



**HAL**  
open science

## The Epigenetic Paradox of Pluripotent ES Cells

Nicola Festuccia, Inma González, Pablo Navarro Gil

► **To cite this version:**

Nicola Festuccia, Inma González, Pablo Navarro Gil. The Epigenetic Paradox of Pluripotent ES Cells. Journal of Molecular Biology, 2017, 429 (10), pp.1476-1503. 10.1016/j.jmb.2016.12.009 . pasteur-02024847

**HAL Id: pasteur-02024847**

**<https://pasteur.hal.science/pasteur-02024847>**

Submitted on 19 Feb 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License



# The Epigenetic Paradox of Pluripotent ES Cells

Nicola Festuccia<sup>†</sup>, Inma Gonzalez<sup>†</sup> 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](mailto:pnavarro@pasteur.fr)  
<http://dx.doi.org/10.1016/j.jmb.2016.12.009>

**Edited by David Laurent**

## 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.

© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 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

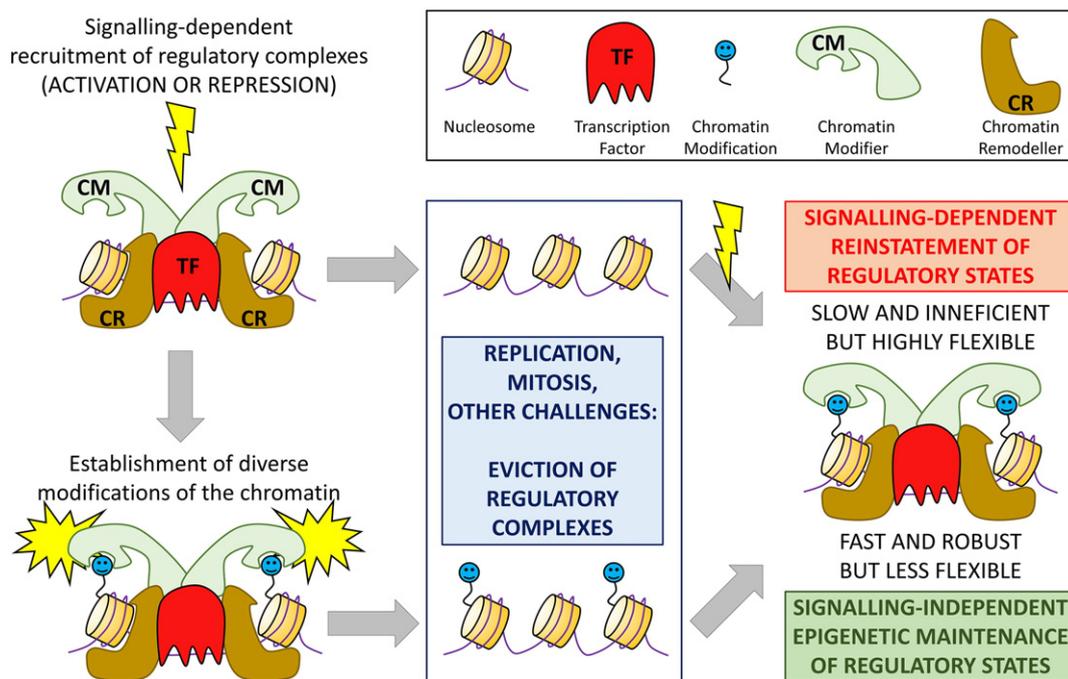
genes to drive and/or enhance their transcriptional activity. In eukaryotes, transcriptional activation is constrained and regulated by the physical accessibility of regulatory elements, which may or may not be permissive to TF binding. While a handful of master TFs, named pioneer TFs [4], are capable 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 hydroxy-methylation 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 remodellers 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



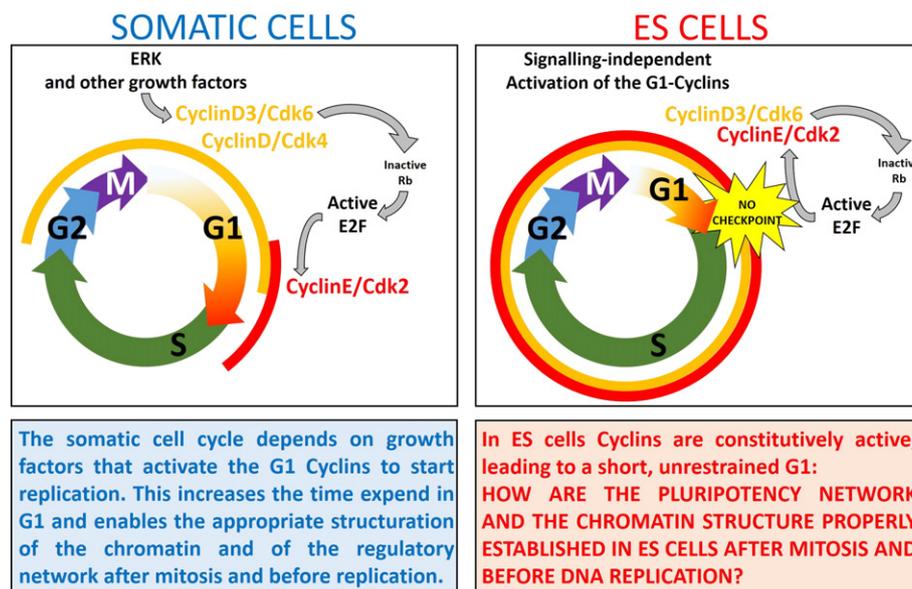
**Fig. 1.** Facing replication and mitosis is the role of epigenetic memory of chromatin states. Gene regulatory processes are often initiated by a signal that culminates in the establishment of a functional complex at a particular genomic location. This process involves TFs that bind the DNA at specific 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, A Hallmark 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 inhibitors 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 multi-protein 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. Nevertheless, 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 trophoblast and endoderm

