 8 days [84,93].	PRC1
H3K9 methyltransferases		
GLP	• GLP−/− embryos are growth retarded and arrest around 9.5 dpc [104]. • GLP−/− ES cells are able to self-renew [104].	GLP/G9a
G9A	• G9a−/− embryos are growth retarded and arrest around 9.5 dpc [103]. • G9a−/− ES cells are able to self-renew, but proliferation and survival of differentiated cells are affected [103]. G9a/GLP dKO cells can also be derived and show de-repression of some bivalent genes and Class III ERVs [115,120,121,129].	GLP/G9a
Setdb1 (ESET)	• Setdb1−/− embryos do not survive past 7.5 dpc due to the almost complete absence of epiblast at 5.5 dpc, but blastocysts can be recovered [130]. • Setdb1−/− blastocyst outgrowth fails to generate ES lines. Acute deletion of Setdb1 results in upregulation of around 600 coding genes (including germline and trophectoderm transcripts), Class I and II ERVs (incuding IAPs), and progressive proliferation defects, eventually resulting in impaired viability and trophectoderm differentiation [115,121–123,131].	
Suv39h1, Suv39h2	• Suv39h1−/− or Suv39h2−/− mice are viable and fertile. Double knockout mice are growth retarded, show increased tumour incidence and signs of genetic instability [105]. • Suv39h1/2 dKO ES cells are able to self-renew, show loss of H3K9me3 and gain of H3K27me3 at pericentric heterochromatin. Satellite repeats, around 500 coding genes, LINE, and few class I and II ERVs are deregulated [106,109,116].	
DNA methyltransferases		
Dnmt1	• Dnmt1−/− embryos are growth retarded; at 10.5 dpc, they just start forming organ rudiments and show 10–20 somites. Embryos die before 12.5 dpc [136]. • Dnmt1−/− ES cells are able to self-renew efficiently, but deletion is lethal in differentiated cells [136,138].	
Dnmt3a, Dnmt3b	• Dnmt3a−/− and Dnmt3b−/− dKO embryos are growth retarded and arrest before 11.5 dpc. Moreover, 9.5-dpc embryos lack somites and fail to undergo turning [137]. • Dnmt3a−/− and Dnmt3b−/− dKO ES cells are able to self-renew but show demethylation of some ERVs and major satellite repeats [137]. Similarly, Dnmt3a−/−, Dnmt3b−/−, and Dnmt1−/− tKO cells can be derived and only show mild de-repression of some classes of repetitive elements [114].	
Tet1, Tet2, Tet3	• Tet1−/−, Tet2−/−, and Tet3−/− tKO ES cells are able to self-renew but show impaired differentiation potential in EB and teratomas. In chimaeric embryos, contribution is almost absent at 9.5 dpc, and in tetraploid complementation assays, no embryo proper is formed [147]. •Tet1−/−, Tet2−/−, and Tet3−/− tKO embryos show primitive streak patterning defects, impaired maturation of axial mesoderm, and failed specification of paraxial mesoderm [148].	

intact elements and of long interspersed elements (LINEs) or short interspersed elements (SINEs; 20% and 8% of the genome, respectively). ERVs can be further split into three classes based on structure and viral origin. Most repetitive elements in the mouse genome have undergone significant levels of sequence degeneration and are inactive, but a fraction (such as young LINEs and the Class-II ERVs Intracisternal A particle (IAPs)) retain the ability to drive the expression of all elements required for efficient transposition. Since transposition has the potential to disrupt overall genomic organisation and alter gene regulation or function, the ability to ensure the silencing of transposable elements is

crucial to maintain genome stability [119]. Different H3K9 methyltransferases collaborate in ensuring the proper repression of these elements. G9a and GLP depose H3K9me2 at Class III murine endogenous retrovirus-L (MERVLs) and directly repress their expression [115,120]. Suv39h1/2 target H3K9me3 at 10–15% of both LTR and non-LTR transposons, but in Suv39h double knockout ES lines, few Class I and II ERVs change expression, and mainly LINE elements are upregulated [116]. Setdb1, by associating with KAP1, is directed by KRAB-Zinc finger proteins to a range of Class I and II ERVs and is required for the deposition of H3K9me3 and H4K20me3 at these elements [121,122], particularly IAP elements, which are pronouncedly upregulated in Setdb1 or KAP1 knockout ES cells [115,121,123]. Mimicking the situation of pericentric heterochromatin, the reprogramming associated with a combination of 2i and Vitamin C leads to an enrichment of H3K27me3 at several repetitive elements [115]. Therefore, ES cells seem to have the ability to use different chromatin repressive pathways to keep non-genic transcription in check and ensure the stability of their genome (Fig. 3). Interestingly, an additional feature of genome stability of major importance for ES cells, the preservation of long telomeres, is also subject to particular regulations in ES cells. The lengthening of ES cell telomeres occurs during transient bursts of activity that take place asynchronously in virtually all cells after long periods of culture. This transient stage is driven by Zscan4 [124], a zinc-finger protein that directly stimulates telomere lengthening and expression of meiotic genes that, in turn, contribute to telomere length regulation and chromosome stability [125]. Interestingly, these Zscan4 events coincide with the upregulation of mERV-L endogenous retrotransposons [126,127] and the reorganisation of pericentric heterochromatin [128], leading to a transient stage that displays several hallmarks of the embryonic two-cell stage, including enhanced contribution of Zscan4-positive cells to extra-embryonic tissues after injection into early embryos [126]. Interestingly, knockdown of Caf1, a major factor to maintain heterochromatin throughout replication [37], leads to the acquisition by ES cells of several properties of the two-cell stage [38]. Therefore, several aspects of heterochromatin regulation in ES cells, including both pericentric heterochromatin and retrotransposon silencing, seem to be connected and correlated with cell plasticity, developmental potency and genome stability.

In agreement with the ability of ES cells to use multiple mechanisms to repress non-genic transcription, G9a [103], GLP [104], G9a/GLP [115,121,129], and Suv39h1/2 single [105] or double [106] knockout ES cells can be maintained in culture. In contrast, Setdb1 blastocyst outgrowths fail to generate ES cell lines [130], and acute deletion or knockdown of Setdb1 in established ES cells leads to progressive proliferation defects [121], eventually resulting in impaired viability and differentiation [122,131]. Interestingly, the failure to derive or maintain Setdb1-deficient ES cells may be linked to its direct control of genes belonging to the trophectoderm lineage. Although GLP, G9a, and Suv39h1/2 have been shown to regulate several hundred genes [116,129], the loss of Setdb1 is associated with major gene expression consequences, including deregulation of imprinted genes, germ cell markers, and a number of key regulators of trophectoderm development such as Cdx2 [122,131]. Moreover, Setdb1 knockdown blastomeres incorporate preferentially into the trophectoderm when aggregated into four-cell stage embryos [131]. Highlighting a direct connection with pluripotency regulators, Oct4—a key factor in repressing trophectoderm differentiation of ES cells [61]—is required to recruit Setdb1 at trophectoderm genes [131]. Of note, however, the depletion of Oct4 leads to very efficient differentiation towards the trophectoderm lineage and is compatible with the derivation of trophectoderm-like stem cells [67]. In contrast, the consequences of Setdb1 depletion are less specific and more progressive, highlighting the dominant role of Oct4 over that of Setdb1 in inhibiting trophectoderm differentiation [131]. Nevertheless, among H3K9 methyltransferases, Setdb1 seems the most closely connected to the maintenance of pluripotency, since only its deletion is characterised by an early developmental phenotype. Setdb1 mutants do not survive after E7.5, and an epiblast is almost absent in implanted E5.5 embryos, but mutant blastocysts can be recovered [130]. In contrast, although they are growth retarded and show increased spontaneous tumour incidence and signs of genetic instability, Suv39h1/2 double knockout animals are viable [105]. G9a or Glp mutants also show a phenotype only after implantation: embryos are delayed and die only after E9.5 [103,104].

Therefore, although superficially only Setdb1 seems to play specific roles in pluripotent cells, as strongly suggested by the phenotype of the knockout for this gene (Table 1), additional work will be required to completely understand whether H3K9 methylation is directly involved in preserving pluripotency during replication and mitosis and whether this function is mediated by shaping global heterochromatin structures or targeting a set of specific genes.

Non-essential DNA Methylation and Hydroxymethylation in ES Cells

Being a direct, heritable modification of the DNA, CpG methylation 5-methylcytosine (5mC) is the prototypical example of an epigenetic modification. It has been widely linked to gene repression and shown to play a mechanistic role in heterochromatin formation, control of transposable elements, X chromosome inactivation, and allelic imprinting [132]. In general,

5mC is a prominent feature of most of the genome except at transcriptionally active promoters containing a CpG island or at other active regulatory elements such as enhancers [133]. The pivotal role played by DNA methylation in ensuring stable gene silencing and heritability of transcriptional identity itself majorly contributed to the identification and characterisation of this epigenetic modification. Seminal studies demonstrated how the treatment of non-myogenic cell lines with the cytosine analogue 5-aza-2′deoxycytidine, later shown to inhibit DNA methyltransferases, results in the spontaneous formation of contractile myotubules [134]. More recently, 5-aza-2′deoxycytidine has been shown to greatly enhance the rate of somatic cell reprogramming [135]. Inactivation of the maintenance Dnmt1 or double knockout of de novo (Dnmt3a and b) DNA methyltransferases leads to severely growth-retarded embryos and is lethal before mid-gestation [136,137]. The importance of DNA methylation for the viability of differentiated cells was further confirmed by inducible deletion of Dnmt1 in mouse embryonic fibroblasts, which results in marked proliferation defects, radical deregulation of gene expression, and overt lethality after 6 days of culture [138].

In stark contrast to the dependence of differentiated cells on DNA methylation, this modification seems to be entirely dispensable in ES cells (Table 1). Inactivation of Dnmt1, Dnmt3a and b, and the compound knockout of all three DNA methyltransferases, leading to the complete loss of CpG methylation, show virtually no phenotype in undifferentiated ES cells [114,136,137]. In fact, displaying a very low level of DNA methylation seems to be a hallmark of pluripotency: growing ES cells in 2i conditions that strongly enhance self-renewal results in the progressive genome-wide reduction of CpG methylation, triggered by the downregulation of Dnmt3a/b/L, enhanced hydroxylation of methylcytosines at specific locations, and global impairment of the DNA methylation maintenance machinery Dnmt1/Uhrf1 [139–143]. 5mC persists at satellite repeats, imprinted genes, and some classes of transposable elements, such as IAPs, in 2i-treated ES cells, but even at these loci, DNA methylation does not play an essential role, as indicated by its nearly complete erasure upon culture in 2i and Vitamin C [115]. In these conditions, and as observed for triple Dnmt1;3a;3b knockout cells, the loss of DNA methylation does not entail immediate detrimental consequences, most likely because, as suggested before, alternative repressive mechanisms are implemented at repetitive elements [113,115].

DNA methylation is counterbalanced by the hydroxylation of 5mC to 5hmC. Hydroxylation has been proposed to be an intermediary of DNA demethylation [144] but also to impair the repressive functions of 5mC, disrupting its interaction with key repressors such as MeCP2 [145], MBD1, or other MBD domain-containing proteins [146]. ES cells grown in the absence of 2i have been shown to display relatively high levels of 5hmC, with increased enrichment across active promoters and enhancers [140], suggesting that 5hmC could be important to preserve the transcriptional identity of ES cells. However, the inactivation of all three 5-methylcytosine dioxygenases Tet1/2/3, which are crucial for the survival of differentiated cells, does not impact on the self-renewal ability of undifferentiated ES cells [147]. In fact, the triple knockout of Tet enzymes has little consequences during pre-implantation development, with only few genes showing deregulated expression at the blastocyst stage [147,148]. In contrast, triple knockout embryos fail to properly gastrulate due to alterations of the Lefty-Nodal signalling axis [148]. Hence, hydroxymethylation is crucial only after the evanescence of pluripotency, when differentiation is established. In agreement, Tet triple knockout ES cells fail to contribute to development in chimeric embryos [147]. In conclusion, neither 5mC nor 5hmC seem to play important epigenetic roles in sustaining ES cell identity (Table 1).

Global Chromatin Accessibility, rather than Epigenetic Silencing, Is at the Foundation of the Maintenance of Pluripotency

The analysis of epiblast stem cells, a pluripotent cell type derived from post-implantation embryos [69,70], has revealed their strict dependence on several epigenetic pathways that play minor roles in ES cells (Table 1). This is demonstrated, for example, in the inactivation of Suz12, Eed, or Dnmt1 [71]. Crucially, this highlights the exquisite specificity of the large independence of ES cells from mechanisms mediating epigenetic repression and reinforces our view that ES cells represent an "*epigenetic paradox*". Indeed, even though both pluripotent cell types need silent genes to be rapidly reactivated, only ES cells exhibit a large independence from canonical epigenetic repression pathways [71]. Strikingly, concomitant inactivation of combinations of repressive systems is compatible with ES cell self-renewal. For instance, triple knockout ES cells harbouring mutations in Suv39h1, Suv39h2, and Eed, showing impaired H3K27 and H3K9 trimethylation, can be derived [115]. In addition, culture of ES cells in 2i medium supplemented with vitamin C results in almost complete erasure of DNA methylation and reduction in H3K9me2 levels, including at imprinted genes [115]. The fact that Suv39h1/2 double knockout, Eed knockout, and G9a/GLP double knockout ES cells can be maintained in these conditions further demonstrates that even after altering simultaneously several epigenetic arms (CpG methylation, and H3K9 and H3K27 methylation), the undifferentiated identity of ES cells remains intact.