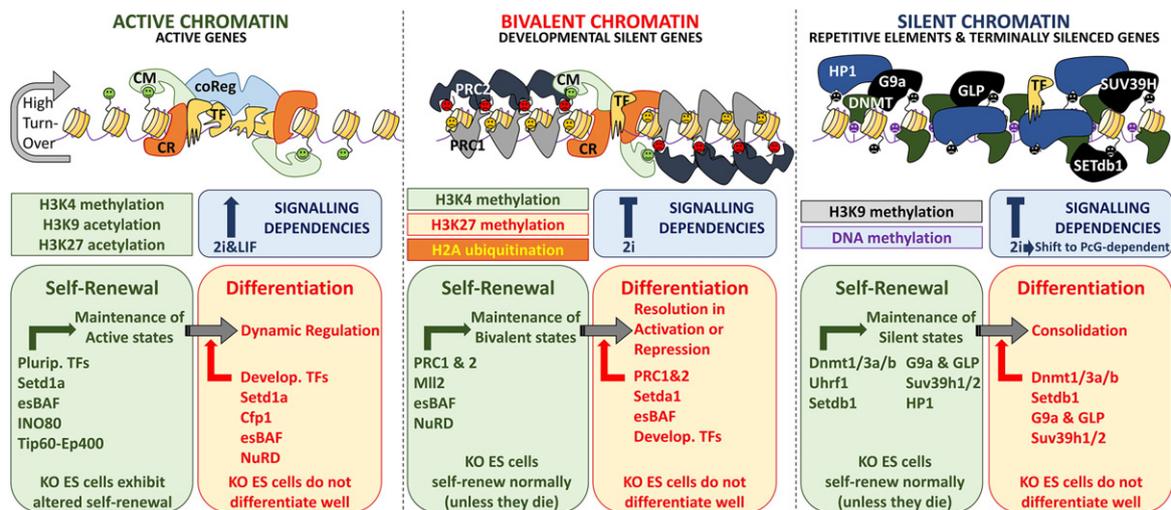
differentiation, respectively. Therefore, specific mechanisms may have evolved to rapidly reconstitute the refractory chromatin state associated with repression of these genes after replication and mitosis. Consequently, one would expect that the highly proliferative ES cells would strongly rely on the 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 spreading or 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 inactivation 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*<sup>−/−</sup> 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*<sup>−/−</sup> 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*<sup>−/−</sup> 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*<sup>−/−</sup> 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*<sup>−/−</sup> 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*<sup>−/−</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup>;*Ring1b*<sup>-/-</sup> ES cells cannot differentiate into all three germ layers *in-vitro*, as also shown for *Ezh2*<sup>-/-</sup> [86] and *Suz12*<sup>-/-</sup> [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*<sup>-/-</sup>, *Ezh2*<sup>-/-</sup> 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
<i>Polycomb repressive complex (PRC) family</i>		
Ezh2	<ul style="list-style-type: none"> <li>• Ezh2<sup>-/-</sup> embryos die around 7.5/8.5 dpc. Severely growth retarded, defects in gastrulation, accumulation of mesoderm in extra-embryonic posterior regions [98]</li> <li>• Ezh2<sup>-/-</sup> 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].</li> </ul>	PRC2
Ezh1	<ul style="list-style-type: none"> <li>• Ezh1 KD in Ezh2<sup>-/-</sup> ES cells results in deregulation of developmental genes [86].</li> </ul>	PRC2
Eed	<ul style="list-style-type: none"> <li>• Eed<sup>-/-</sup> embryos die around 8.5 dpc. Defects in gastrulation, morphogenetic movements and reduction of embryonic mesoderm [99,100,243]</li> <li>• Eed<sup>-/-</sup> ESC are able to self-renew but de-repress bivalent developmental genes and show defective contribution to chimeras after 9.5 dpc [88,89].</li> </ul>	PRC2
Suz12	<ul style="list-style-type: none"> <li>• Suz12<sup>-/-</sup> embryos are growth retarded and arrest around 8.5 dpc. Defects in embryonic and extra-embryonic tissues, no neural ectoderm nor signs of organogenesis [87]</li> <li>• Suz12<sup>-/-</sup> 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].</li> </ul>	PRC2
Rinb1b	<ul style="list-style-type: none"> <li>• Ring1b<sup>-/-</sup> 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]</li> <li>• Ring1b<sup>-/-</sup> ES cells are able to self-renew, de-repress developmental genes, and show impaired ability to differentiate in EBs [91–93,244].</li> </ul>	PRC1
Ring1a	<ul style="list-style-type: none"> <li>• Ring1a<sup>-/-</sup> mice are viable and fertile but show skeletal abnormalities [245].</li> <li>• Ring1a<sup>-/-</sup> 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].</li> </ul>	PRC1
<i>H3K9 methyltransferases</i>		
GLP	<ul style="list-style-type: none"> <li>• GLP<sup>-/-</sup> embryos are growth retarded and arrest around 9.5 dpc [104].</li> <li>• GLP<sup>-/-</sup> ES cells are able to self-renew [104].</li> </ul>	GLP/G9a
G9A	<ul style="list-style-type: none"> <li>• G9a<sup>-/-</sup> embryos are growth retarded and arrest around 9.5 dpc [103].</li> <li>• G9a<sup>-/-</sup> 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].</li> </ul>	GLP/G9a
Setdb1 (ESET)	<ul style="list-style-type: none"> <li>• Setdb1<sup>-/-</sup> 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].</li> <li>• Setdb1<sup>-/-</sup> 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 (including IAPs), and progressive proliferation defects, eventually resulting in impaired viability and trophectoderm differentiation [115,121–123,131].</li> </ul>	
Suv39h1, Suv39h2	<ul style="list-style-type: none"> <li>• Suv39h1<sup>-/-</sup> or Suv39h2<sup>-/-</sup> mice are viable and fertile. Double knockout mice are growth retarded, show increased tumour incidence and signs of genetic instability [105].</li> <li>• 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].</li> </ul>	
<i>DNA methyltransferases</i>		
Dnmt1	<ul style="list-style-type: none"> <li>• Dnmt1<sup>-/-</sup> 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].</li> <li>• Dnmt1<sup>-/-</sup> ES cells are able to self-renew efficiently, but deletion is lethal in differentiated cells [136,138].</li> </ul>	
Dnmt3a, Dnmt3b	<ul style="list-style-type: none"> <li>• Dnmt3a<sup>-/-</sup> and Dnmt3b<sup>-/-</sup> dKO embryos are growth retarded and arrest before 11.5 dpc. Moreover, 9.5-dpc embryos lack somites and fail to undergo turning [137].</li> <li>• Dnmt3a<sup>-/-</sup> and Dnmt3b<sup>-/-</sup> dKO ES cells are able to self-renew but show demethylation of some ERVs and major satellite repeats [137]. Similarly, Dnmt3a<sup>-/-</sup>, Dnmt3b<sup>-/-</sup>, and Dnmt1<sup>-/-</sup> tKO cells can be derived and only show mild de-repression of some classes of repetitive elements [114].</li> </ul>	
Tet1, Tet2, Tet3	<ul style="list-style-type: none"> <li>• Tet1<sup>-/-</sup>, Tet2<sup>-/-</sup>, and Tet3<sup>-/-</sup> 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].</li> <li>• Tet1<sup>-/-</sup>, Tet2<sup>-/-</sup>, and Tet3<sup>-/-</sup> tKO embryos show primitive streak patterning defects, impaired maturation of axial mesoderm, and failed specification of paraxial mesoderm [148].</li> </ul>	

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 deposit H3K9me2 at Class III murine endogenous retrovirus-L (MERVs) 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 prolifera-

tion 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
<i>Mixed lineage leukaemia (MLL) family</i>		
SET1A (SET domain-containing protein 1A)	<ul style="list-style-type: none"> <li>• SET1A<sup>-/-</sup> embryos fail to pass the epiblast stage and arrest around 7.5 dpc [170].</li> <li>• Required for ES cell self-renewal, pluripotency, and proliferation [170]</li> </ul>	COMPASS ATPase subunits
SET1B (SET domain-containing protein 1B)	<ul style="list-style-type: none"> <li>• SET1B<sup>-/-</sup> embryos arrest around 11.5 dpc [170].</li> </ul>	COMPASS ATPase subunits
MLL1 (KMT2A)	<ul style="list-style-type: none"> <li>• MLL1<sup>-/-</sup> embryos arrest after 10.5 dpc [171].</li> </ul>	COMPASS-like ATPase subunits
MLL2 (KMT2B)	<ul style="list-style-type: none"> <li>• MLL2<sup>-/-</sup> embryos results in severe developmental retardation and lethality at ~ 10.5 dpc [168,169].</li> <li>• MLL2<sup>-/-</sup> ES cells are viable and retain pluripotency, but they display cell proliferation defects due to an enhanced rate of apoptosis [175].</li> </ul>	COMPASS-like ATPase subunits
MLL3 (KMT2C)	<ul style="list-style-type: none"> <li>• MLL3<sup>-/-</sup> embryos die perinatally [172].</li> </ul>	COMPASS-like ATPase subunits
MLL4 (KMT2D)	<ul style="list-style-type: none"> <li>• MLL4<sup>-/-</sup> embryos arrest around 9.5 dpc [172].</li> </ul>	COMPASS-like ATPase subunits
Cfp1 (CxxC finger protein 1)	<ul style="list-style-type: none"> <li>• Cfp1<sup>-/-</sup> embryos die around implantation [181].</li> <li>• Cfp1 is required for ESC differentiation [246].</li> </ul>	COMPASS complex
Ash2 (Absent, small, or homeotic discs 2)	<ul style="list-style-type: none"> <li>• Ash2<sup>-/-</sup> embryos arrest before 8.5 dpc [180].</li> <li>• Required for ES cell self-renewal and pluripotency [179]</li> </ul>	COMPASS and COMPASS-like complexes
DPY30 (Dumpy-30)	<ul style="list-style-type: none"> <li>• DPY30<sup>-/-</sup> embryos arrest after 8.5 dpc [247].</li> </ul>	COMPASS and COMPASS-like complexes
<i>Chromatin remodelling factors</i>		
Brg1 (Smarca4)	<ul style="list-style-type: none"> <li>• Brg1<sup>-/-</sup> embryos die around implantation [164]. Role in zygotic gene activation after fertilisation [187]</li> <li>• Required for ES cell self-renewal and pluripotency [193,191]</li> </ul>	SWI/SNF complex ATPase subunits
BRM (Smarca2) ARID1 BAF250A (Arid1a)	<ul style="list-style-type: none"> <li>• Normal embryogenesis [248]</li> <li>• BAF250A<sup>-/-</sup> embryos arrest around E6.5 and fail to gastrulate without the formation of mesoderm [189].</li> <li>• Required for maintenance of ESC pluripotency [189]</li> </ul>	SWI/SNF complex ATPase subunits SWI/SNF complex
BAF250B (Arid1b) BAF155 (Smarcc1)	<ul style="list-style-type: none"> <li>• Regulates nucleosome occupancy during ESC differentiation [197]</li> <li>• Required for proliferation and maintenance of ESC pluripotency [192]</li> <li>• BAF155<sup>-/-</sup> embryos die during the implantation stage [162].</li> <li>• Required for ESC differentiation [249]</li> </ul>	SWI/SNF complex SWI/SNF complex Scaffolding proteins BAF60

(continued on next page)

Table 2 (continued)