These observations strongly indicate that the control exerted by TFs over gene expression is dominant in ES

Table 2. Major phenotypes associated with loss of function of regulators of chromatin remodelling and H3K4 methylation in ES cells and during early embryogenesis

Factor	Role in development and ESC	Complexes
Mixed lineage leukaemia (MLL) family		
SET1A (SET domain-containing protein 1A)	• SET1A –/– embryos fail to pass the epiblast stage and arrest around 7.5 dpc [170]. • Required for ES cell self-renewal, pluripotency, and proliferation [170]	COMPASS ATPase subunits
SET1B (SET domain-containing protein 1B)	• SET1B –/– embryos arrest around 11.5 dpc [170].	COMPASS ATPase subunits
MLL1 (KMT2A)	• MLL1 –/– embryos arrest after 10.5 dpc [171].	COMPASS-like ATPase subunits
MLL2 (KMT2B)	• MLL2 –/– embryos results in severe developmental retardation and lethality at ~10.5 dpc [168,169]. • MLL2 –/– ES cells are viable and retain pluripotency, but they display cell proliferation defects due to an enhanced rate of apoptosis [175].	COMPASS-like ATPase subunits
MLL3 (KMT2C)	• MLL3 –/– embryos die perinatally [172].	COMPASS-like ATPase subunits
MLL4 (KMT2D)	• MLL4 –/– embryos arrest around 9.5 dpc [172].	COMPASS-like ATPase subunits
Cfp1 (CxxC finger protein 1)	• Cfp1 –/– embryos die around implantation [181]. Cfp1 is required for ESC differentiation [246].	COMPASS complex
Ash2 (Absent, small, or homeotic discs 2)	• Ash2 –/– embryos arrest before 8.5 dpc [180]. • Required for ES cell self-renewal and pluripotency [179]	COMPASS and COMPASS-like complexes
DPY30 (Dumpy-30)	• DPY30 –/– embryos arrest after 8.5 dpc [247].	COMPASS and COMPASS-like complexes
Chromatin remodelling factors		
Brg1 (Smarca4)	• Brg1 –/– embryos die around implantation [164]. Role in zygotic gene activation after fertilisation [187] • Required for ES cell self-renewal and pluripotency [193,191]	SWI/SNF complex ATPase subunits
BRM (Smarca2) ARID1	• Normal embryogenesis [248]	SWI/SNF complex ATPase subunits
BAF250A (Arid1a)	• BAF250A –/– embryos arrest around E6.5 and fail to gastrulate without the formation of mesoderm [189]. • Required for maintenance of ESC pluripotency [189] • Regulates nucleosome occupancy during ESC differentiation [197]	SWI/SNF complex
BAF250B (Arid1b)	• Required for proliferation and maintenance of ESC pluripotency [192]	SWI/SNF complex
BAF155 (Smarcc1)	• BAF155 –/– embryos die during the implantation stage [162]. • Required for ESC differentiation [249]	SWI/SNF complex Scaffolding proteins BAF60

(continued on next page)

Table 2 (*continued*)

Factor	Role in development and ESC	Complexes
hSNF5/BAF47/INI1 (Smarcb1)	• hSNF5 –/– embryos die between 3.5 and 5.5 dpc [165,188]. • Required for ESC differentiation [249]	SWI/SNF complex
BRD7	• Activation and repression of target genes in ES cells [250]	SWI/SNF complex
BAF57 (Smarce1)	• Required for ESC differentiation [249]	SWI/SNF complex
CHD1	• CHD1 –/– embryos show proliferation defects and lethality after implantation [251,252]. • Essential for ES cell self-renewal [203]	CHD family ATPase subunits
CHD2	• CHD2 –/– embryos exhibit growth delays late in embryogenesis and perinatal lethality [253]. • CHD2 and CHD1 influence chromatin accessibility and histone H3.3 deposition at active chromatin regions [254].	CHD family ATPase subunits
CHD3/Mi2-α	• NuRD functions to regulate ESC differentiation [210] andlineage specification [209].	CHD family NURD complex ATPase subunits
Mbd3	• NuRD subunit MBD3 is required for ESCs to differentiate [205]. • Required to avoid ESC differentiation to trophectoderm [255]	CHD family NURD complex
CHD4/Mi-2β	• CHD4 is required to restrict the expression of lineage-specific genes [256] and has been associated with maintaining nucleosome density at bivalent promoters [196].	CHD family NURD complex ATPase subunits and Independently of NURD complex
TIP60 (Kat5), ING3, BRD8, MRG15 (Morf40l1), MRGBP, P400 (Ep400), TRRAP, Eaf6 (Meaf6), YL1 (Vps72), GAS41 (Yeats4), DMAP1, RVB1 (Ruvbl1), RVB2 (Ruvbl2), β-actin (Actb), and ARP4	• Embryos lacking Tip60 and Trrap die before implantation [211,212]. • The Tip60–p400 complex is required for ESC self-renewal, pluripotency, and differentiation [214]. Tip60–p400 deposits H3.3 and H2A.Z on promoters and enhancers during gene activation [215].	The INO80 family TIP60/p400 complex
INO80, ARP4 (Actl6a), ARP5 (Angptl6), ARP8 (Actr8), IES2 (Ino80b), IES6 (Ino80c), RVB1 (Ruvbl1), RVB2 (Ruvbl2), YY1, Amida (Tfpt), FLJ20309 (Ino80d), MCRS1, UCH37 (Uchl5), NFRKB, and FLJ90652 (Ino80e)	• Required for ESC self-renewal and blastocyst formation [217]	The INO80 family INO80 complex (INO80 removes the H2A.Z from coding region nucleosomes)
SNF2H (Smarca5)	• Snf2h –/– embryos die around implantation stage due to proliferation defects [201].	ISWI family Complex: ACF, CHRAC, WICH
Bptf	• Bptf protein (NURF complex) is required during post-implantation embryonic development. Bptf –/– embryos die around E6.5 [257]. • Bptf is required for ESC differentiation [257].	ISWI family Complex: NURF, CERF