Factor	Role in development and ESC	Complexes
hSNF5/BAF47/INI1 (Smarcb1)	<ul style="list-style-type: none"> <li>• hSNF5<sup>-/-</sup> embryos die between 3.5 and 5.5 dpc [165,188].</li> <li>• Required for ESC differentiation [249]</li> </ul>	SWI/SNF complex
BRD7	<ul style="list-style-type: none"> <li>• Activation and repression of target genes in ES cells [250]</li> </ul>	SWI/SNF complex
BAF57 (Smarce1)	<ul style="list-style-type: none"> <li>• Required for ESC differentiation [249]</li> </ul>	SWI/SNF complex
CHD1	<ul style="list-style-type: none"> <li>• CHD1<sup>-/-</sup> embryos show proliferation defects and lethality after implantation [251,252].</li> <li>• Essential for ES cell self-renewal [203]</li> </ul>	CHD family ATPase subunits
CHD2	<ul style="list-style-type: none"> <li>• CHD2<sup>-/-</sup> embryos exhibit growth delays late in embryogenesis and perinatal lethality [253].</li> <li>• CHD2 and CHD1 influence chromatin accessibility and histone H3.3 deposition at active chromatin regions [254].</li> </ul>	CHD family ATPase subunits
CHD3/Mi2- $\alpha$	<ul style="list-style-type: none"> <li>• NuRD functions to regulate ESC differentiation [210] and lineage specification [209].</li> </ul>	CHD family NURD complex ATPase subunits
Mbd3	<ul style="list-style-type: none"> <li>• NuRD subunit MBD3 is required for ESCs to differentiate [205].</li> <li>• Required to avoid ESC differentiation to trophectoderm [255]</li> </ul>	CHD family NURD complex
CHD4/Mi-2 $\beta$	<ul style="list-style-type: none"> <li>• CHD4 is required to restrict the expression of lineage-specific genes [256] and has been associated with maintaining nucleosome density at bivalent promoters [196].</li> </ul>	CHD family NURD complex ATPase subunits and Independently of NURD complex
TIP60 (Kat5), ING3, BRD8, MRG15 (Morf40I1), MRGBP, P400 (Ep400), TRRAP, Eaf6 (Meaf6), YL1 (Vps72), GAS41 (Yeats4), DMAP1, RVB1 (Ruvbl1), RVB2 (Ruvbl2), $\beta$ -actin (Actb), and ARP4	<ul style="list-style-type: none"> <li>• Embryos lacking Tip60 and Trrap die before implantation [211,212].</li> <li>• 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].</li> </ul>	The INO80 family INO80 complex (INO80 removes the H2A.Z from coding region nucleosomes)
INO80, ARP4 (Act16a), ARP5 (Angpt16), ARP8 (Actr8), IES2 (Ino80b), IES6 (Ino80c), RVB1 (Ruvbl1), RVB2 (Ruvbl2), YY1, Amida (Tfpt), FLJ20309 (Ino80d), MCRS1, UCH37 (Uchl5), NFRKB, and FLJ90652 (Ino80e)	<ul style="list-style-type: none"> <li>• Required for ESC self-renewal and blastocyst formation [217]</li> </ul>	The INO80 family INO80 complex (INO80 removes the H2A.Z from coding region nucleosomes)
SNF2H (Smarca5)	<ul style="list-style-type: none"> <li>• Snf2h<sup>-/-</sup> embryos die around implantation stage due to proliferation defects [201].</li> </ul>	ISWI family Complex: ACF, CHRAC, WICH
Bptf	<ul style="list-style-type: none"> <li>• Bptf protein (NURF complex) is required during post-implantation embryonic development.</li> <li>• Bptf<sup>-/-</sup> embryos die around E6.5 [257].</li> <li>• Bptf is required for ESC differentiation [257].</li> </ul>	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 depositing 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<sup>-/-</sup> 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 H3K4me<sub>3</sub>-rich promoters in ES cells, including those of bivalent genes [174], but its depletion leads to the loss of H3K4me<sub>3</sub> exclusively at bivalent promoters. Thus, while Mll2 may be the key factor defining the set of H3K4me<sub>3</sub>-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<sup>-/-</sup> 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 H3K4me<sub>3</sub> in ES cells [170], leading to major gene expression consequences affecting in particular genes controlled by Oct4 [176]. Accordingly, Setd1a<sup>-/-</sup> ES cells cannot be derived from mutant blastocysts. However, inducible Setd1a<sup>-/-</sup> 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 H3K4me<sub>3</sub> 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<sup>-/-</sup> 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 H3K9me<sub>3</sub> levels. This is likely to be the consequence of downregulation of the H3K9me<sub>3</sub> 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<sup>-/-</sup> ES cells can be derived. Cfp1<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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 manifests (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<sup>-/-</sup> 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 Tcf1 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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 H3K27me<sub>3</sub>, H3K9me<sub>3</sub>, 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 H3K27me<sub>3</sub> [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 decondensing 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 understand-

ing the role of TFs as carriers of regulatory information across ES cell generations.

## Acknowledgements

We acknowledge H. Leitch and N. Owens for the critical reading of the manuscript. Research in P.N. laboratory is supported by the Institut Pasteur, the CNRS, the ANR Laboratoire d'Excellence Revive (Investissement d'Avenir; ANR-10-LABX-73), and the FSER. N.F. was funded by an EMBO Long Term Fellowship (ALTF 876-2013) and a Marie-Curie Intra-European Fellowship (EFIMB – 626705). I.G. is supported by Revive.

Received 11 November 2016;

Received in revised form 2 December 2016;

Accepted 5 December 2016

Available online 15 December 2016

### Keywords:

pluripotent embryonic stem cells;  
ES cell self-renewal;  
epigenetics;  
transcription factor;  
chromatin

†N.F. and I.G. contributed equally to this work.