cells, as illustrated by the fact that forced expression of pluripotency TFs such as Nanog and Esrrb leads to LIF-independent self-renewal even in the absence of 2i [64,149]. This implies that chromatin accessibility, in relation with TF binding, may play a preponderant role (Fig. 3). In agreement, it has been proposed that ES cells display a plastic and lax chromatin organisation, characterised by transcriptional permissiveness and abundance of activating histone modifications [112,150,151]. In addition to the loose state of centromeres, the coalescence of silent heterochromatin into spatially confined blocks of condensed material in proximity of the nuclear envelope, characteristic of differentiated cells, is not observed in ES cells [151–154], reminiscent of what is observed in the ICM of pre-implantation embryos [152]. Accordingly, blocks of repressive histone modifications accumulate during differentiation genome-wide. In particular, H3K9me2 absolute levels pronouncedly increase upon loss of pluripotency *in vivo* and *in vitro* [155], and large domains of enrichment appear in differentiated cell types, contributing to the establishment of tissue-specific regions of silent chromatin [156]. The existence of a generally decondensed chromatin state is also indicated by the contraction of the volume occupied by chromosome territories upon differentiation. Moreover, particular loci such as those of pluripotency genes can be located on extended chromatin loops, far away from the respective territories, specifically in pluripotent cells [157]. As a consequence of their chromatin configuration, a number of structural chromatin proteins, including core and linker histones, Lamin b, and the heterochromatic protein HP1, display a hyperdynamic behaviour in ES cells, as shown by FRAP and salt extraction experiments [112,158,159]. Possibly explaining the structural properties of the chromatin of ES cells, H3 and H4 acetylation, and H3K4me3, histone modifications associated with gene activity, show higher levels in ES compared to differentiated cells [112,150,151]. These structural changes result in overall higher levels of transcription in pluripotent cells, evidenced by the widespread detection of low levels of transcripts originating from genic and intergenic regions, including repetitive elements [151]. Altogether, these observations suggest that a relatively unrestricted accessibility of promoters and regulatory elements may enable pluripotency TFs to rapidly reestablish the appropriate regulatory architecture required to maintain ES cell identity after replication and mitosis. Furthermore, being strictly dependent on TFs may enable ES cells to rapidly change cell fate in response to developmental cues.

Given the major role played by pluripotency TFs in ES cells and the peculiar properties of ES cell chromatin, ATP-dependent chromatin-remodelling complexes that hydrolyse ATP to restructure, mobilise, or eject nucleosomes [160,161], together with histone modifiers associated with transcriptional activity, such as those deposing H3K4me3 or H3K27ac, may be of critical importance in controlling when and where these TFs act (Fig. 3). In agreement with this view, and in stark contrast to what is observed for repressive epigenetic pathways, several chromatin remodelling factors and chromatin modifiers establishing active marks appear to be more important in ES than in differentiated cells. Indeed, several remodellers and regulators of euchromatin can be depleted in primary embryonic fibroblasts without affecting survival or proliferation [162–165]. In contrast, embryos deficient in the function of several chromatin remodelling complexes and euchromatin regulators show embryonic lethality around the blastocyst stage (Table 2). ES cells cannot be established from these mutants, indicating that chromatin remodellers are required for either the establishment or maintenance of ES cells (Table 2). This strongly indicates that, as reviewed in the following sections, chromatin remodelling factors, together with complexes responsible for the deposition of active histone marks, play important roles in the maintenance of ES cell identity (Fig. 3). This ultimately suggests that ES cells may rely on a memory of gene activation rather than repression.

MLL Family and H3K4 Methylation, Marking Promoters for Activity

Several studies in diverse biological systems have pointed to H3K4 methylation as a key mark contributing to an epigenetic memory of gene activity [73]. Consistently, imaging approaches showed that H3K4 methylation is enriched at gene-dense regions of human metaphasic chromosomes [52]. More recently, it was shown that mitotic human ES cells and Hela cells display an H3K4 methylation profile highly similar to that of interphase cells, indicating a nearly full mitotic preservation of this mark [54,55]. Combinations of H3K4 methylation mark regulatory elements in different states: if H3K4me1 and me2 are associated with all regulatory regions and mark competence for activity rather than transcription per se, H3K4me3 is restricted to transcriptionally active promoters. In mammals, H3K4 methylation is established by six H3K4 methyltransferases (Mll1, Mll2, Mll3, Mll4, Setd1a, and Setd1b), which belong to the mixed lineage leukaemia (MLL) family bearing homology with yeast Set1 and *Drosophila* Trx. They form six multisubunit COMPASS and COMPASS-related complexes, sharing a core subcomplex composed of Wdr5 (WD repeat domain 5), Rbpb5 (retinoblastoma binding protein 5), absent, small, or homeotic discs 2-like (Ash2l), and Dpy30 factors [166].

The presence of several H3K4 methyltransferases in mammals might be related to the temporal regulation of their activity during development. Mll2 controls the deposition of most of H3K4me3 during oogenesis and early cleavage stages following fertilisation [167]. In agreement, Mll2 is expressed early during development, even though its loss of

function results in severe developmental delay only after E7.5 and lethality at E10.5 [168,169]. Using an oocyte driver to induce its deletion, it was shown that Mll2 is strictly required for oogenesis, with its deletion leading to complete infertility [167]. The activity of Setd1a/b, the central H3K4 trimethylases, is also temporally controlled during development: while Setd1a is required immediately before gastrulation, Setd1b is required during organogenesis [170]. Finally, the other Mll proteins, Mll1, Mll3, and Mll4 are required at later developmental stages [171–173]. Therefore, from a superficial perspective, it may appear that Mll proteins are not generally required during pre-implantation development and, hence, may play minor roles in the control of pluripotency (Table 2). However, careful examination of Setd1a –/– embryos indicates that the pluripotent compartment of implanted embryos is completely exhausted [170], strongly indicating that Setd1a is specifically required to maintain pluripotent cells. Moreover, the effects of the loss of Mll2 during blastocyst formation have not been directly studied, due to the defects of Mll2 knockout oocytes. In addition, before implantation, Setd1a/b, Mll2, and other Mll proteins could display compensatory roles. Despite these uncertainties, a picture has emerged in which Mll2 is the major H3K4 methyltransferase during early cleavage stages until the formation of the blastocyst when, around implantation, H3K4 methylation becomes dependent on Setd1a in the epiblast.

This simple model is also supported by the role of Mll2, Setd1a, and Setd1b in mouse ES cells. Mll2 binds to most H3K4me3-rich promoters in ES cells, including those of bivalent genes [174], but its depletion leads to the loss of H3K4me3 exclusively at bivalent promoters. Thus, while Mll2 may be the key factor defining the set of H3K4me3-enriched promoters in ES cells, these results show how active and bivalent promoters use different mechanisms to maintain their trimethyl H3K4 status. However, it was also shown that Mll2 loss has no effect on the upregulation of bivalent genes upon differentiation [173,174], indicating that bivalency in ES cells may not be functional. In fact, Mll2 –/– ES cells are viable, self-renew, and retain pluripotency and differentiation capacity while only displaying increased apoptosis [175]. In contrast, the inducible knockout of Setd1a (but not of Setd1b) leads to a drastic depletion of bulk H3K4me3 in ES cells [170], leading to major gene expression consequences affecting in particular genes controlled by Oct4 [176]. Accordingly, Setd1a –/– ES cells cannot be derived from mutant blastocysts. However, inducible Setd1a –/– ES cells do not directly differentiate despite expressing several lineage-specific markers [176]. Rather, they experience an abrupt proliferative arrest in G1 accompanied by dramatic apoptosis [170]. Overall, Setd1a may be a key component sustaining the permanent activity of the pluripotency network that drives self-renewal but might also be required to execute the early differentiation program in ES cells and in the epiblast (Table 2).

As seen above, a major difficulty in the study of Mll complexes is the fact that the dependence of the cells on these enzymatic activities is developmentally regulated. Therefore, to address the global role of H3K4 methylation, several studies have focused on the analysis of common partners of all Mll complexes such as Ash2l and Wdr5 (Table 2). For instance, Wdr5, a key mediator of the transition from di- to trimethylation [177] maintains H3K4me3 levels at pluripotency-associated gene promoter and bivalent promoters in ESCs. Wdr5 depletion results in the reduction of expression of pluripotency factors such as Oct4 and Nanog, leading to reduced self-renewal efficiency and increased spontaneous differentiation. In line with the preferential alteration of the Oct4-centred network in Setd1a –/– ES cells [176], Wdr5 interacts and cooperates with Oct4 to activate transcription [178]. Moreover, the knockdown of another key core component of Mll complexes, Ash2l, results in a global decrease in H3K4 methylation and increased H3K9me3 levels. This is likely to be the consequence of downregulation of the H3K9me3 demethylase Kdm4c. Consequently, the chromatin structure of Ash2l knockdown cells switches from an open to a more closed conformation. These changes are accompanied by loss of self-renewal associated with decreased expression of pluripotency factors such as Nanog, Oct4, and Esrrb, together with enhanced expression of differentiation genes [179]. These results are consistent with an essential role of Ash2l in early embryogenesis, as Ash2l-null embryos die before implantation [180]. Similarly, it has been shown that embryos lacking the Set1a subunit CXXC finger protein 1 (Cfp1) die around the peri-implantation stage [181]. In contrast to the results described for Setd1a, Cfp1 –/– ES cells can be derived. Cfp1 –/– ES cells show a reduction of H3K4 methylation at a subset of CpG island promoters, particularly downstream of transcription start sites, although minor alterations of gene expression are detected [182]. Surprisingly, Cfp1 –/– cells are somehow blocked in an undifferentiated state, and their differentiation potential seems severely compromised. In conclusion, Cfp1 seemingly plays a different role than other components of the Mll complexes.

esBAF, an ES-Specific SWI/SNF Complex Orchestrating the Activity of the Gene Regulatory Network

SWI/SNF complexes (also called BRG1/BRM associated factors (BAF) complexes) are composed of more than 20 proteins that can repress or activate gene expression [183,184]. Two alternative ATPases, Brg1

(Brahma-related gene 1) and Brm (Brahma), can be found in the two main core complexes, together with additional and variable subunits such as BAF170, BAF47, BAF155, or BAF250. Even though both ATPase subunits exhibit intrinsic chromatin remodelling activity *in vitro*, full activity is achieved only when the subunits Baf170/Baf155 and hSnf5/Baf47 are provided [185]. Specific components confer specialised activities and selectively target distinct BAF complexes to specific genes. For example, complexes containing Brg1 or Brm have different functions during early development: only the loss of Brg1 results in embryonic lethality around implantation (Table 2), with mutant blastocysts being unable to expand as *in vitro* outgrowths [164,186]. In addition, invalidation of Brg1 specifically in the oocyte results in developmental arrest at the two-cell stage, possibly highlighting a functional interaction between Brg1 and H3K4 methylation deposited by Mll2 in the context of zygotic gene activation [187]. Furthermore, embryos mutated for other proteins associated with BAF complexes display severe embryonic phenotypes: Baf47 –/– embryos develop successfully to the blastocyst stage but die shortly after implantation [165,188]; deficiency in Baf155 expression results in early embryonic lethality around implantation due to defects in the formation of the ICM and primitive endoderm [162]; complete loss of Baf250a causes developmental arrest around E6.5, with embryos failing to undergo gastrulation [189]. Overall, SWI/SNF complexes have been proven to be essential for the correct progression of early embryogenesis, during the time window in which pluripotency manisfests (Table 2). In contrast to Mll complexes, however, the function of BAF complexes seems less specific to the pluripotent compartment, as extra-embryonic lineages are also affected by their inactivation. Nevertheless, Brg1 is not necessary for the survival or proliferation of fibroblasts or glial cells, suggesting that it is not a general cell viability factor [187,190].

The exact mechanisms by which BAF complexes support early development remain incompletely understood. ES cells possess a specialised SWI/SNF complex, esBAF, critical for their maintenance [191]. As described, different components of esBAF (Brg1, Baf155, Baf47, Baf250a, and Baf250b) have been implicated in early embryogenesis. Moreover, the composition of the BAF complexes is dynamically regulated upon induction of ES cell differentiation; particularly, undifferentiated ES cells display high expression levels of Brg1, Baf155, and Baf250 that are attenuated during differentiation, when Brm replaces Brg1 [192]. These observations, coupled to the phenotype observed in knockout models, suggest that esBAF is required for ES cell self-renewal and differentiation by regulating both pluripotency factors and developmental genes (Fig. 3). Indeed, genome-wide profiling of Brg1 recruitment [193,194] demonstrated that it binds in the vicinity of gene promoters of the core ES transcriptional circuitry. Moreover, it colocalises extensively with the pluripotency transcription factors Oct4, Sox2, and Nanog [193,194], leading to the proposal that esBAF complexes represent integral components of the core gene regulatory network sustaining pluripotency. In agreement, both knockdown and inducible deletion of Brg1 lead to the downregulation of pluripotency genes and the upregulation of differentiation-associated genes [191,193]. The loss of Brg1 leads to a complete loss of self-renewal in clonogenicity assays [191], with most colonies showing clear morphological signs of differentiation and lacking expression of Oct4, Sox2, or Nanog [191]. Providing further support to these observations, mutations in BAF-associated proteins (Baf250a/b) also result in the downregulation of pluripotency genes and the upregulation of differentiation genes, leading to impaired self-renewal [189,192].