### 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.

## References

- [1] A.B. Stergachis, S. Neph, A. Reynolds, R. Humbert, B. Miller, S.L. Paige, et al., Developmental fate and cellular maturity encoded in human regulatory DNA landscapes, *Cell* 154 (2013) 888–903.
- [2] S. Heinz, C.E. Romanoski, C. Benner, C.K. Glass, The selection and function of cell type-specific enhancers, *Nat. Rev. Mol. Cell Biol.* 16 (2015) 144–154.
- [3] J.S. Becker, D. Nicetto, K.S. Zaret, H3K9me3-dependent heterochromatin: barrier to cell fate changes, *Trends Genet.* 32 (2016) 29–41.

- [4] K.S. Zaret, S.E. Mango, Pioneer transcription factors, chromatin dynamics, and cell fate control, *Curr. Opin. Genet. Dev.* 37 (2016) 76–81.
- [5] T. Chen, S.Y. Dent, Chromatin modifiers and remodellers: regulators of cellular differentiation, *Nat. Rev. Genet.* 15 (2014) 93–106.
- [6] V.W. Zhou, A. Goren, B.E. Bernstein, Charting histone modifications and the functional organization of mammalian genomes, *Nat. Rev. Genet.* 12 (2011) 7–18.
- [7] F. Wang, J.M. Higgins, Histone modifications and mitosis: countermarks, landmarks, and bookmarks, *Trends Cell Biol.* 23 (2013) 175–184.
- [8] E.I. Campos, J.M. Stafford, D. Reinberg, Epigenetic inheritance: histone bookmarks across generations, *Trends Cell Biol.* 24 (2014) 664–674.
- [9] S.L. Berger, T. Kouzarides, R. Shiekhattar, A. Shilatifard, An operational definition of epigenetics, *Genes Dev.* 23 (2009) 781–783.
- [10] S. Henikoff, J.M. Gready, Epigenetics, cellular memory and gene regulation, *Curr. Biol.* 26 (2016) R644–R648.
- [11] I. Bedzhov, S.J. Graham, C.Y. Leung, M. Zernicka-Goetz, Developmental plasticity, cell fate specification and morphogenesis in the early mouse embryo, *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 369 (2014).
- [12] C. Marcho, W. Cui, J. Mager, Epigenetic dynamics during preimplantation development, *Reproduction* 150 (2015) R109–R120.
- [13] R. Osorno, A. Tsakiridis, F. Wong, N. Cambray, C. Economou, R. Wilkie, et al., The developmental dismantling of pluripotency is reversed by ectopic Oct4 expression, *Development* 139 (2012) 2288–2298.
- [14] G.R. Martin, Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells, *Proc. Natl. Acad. Sci. U. S. A.* 78 (1981) 7634–7638.
- [15] M.J. Evans, M.H. Kaufman, Establishment in culture of pluripotential cells from mouse embryos, *Nature* 292 (1981) 154–156.
- [16] I. Chambers, S.R. Tomlinson, The transcriptional foundation of pluripotency, *Development* 136 (2009) 2311–2322.
- [17] G. Martello, A. Smith, The nature of embryonic stem cells, *Annu. Rev. Cell Dev. Biol.* 30 (2014) 647–675.
- [18] N. Festuccia, A. Dubois, S. Vandormael-Pourin, E. Gallego Tejada, A. Mouren, S. Bessonard, et al., Mitotic binding of Esrrb marks key regulatory regions of the pluripotency network, *Nat. Cell Biol.* 18 (2016) 1139–1148.
- [19] P. Savatier, S. Huang, L. Szekely, K.G. Wiman, J. Samarut, Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts, *Oncogene* 9 (1994) 809–818.
- [20] Q.L. Ying, J. Wray, J. Nichols, L. Battle-Morera, B. Doble, J. Woodgett, et al., The ground state of embryonic stem cell self-renewal, *Nature* 453 (2008) 519–523.
- [21] T. Kunath, M.K. Saba-El-Leil, M. Almousailleakh, J. Wray, S. Meloche, A. Smith, FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment, *Development* 134 (2007) 2895–2902.