However, careful analysis of Brg1 knockdown and inducible knockout lines suggests further complexity. Loss of pluripotency gene expression was observed only after several passages in regular cultures or after several days in clonal assays [191,193]. Therefore, the attenuation of the pluripotency network observed in the absence of Brg1 seems rather a progressive consequence of the induction of differentiation genes than a direct effect. Additional analysis of the inducible Brg1 –/– ES cells suggested that esBAF may sustain pluripotency by controlling chromatin accessibility in two molecularly opposed but functionally complementary manners (Fig. 3). On the one hand, esBAF maintains open and refractory to PcG-mediated repression key regulatory regions that are directly targeted by the LIF signalling pathway; on the other hand, esBAF enables PcG-mediated repression of differentiation genes. First, it was observed that Brg1 colocalises with Stat3 genome-wide [194]. Then, by analysing the gene expression changes occurring before the complete collapse of the pluripotency network, both in the context of the inducible deletion of Brg1 and after the withdrawal of LIF, it was shown that esBAF and LIF/Stat3 co-regulate a large number of genes [195]. Strikingly, key TFs regulating self-renewal such as Tbx3, Tfcp2l1, Esrrb, Socs3, and Tcl1 require both LIF and Brg1 to be expressed at levels sufficiently high to enable self-renewal. This strongly suggests that, at least at these targets, the recruitment of Stat3 and Brg1 is mutually dependent, although Stat3 binding appears to rely on Brg1 more than the opposite [195]. In agreement with this, Brg1 has been shown to bind the two nucleosomes flanking relatively long nucleosome-free promoter regions in ES cells [196], most likely enabling the binding of Stat3 and perhaps of additional pluripotency TFs. In the absence of Brg1, however, PcG proteins seem to gain control over these key self-renewal genes leading to H3K27me3 accumulation [195]. Interestingly, and somehow surprisingly, at repressed and bivalent genes where Brg1 binds in the context of a

narrow nucleosome-free promoter region [196], the loss of Brg1 or of Baf250a is accompanied by a reduction of Suz12 recruitment and decreased H3K27me3 levels [195,197].

In conclusion, the requirements of ES cells regarding the esBAF complex seem to rely on complex molecular mechanisms in which Brg1 controls chromatin accessibility to promote either the binding of pluripotency TFs, particularly Stat3, or the binding of PRC2 complexes at repressed genes (Fig. 3). How esBAF is initially recruited at these locations remains unclear, although an exciting possibility is the use of a preexisting histone modification, such as acetylation [198]. The presence of such acetylated histones could result from the inherent propensity of replication to incorporate acetylated H3 and H4. However, when MEK/ERK and GSK3b are chemically inhibited, self-renewal can be preserved in the absence of LIF [20], and H3K27me3 is erased from the vast majority of bivalent regions [85,90,115]. Studying the role of Brg1 and nucleosome positioning in these conditions may provide further insights.

Other Chromatin Remodellers Potentially Important to Preserve ES Cell Identity

Brg1 is the chromatin remodeller that has been more deeply studied in the context of pluripotency. However, a large number of chromatin remodellers exist in mammals [199,200]. While their function has not yet been analysed in detail in ES cells, some of these complexes could prove to play an important role in ES cells (Fig. 3 and Table 2). For instance, Snf2h, one of the two highly homologous ATPases of the ISWI family (such as CHRAC, CERF, ACF, WICH, and NURF) is required for early embryogenesis: mice mutant for Snf2h have proliferation defects and die during the pre-implantation stage, and Snf2h –/– blastocyst outgrowths fail to give rise to ES cells [201]. Other notable chromatin remodellers belong to the chromodomain helicase DNA-binding (CHD) family, characterised by the presence of a tandem chromodomain at the N terminal of the ATPase domain, which functions as an interaction surface for a variety of chromatin components [202]. Among its several members, some have been studied in ES cells. Whereas Chd1 associates with promoters of active genes [203], Chd7 is mainly bound at enhancers [204], together with p300, Oct4, Sox2, and Nanog, and fine-tunes expression levels of genes that are specifically expressed in mouse ES cells. However, neither Chd1- nor Chd7-deficient ES cells exhibit major alterations in self-renewal efficiency. Chd3 and Chd4 comprise the catalytic subunits of the nucleosome remodelling and deacetylation (NuRD) complex, which has been involved in the regulation of ES cell self-renewal [205,206] and has a function in early embryogenesis [207]. Even though NuRD is composed of several proteins, most of the studies have focused on Mbd3, in the absence of which the complex disassembles [205]. Mbd3 –/– embryos die between implantation and E8.5 [207] because the pluripotent epiblast is developmentally arrested at a stage resembling the pre-implantation ICM [206]. Interestingly, mutant ES cells can be derived only in the presence of 2i inhibitors [206,208], further highlighting the inter-relationship existing between signalling and chromatin remodelling activities in the context of pluripotency (Fig. 3). Moreover, the deletion of Mbd3 in ES cells is viable, demonstrating that NuRD is not required to maintain the undifferentiated state but, rather, for effective differentiation [205]. At the molecular level, NuRD activity serves to both attenuate the expression of pluripotency genes in self-renewal conditions and reinforce their downregulation as cells differentiate [209]. Since Mbd3 –/– ES cells self-renew in the absence of LIF [205], it was proposed that NuRD counterbalances the activity of the LIF/Stat3 pathway [209]. Notably, at NuRD target promoters, the loss of Mbd3 is associated with a shift from H3K27me3 to H3K27ac, suggesting that NuRD deacetylates H3 to facilitate PRC2 activity [210].

Additional chromatin remodellers, such as the Tip60–E1A-associated p400 protein (Ep400) complex, might have a function in ES cells. Tip60–Ep400 comprises around 16 subunits, including Ep400, the Tip60 histone acetyltransferase (HAT), the phosphatidylinositol 3- kinase family homologue Trrap, and Brd8 [200]. Embryos lacking Tip60 and Trrap die before implantation [211,212]. Mutant blastocysts outgrowths also display high levels of cell death [212,213]. Moreover, Trrap –/– ES cells cannot be established by gene targeting [212]. Accordingly, upon individual knockdown of seven members of the Tip60–Ep400 complex in ES cells, a similar phenotype is observed: ES cell colonies flattened out, grow as monolayers, reduce their proliferation rate, and display low levels of alkaline–phosphatase staining, a marker of undifferentiated cells [214]. Hence, the Tip60–Ep400 complex is important for maintaining ES cells. The major functions of Tip60–Ep400 include H2AZ/H3.3 incorporation and histone acetylation [215], therefore contributing to gene activation [216]. Different studies demonstrated that Ep400 occupies mainly the –1 nucleosome at active promoters in ES cells and may be involved in the recruitment of other TFs, including the RNAPII machinery [196,214]. Accordingly, the knockdown of Ep400 results in a reduction of RNAPII levels at these promoters [196]. However, most of the genes responding to a loss of function of Ep400 appear to be differentiation-associated genes that overlap considerably with the targets of Nanog [214]. Therefore, in addition to its more canonical function as an activator of transcription in ES cells, Tip60–Ep400 may also be acting as a repressor.

Finally, the INO80 complex has been shown to be required for ES cell self-renewal and blastocyst development. INO80 is expressed at higher levels in ES cells compared to other cell types and is downregulated during differentiation. Knockdown of INO80 results in decreased expression of key pluripotency factors, including Oct4, Nanog, Sox2, Klf4, and Esrrb, as well as increased expression of lineage markers. Ino80 and pluripotency TFs co-occupy the promoters of pluripotency genes that tend to be downregulated during differentiation, and the binding of INO80 at these positions is dependent on Oct4 and the H3K4 methyltransferase complex component Wdr5. It has been suggested that INO80 is required for the maintenance of nucleosome-depleted regions and open chromatin structure at pluripotency promoters, possibly inducing expression by facilitating the recruitment of the transcriptional machinery [217].

Concluding Remarks and Perspectives: A Memory of TF Binding at the Heart of Pluripotency?

As every cell, undifferentiated ES cells need to express only a specific subset of their genes. However, and in sharp contrast to most of the other cell types, ES cells maintain the ability to activate the genic configurations associated with different somatic identities. Classical epigenetic pathways of gene repression may not be suited to rapidly respond to developmental cues. Indeed, repression mediated by H3K27me3, H3K9me3, or 5mC, once established, is transmitted across many cell divisions after the removal of the instructive signals [218]—in this sense, these mechanisms can be considered strictly epigenetic (Fig. 1). This stability is based on the very robust mechanisms through which the cells maintain and reestablish repressive marks after replication [73]. In ES cells, however, and as reviewed here, chromatin repression mechanisms seem more important to establish new cellular identities during differentiation than to preserve the undifferentiated state (Table 1). Accordingly, the current data suggest that signalling-dependent deposition of repressive marks is involved in lineage priming, as shown for H3K27me3 [219]. In ES cells, therefore, chromatin repression marks are reconfigured in response to changes in the signalling environment, and such dependence is likely to be a determinant component enabling ES cells to exit self-renewal (Fig. 3). The contribution and function of the individual signalling pathways altered in 2i need to be carefully analysed.

The key question of why in ES cells the chromatin marks associated with gene repression do not strictly show epigenetic properties now needs attention. Are the systems responsible for the maintenance of such marks, particularly during replication, operational? Maintenance mechanisms may be functional, but the expression of key enzymes in these complexes may be directly controlled by MEK/ERK, GSK3b, or other signalling pathways. For instance, the nearly complete erasure of 5mC observed in 2i conditions is associated with the downregulation of Dnmt3a/b/L, locus-specific hydroxylation, and global impairment of the DNA methylation maintenance machinery. Moreover, several other triggers of repressive marks, particularly of H3K9 methylation, are also downregulated in 2i [220]. An alternative, but not mutually exclusive, possibility is that during the fast ES cell cycle, repressive marks do not have sufficient time to be established or consolidated, especially due to a short G1 (Fig. 2). Understanding when, during differentiation, chromatin repressive marks become genuinely epigenetic and signalling-independent should be a key aspect of future research. It is possible that this important transition takes place around the time of commitment and so ensures an irreversible exit from the undifferentiated state with the establishment of the first epigenetic marks preserving lineage identity.

If repressive mechanisms based on chromatin modifications are not strictly required to maintain differentiation genes in a silent state, how is their expression kept in check? The answer to this question remains elusive. However, strong lines of evidence indicate that pluripotency TFs themselves play a central role. Oct4 is directly involved in the inhibition of trophectoderm and Nanog of (primitive) endoderm differentiation. Other factors such as Esrrb, Klf4, Prdm14, and Tfcp2l1, which are rapidly silenced at implantation [221,222], may also be directly involved in the repression of genes required for the specification of the three germ layers. But are TFs themselves sufficient to recreate the architecture of the pluripotency gene regulatory network after each replication and mitosis? This may well be the case, given that a handful of pluripotency TFs have been shown to be capable of reprogramming the somatic epigenome to pluripotency [223]. In this regard, the notion that certain pluripotency TFs, such as Oct4, have been shown to act as “pioneer” factors may be instrumental [4]. This leads to the radical hypothesis that the long-term preservation of pluripotency could result not from the maintenance of gene expression signatures but simply from a permanent recreation of the regulatory architecture associated with pluripotency after each cycle of DNA replication and mitosis [224]. However, the acquisition of pluripotency in reprogramming experiments remains a long and inefficient process, and a parallel with the regular constraints posed by replication and mitosis in self-renewing conditions is, perhaps, not appropriate. We propose here that

the long-term preservation of pluripotency relies on alternative mechanisms of inheritance, not based on classical epigenetic repression but on the memory of gene activation or, more precisely, the memory of TF binding. In line with this, and as discussed, ES cells are particularly sensitive to the invalidation of several systems associated with gene activity and required for TF binding such as Setd1a, esBAF, Ino80, and Tip60–Ep400 (Table 2). However, it is essential to highlight that it remains unknown whether the alterations observed upon the inactivation of these complexes are rescued in 2i conditions. This major caveat needs to be addressed should we want to rigorously conclude that ES cells show a structural dependence on chromatin activation mechanisms.