- [22] P. Savatier, H. Lapillonne, L.A. van Grunsven, B.B. Rudkin, J. Samarut, Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells, *Oncogene* 12 (1996) 309–322.
- [23] J. White, E. Stead, R. Faast, S. Conn, P. Cartwright, S. Dalton, Developmental activation of the Rb-E2F pathway and establishment of cell cycle-regulated cyclin-dependent kinase activity during embryonic stem cell differentiation, *Mol. Biol. Cell* 16 (2005) 2018–2027.
- [24] H. Fujii-Yamamoto, J.M. Kim, K. Arai, H. Masai, Cell cycle and developmental regulations of replication factors in mouse embryonic stem cells, *J. Biol. Chem.* 280 (2005) 12,976–12,987.
- [25] R. Faast, J. White, P. Cartwright, L. Crocker, B. Sarcevic, S. Dalton, Cdk6-cyclin D3 activity in murine ES cells is resistant to inhibition by p16(INK4a), *Oncogene* 23 (2004) 491–502.
- [26] E. Stead, J. White, R. Faast, S. Conn, S. Goldstone, J. Rathjen, et al., Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities, *Oncogene* 21 (2002) 8320–8333.
- [27] A. Ballabeni, I.H. Park, R. Zhao, W. Wang, P.H. Lerou, G.Q. Daley, et al., Cell cycle adaptations of embryonic stem cells, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 19,252–19,257.
- [28] L. Jirmanova, M. Afanassieff, S. Gobert-Gosse, S. Markossian, P. Savatier, Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells, *Oncogene* 21 (2002) 5515–5528.
- [29] C.L. Mummery, C.E. van den Brink, S.W. de Laat, Commitment to differentiation induced by retinoic acid in P19 embryonal carcinoma cells is cell cycle dependent, *Dev. Biol.* 121 (1987) 10–19.
- [30] S. Pauklin, L. Vallier, The cell-cycle state of stem cells determines cell fate propensity, *Cell* 155 (2013) 135–147.
- [31] A.M. Singh, J. Chappell, R. Trost, L. Lin, T. Wang, J. Tang, et al., Cell-cycle control of developmentally regulated transcription factors accounts for heterogeneity in human pluripotent cells, *Stem Cell Rep.* 1 (2013) 532–544.
- [32] D. Coronado, M. Godet, P.Y. Bourillot, Y. Taponnier, A. Bernat, M. Petit, et al., A short G1 phase is an intrinsic determinant of naive embryonic stem cell pluripotency, *Stem Cell Res.* 10 (2013) 118–131.
- [33] M. Roccio, D. Schmitter, M. Knobloch, Y. Okawa, D. Sage, M.P. Lutolf, Predicting stem cell fate changes by differential cell cycle progression patterns, *Development* 140 (2013) 459–470.
- [34] S. Pauklin, P. Madrigal, A. Bertero, L. Vallier, Initiation of stem cell differentiation involves cell cycle-dependent regulation of developmental genes by Cyclin D, *Genes Dev.* 30 (2016) 421–433.
- [35] A.M. Singh, Y. Sun, L. Li, W. Zhang, T. Wu, S. Zhao, et al., Cell-cycle control of bivalent epigenetic domains regulates the exit from pluripotency, *Stem Cell Rep.* 5 (2015) 323–336.
- [36] W.L. Fangman, B.J. Brewer, A question of time: replication origins of eukaryotic chromosomes, *Cell* 71 (1992) 363–366.
- [37] C. Alabert, A. Groth, Chromatin replication and epigenome maintenance, *Nat. Rev. Mol. Cell Biol.* 13 (2012) 153–167.
- [38] T. Ishiuchi, R. Enriquez-Gasca, E. Mizutani, A. Boskovic, C. Ziegler-Birling, D. Rodriguez-Terrones, et al., Early embryonic-like cells are induced by downregulating replication-dependent chromatin assembly, *Nat. Struct. Mol. Biol.* 22 (2015) 662–671.
- [39] L. Schermelleh, A. Haemmer, F. Spada, N. Rosing, D. Meilinger, U. Rothbauer, et al., Dynamics of Dnmt1 interaction with the replication machinery and its role in

- postreplicative maintenance of DNA methylation, *Nucleic Acids Res.* 35 (2007) 4301–4312.
- [40] S. Milutinovic, Q. Zhuang, M. Zsyzf, Proliferating cell nuclear antigen associates with histone deacetylase activity, integrating DNA replication and chromatin modification, *J. Biol. Chem.* 277 (2002) 20,974–20,978.
- [41] P.O. Esteve, H.G. Chin, A. Smallwood, G.R. Feehery, O. Gangisetty, A.R. Karpf, et al., Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication, *Genes Dev.* 20 (2006) 3089–3103.
- [42] A. Loyola, H. Tagami, T. Bonaldi, D. Roche, J.P. Quivy, A. Imhof, et al., The HP1alpha-CAF1-SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin, *EMBO Rep.* 10 (2009) 769–775.
- [43] S. Hasan, P.O. Hassa, R. Imhof, M.O. Hottiger, Transcription coactivator p300 binds PCNA and may have a role in DNA repair synthesis, *Nature* 410 (2001) 387–391.
- [44] R.J. Burgess, Z. Zhang, Histone chaperones in nucleosome assembly and human disease, *Nat. Struct. Mol. Biol.* 20 (2013) 14–22.
- [45] S. Ramachandran, S. Henikoff, Transcriptional regulators compete with nucleosomes post-replication, *Cell* 165 (2016) 580–592.
- [46] P. Vasseur, S. Tonazzini, R. Ziane, A. Camasses, O.J. Rando, M. Radman-Livaja, Dynamics of nucleosome positioning maturation following genomic replication, *Cell Rep.* 16 (2016) 2651–2665.
- [47] I.J. de Castro, E. Gokhan, P. Vagnarelli, Resetting a functional G1 nucleus after mitosis, *Chromosoma* 125 (2016) 607–619.
- [48] J. Shin, T.W. Kim, H. Kim, H.J. Kim, M.Y. Suh, S. Lee, et al., Aurkb/PP1-mediated resetting of Oct4 during the cell cycle determines the identity of embryonic stem cells, *Elife* 5 (2016), e10877.
- [49] C. Muchardt, J.C. Reyes, B. Bourachot, E. Leguoy, M. Yaniv, The hbrm and BRG-1 proteins, components of the human SNF/SWI complex, are phosphorylated and excluded from the condensed chromosomes during mitosis, *EMBO J.* 15 (1996) 3394–3402.
- [50] S. Sif, P.T. Stukenberg, M.W. Kirschner, R.E. Kingston, Mitotic inactivation of a human SWI/SNF chromatin remodeling complex, *Genes Dev.* 12 (1998) 2842–2851.
- [51] S. Kadauke, G.A. Blobel, Mitotic bookmarking by transcription factors, *Epigenet. Chromatin* 6 (2013) 6.
- [52] E. Terrenoire, F. McDonald, J.A. Halsall, P. Page, R.S. Illingworth, A.M. Taylor, et al., Immunostaining of modified histones defines high-level features of the human metaphase epigenome, *Genome Biol.* 11 (2010), R110.
- [53] C.C. Hsiung, C.R. Bartman, P. Huang, P. Ginart, A.J. Stonestrom, C.A. Keller, et al., A hyperactive transcriptional state marks genome reactivation at the mitosis-G1 transition, *Genes Dev.* 30 (2016) 1423–1439.
- [54] R.A. Grandy, T.W. Whitfield, H. Wu, M.P. Fitzgerald, J.J. VanOudenhove, S.K. Zaidi, et al., Genome-wide studies reveal that H3K4me3 modification in bivalent genes is dynamically regulated during the pluripotent cell cycle and stabilized upon differentiation, *Mol. Cell. Biol.* 36 (2015) 615–627.
- [55] K. Liang, A.R. Woodfin, B.D. Slaughter, J.R. Unruh, A.C. Box, R.A. Rickels, et al., Mitotic transcriptional activation: clearance of actively engaged Pol II via transcriptional elongation control in mitosis, *Mol. Cell* 60 (2015) 435–445.
- [56] G. Juan, W. Pan, Z. Darzynkiewicz, DNA segments sensitive to single-strand-specific nucleases are present in chromatin of mitotic cells, *Exp. Cell Res.* 227 (1996) 197–202.
- [57] A. Dey, A. Nishiyama, T. Karpova, J. McNally, K. Ozato, Brd4 marks select genes on mitotic chromatin and directs postmitotic transcription, *Mol. Biol. Cell* 20 (2009) 4899–4909.
- [58] G.A. Blobel, S. Kadauke, E. Wang, A.W. Lau, J. Zuber, M.M. Chou, et al., A reconfigured pattern of MLL occupancy within mitotic chromatin promotes rapid transcriptional reactivation following mitotic exit, *Mol. Cell* 36 (2009) 970–983.
- [59] H. Xing, N.L. Vanderford, K.D. Sarge, The TBP-PP2A mitotic complex bookmarks genes by preventing condensin action, *Nat. Cell Biol.* 10 (2008) 1318–1323.
- [60] S. Kadauke, M.I. Udugama, J.M. Pawlicki, J.C. Achtman, D.P. Jain, Y. Cheng, et al., Tissue-specific mitotic bookmarking by hematopoietic transcription factor GATA1, *Cell* 150 (2012) 725–737.
- [61] H. Niwa, J. Miyazaki, A.G. Smith, Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells, *Nat. Genet.* 24 (2000) 372–376.
- [62] S. Masui, Y. Nakatake, Y. Toyooka, D. Shimosato, R. Yagi, K. Takahashi, et al., Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells, *Nat. Cell Biol.* 9 (2007) 625–635.
- [63] J. Jiang, Y.S. Chan, Y.H. Loh, J. Cai, G.Q. Tong, C.A. Lim, et al., A core Klf circuitry regulates self-renewal of embryonic stem cells, *Nat. Cell Biol.* 10 (2008) 353–360.
- [64] N. Festuccia, R. Osorno, F. Halbritter, V. Karwacki-Neisius, P. Navarro, D. Colby, et al., Esrrb is a direct Nanog target gene that can substitute for Nanog function in pluripotent cells, *Cell Stem Cell* 11 (2012) 477–490.
- [65] I. Chambers, J. Silva, D. Colby, J. Nichols, B. Nijmeijer, M. Robertson, et al., Nanog safeguards pluripotency and mediates germline development, *Nature* 450 (2007) 1230–1234.
- [66] K. Mitsui, Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, et al., The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells, *Cell* 113 (2003) 631–642.
- [67] H. Niwa, Y. Toyooka, D. Shimosato, D. Strumpf, K. Takahashi, R. Yagi, et al., Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation, *Cell* 123 (2005) 917–929.
- [68] D. Shimosato, M. Shiki, H. Niwa, Extra-embryonic endoderm cells derived from ES cells induced by GATA factors acquire the character of XEN cells, *BMC Dev. Biol.* 7 (2007) 80.
- [69] I.G. Brons, L.E. Smithers, M.W. Trotter, P. Rugg-Gunn, B. Sun, S.M. Chuva de Sousa Lopes, et al., Derivation of pluripotent epiblast stem cells from mammalian embryos, *Nature* 448 (2007) 191–195.
- [70] P.J. Tesar, J.G. Chenoweth, F.A. Brook, T.J. Davies, E.P. Evans, D.L. Mack, et al., New cell lines from mouse epiblast share defining features with human embryonic stem cells, *Nature* 448 (2007) 196–199.
- [71] S. Geula, S. Moshitch-Moshkovitz, D. Dominissini, A.A. Mansour, N. Kol, M. Salmon-Divon, et al., Stem cells. m6A mRNA methylation facilitates resolution of naive pluripotency toward differentiation, *Science* 347 (2015) 1002–1006.
- [72] M. Entrevan, B. Schuettengruber, G. Cavalli, Regulation of genome architecture and function by polycomb proteins, *Trends Cell Biol.* 26 (2016) 511–525.