The central question is, to our understanding, to elucidate how pluripotency TF binding is rapidly reinstated after replication and cell division (Fig. 1), such that the expression of factors required to maintain self-renewal is kept relatively constant. Therefore, it is important to deepen our knowledge of how pluripotency TFs functionally and biochemically interact with key activities known to facilitate their binding to chromatin, particularly during these two phases of the cell cycle. It is known that replicated strands do not contain underrepresented histone modifications as compared to parental chromatin, suggesting that nucleosomes with active histone marks are efficiently recycled during replication [225]. This permanence of active marks at promoters and enhancers may be of critical importance to redirect TF binding just after replication. Moreover, several chromatin remodellers required for the maintenance of ES cell identity, such as INO80 and Brg1, have been involved in DNA replication [226,227]. This may provide direct means, in collaboration with inherited histone marks, to locally reorganise the nucleosomal array at regulatory regions after the passage of the replication fork. In ES cells, this process must be tightly controlled to ensure that the general permissiveness of the replicated chromatin is not associated with spurious transcription of differentiation genes. Alternatively, mechanisms specific to ES cells may be in place to enable the rapid scanning of the daughter chromatin fibres by pluripotency TFs. For instance, it may be highly relevant that, at least in human ES cells, the origins of replication are marked by the binding of pluripotency TFs [228]. This would constitute an elegant and simple means to provide a nucleation centre for pluripotency TF binding in the vicinity of replicated chromatin, from which scanning could occur in search of newly replicated targets. In ES cells, where many more replication origins than needed are established [229], this mechanism could greatly accelerate the dynamics of binding post-replication. Prompted by the observation that some TFs such as Nanog interact with proteins involved in DNA replication [230] such as Rfc4—a replication elongation factor—or Smc3—a Cohesin component involved in sister chromatid cohesion, it could be interesting to investigate whether pluripotency TF can directly travel with the replication machinery or interact locally with it upon the passage of the fork across their binding sites. Moreover, the fact that Cohesin, involved in both replication and mitosis, binds with clustered TFs at active enhancers, including those determinant in ES cells [231], may provide a simple means to redirect TFs binding after replication and cell division [232].

In addition to replication, mitosis has long been recognised as a major obstacle for the continuity of TF-mediated control [233]. Given that ES cells divide frequently, and a short G1 divides mitosis from replication, it seems critically important that in these cells, TFs promptly localise their targets on the decondesing chromatin. Similar to replication, several mechanisms have been proposed to canalise TF binding post-mitosis, including the maintenance of histone marks associated with gene activity [7], the preservation of a locally accessible chromatin environment [234], and the presence of Cohesin at key regulatory regions [232]. All these mechanisms are yet to be thoroughly explored in ES cells. Strikingly, some of the components of esBAF have been previously shown to be targeted by ERK during mitosis in somatic cells, leading to the disruption of BAF complexes [50]. Whether this occurs to esBAF and/or other remodelling complexes in ES cells is a very relevant question. It could be hypothesised that in self-renewing conditions, one or few key chromatin remodellers remain active during mitosis, particularly in 2i conditions, to allow the daughter cells to inherit a transcriptionally competent chromatin fibre. While these would still remain a relatively indirect means to enhance TF binding post-mitosis, other mechanisms have been proposed to preserve the stability of regulatory architectures throughout cell division, most notably mitotic bookmarking by TFs. While several TFs are excluded from chromatin during mitosis, others have the ability to resist condensation and maintain binding at specific loci on mitotic chromatin [51], as initially proposed nearly 2 decades ago [233]. More recently, mitotic bookmarking has been shown to be an attribute shared by a number of TFs, operating in different cell types [51,60,235–237]. In this light, binding of pluripotency TFs would not need to be reestablished after each cell division: one or more bookmarking factors would continuously keep key regulatory elements in a state that would directly assist the rapid nucleation of binding of other components of the network in early G1. In turn, this would elicit an accelerated resumption of transcription at bookmarked genes, as shown using artificial transcriptional arrays controlled by a canonical bookmarking factor, Brd4 [238], and at endogenous genes bookmarked by Gata1 and FoxA1 [60,236]. Supporting this possibility,

Esrrb, an orphan oestrogen receptor, which expression tightly controls the self-renewal efficiency of ES cells [64,239], has been recently shown to behave as a canonical bookmarking factor [18]. Further investigation is now urgently required to establish if this ability is shared by other pluripotency regulators, as suggested by imaging approaches [240]. Also, the role of Esrrb (and other potential bookmarking factors in ES cells) in maintaining an accessible chromatin configuration at bookmarked positions during mitosis, and how this might facilitate the binding of other TFs after division, needs to be fully explored. Finally, if bookmarking pluripotency TFs plays a central role in maintaining ES cell identity, the events accompanying early differentiation must be characterised in detail to assess the molecular consequences of the loss of bookmarking at key regulatory elements. In fact, the regulatory challenges posed by cell division make mitosis an ideal window of opportunity to change cell identity. In agreement, mitotic cells have been demonstrated to be more easily reprogrammed upon nuclear transfer into amphibian oocytes [241]. Moreover, mitotic debookmarking has recently been identified as an early key event during TF-mediated reprogramming [242]. More generally, it will also be important to address whether the acquisition of pluripotency *in vivo* and *in vitro* only culminates to full reprogramming when the pluripotency network becomes mitotically self-sustainable through mitotic bookmarking by pluripotency TFs. Despite the standing gaps in our knowledge of the molecular consequences of Esrrb bookmarking, the discovery of this process provides a new framework for conceptualising how pluripotency TF can exert a dominant role over other systems of memory of gene regulation in ES cells.

ES cells represent a relatively unique context in mammals that is not even shared by other pluripotent cell types [71]: they maintain their identity for virtually infinite cell divisions without relying on canonical epigenetic systems of gene repression. This represents a conceptual challenge to our understanding of gene regulation that is defined here as the "*epigenetic paradox of ES cells*". In addition to reviewing the limited dependence on chromatin repression mechanisms in the light of the particular ES cell cycle structure and their ability to rapidly respond to signalling cues and embark on differentiation, we have also described several key properties of the ES cell chromatin that may explain how pluripotency TFs perform a major role in preserving the identity of these cells. We believe that a full understanding of the mechanisms enabling ES cell self-renewal will only be gained by analysing the behaviour of pluripotency TFs in the context of replication and mitosis (Fig. 1). We hope that the hypotheses presented here will inspire new research aimed at addressing these questions in a direct manner, such as the discovery of mitotic bookmarking by Esrrb, which is a firm first step towards understanding the role of TFs as carriers of regulatory information across ES cell generations.

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Abbreviations used:
TF, transcription factor; ES, embryonic stem; PcG, Polycomb Group; PRC, polycomb repressive complex; ERV, endogenous retrovirus; LTR, long terminal repeat; LINE, long interspersed elements; Brg1, Brahma-related gene 1; MLL, mixed lineage leukaemia; Ash2, absent, small, or homeotic discs 2; Cfp1, CXXC finger protein 1; Brm, Brahma; CHD, chromodomain helicase DNA-binding; NuRD, nucleosome remodelling and deacetylation; Ep400, E1A-associated p400 protein; EB, Embryoid Bodies; ERK, Extracellular signal-regulated kinase-2; Rb, retinoblastoma; LIF, Leukaemia Inhibitory Factor; ICM, Inner Cell Mass; IAP, Intracisternal A particle; BAF, BRG1/BRM associated factors.

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