- [73] P.A. Steffen, L. Ringrose, What are memories made of? How polycomb and trithorax proteins mediate epigenetic memory, *Nat. Rev. Mol. Cell Biol.* 15 (2014) 340–356.
- [74] K.H. Hansen, A.P. Bracken, D. Pasini, N. Dietrich, S.S. Gehani, A. Monrad, et al., A model for transmission of the H3K27me3 epigenetic mark, *Nat. Cell Biol.* 10 (2008) 1291–1300.
- [75] M. Arora, C.Z. Packard, T. Banerjee, J.D. Parvin, RING1A and BMI1 bookmark active genes via ubiquitination of chromatin-associated proteins, *Nucleic Acids Res.* 44 (2016) 2136–2144.
- [76] N.E. Follmer, A.H. Wani, N.J.A. Francis, Polycomb group protein is retained at specific sites on chromatin in mitosis, *PLoS Genet.* 8 (2012), e1003135.
- [77] T.S. Mikkelsen, M. Ku, D.B. Jaffe, B. Issac, E. Lieberman, G. Giannoukos, et al., Genome-wide maps of chromatin state in pluripotent and lineage-committed cells, *Nature* 448 (2007) 553–560.
- [78] L.A. Boyer, K. Plath, J. Zeitlinger, T. Brambrink, L.A. Medeiros, T.I. Lee, et al., Polycomb complexes repress developmental regulators in murine embryonic stem cells, *Nature* 441 (2006) 349–353.
- [79] B.E. Bernstein, T.S. Mikkelsen, X. Xie, M. Kamal, D.J. Huebert, J. Cuff, et al., A bivalent chromatin structure marks key developmental genes in embryonic stem cells, *Cell* 125 (2006) 315–326.
- [80] V. Azuara, P. Perry, S. Sauer, M. Spivakov, H.F. Jorgensen, R.M. John, et al., Chromatin signatures of pluripotent cell lines, *Nat. Cell Biol.* 8 (2006) 532–538.
- [81] M. Ku, R.P. Koche, E. Rheinbay, E.M. Mendenhall, M. Endoh, T.S. Mikkelsen, et al., Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains, *PLoS Genet.* 4 (2008), e1000242.
- [82] M. Endoh, T.A. Endo, T. Endo, K. Isono, J. Sharif, O. Ohara, et al., Histone H2A mono-ubiquitination is a crucial step to mediate PRC1-dependent repression of developmental genes to maintain ES cell identity, *PLoS Genet.* 8 (2012), e1002774.
- [83] O. Alder, F. Laval, A. Helness, E. Brookes, S. Pinho, A. Chandrashekran, et al., Ring1B and Suv39h1 delineate distinct chromatin states at bivalent genes during early mouse lineage commitment, *Development* 137 (2010) 2483–2492.
- [84] J.K. Stock, S. Giadrossi, M. Casanova, E. Brookes, M. Vidal, H. Koseki, et al., Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells, *Nat. Cell Biol.* 9 (2007) 1428–1435.
- [85] W.W. Tee, S.S. Shen, O. Oksuz, V. Narendra, D. Reinberg, Erk1/2 activity promotes chromatin features and RNAPII phosphorylation at developmental promoters in mouse ESCs, *Cell* 156 (2014) 678–690.
- [86] X. Shen, Y. Liu, Y.J. Hsu, Y. Fujiwara, J. Kim, X. Mao, et al., EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency, *Mol. Cell* 32 (2008) 491–502.
- [87] D. Pasini, A.P. Bracken, M.R. Jensen, E. Lazzerini Denchi, K. Helin, Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity, *EMBO J.* 23 (2004) 4061–4071.
- [88] S.J. Chamberlain, D. Yee, T. Magnuson, Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency, *Stem Cells* 26 (2008) 1496–1505.
- [89] N.D. Montgomery, D. Yee, A. Chen, S. Kalantry, S.J. Chamberlain, A.P. Otte, et al., The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation, *Curr. Biol.* 15 (2005) 942–947.
- [90] H. Marks, T. Kalkan, R. Menafrá, S. Denissov, K. Jones, H. Hofemeister, et al., The transcriptional and epigenomic foundations of ground state pluripotency, *Cell* 149 (2012) 590–604.
- [91] M. Leeb, A. Wutz, Ring1B is crucial for the regulation of developmental control genes and PRC1 proteins but not X inactivation in embryonic cells, *J. Cell Biol.* 178 (2007) 219–229.
- [92] P. van der Stoep, E.A. Boutsma, D. Hulsman, S. Noback, M. Heimerikx, R.M. Kerkhoven, et al., Ubiquitin E3 ligase Ring1b/Rnf2 of polycomb repressive complex 1 contributes to stable maintenance of mouse embryonic stem cells, *PLoS One* 3 (2008), e2235.
- [93] M. Endoh, T.A. Endo, T. Endo, Y. Fujimura, O. Ohara, T. Toyoda, et al., Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity, *Development* 135 (2008) 1513–1524.
- [94] M. Leeb, D. Pasini, M. Novatchkova, M. Jaritz, K. Helin, A. Wutz, Polycomb complexes act redundantly to repress genomic repeats and genes, *Genes Dev.* 24 (2010) 265–276.
- [95] A.R. Pengelly, R. Kalb, K. Finkl, J. Muller, Transcriptional repression by PRC1 in the absence of H2A monoubiquitylation, *Genes Dev.* 29 (2015) 1487–1492.
- [96] R.S. Illingworth, M. Moffat, A.R. Mann, D. Read, C.J. Hunter, M.M. Pradeepa, et al., The E3 ubiquitin ligase activity of RING1B is not essential for early mouse development, *Genes Dev.* 29 (2015) 1897–1902.
- [97] J.W. Voncken, B.A. Roelen, M. Roefs, S. de Vries, E. Verhoeven, S. Marino, et al., Rnf2 (Ring1b) deficiency causes gastrulation arrest and cell cycle inhibition, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 2468–2473.
- [98] D. O'Carroll, S. Erhardt, M. Pagani, S.C. Barton, M.A. Surani, T. Jenuwein, The polycomb-group gene *Ezh2* is required for early mouse development, *Mol. Cell. Biol.* 21 (2001) 4330–4336.
- [99] C. Faust, K.A. Lawson, N.J. Schork, B. Thiel, T. Magnuson, The polycomb-group gene *eed* is required for normal morphogenetic movements during gastrulation in the mouse embryo, *Development* 125 (1998) 4495–4506.
- [100] E.M. Morin-Kensicki, C. Faust, C. LaMantia, T. Magnuson, Cell and tissue requirements for the gene *eed* during mouse gastrulation and organogenesis, *Genesis* 31 (2001) 142–146.
- [101] D. Pasini, A.P. Bracken, J.B. Hansen, M. Capillo, K. Helin, The polycomb group protein *Suz12* is required for embryonic stem cell differentiation, *Mol. Cell. Biol.* 27 (2007) 3769–3779.
- [102] E.M. Riising, I. Comet, B. Leblanc, X. Wu, J.V. Johansen, K. Helin, Gene silencing triggers polycomb repressive complex 2 recruitment to CpG islands genome wide, *Mol. Cell* 55 (2014) 347–360.
- [103] M. Tachibana, K. Sugimoto, M. Nozaki, J. Ueda, T. Ohta, M. Ohki, et al., G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis, *Genes Dev.* 16 (2002) 1779–1791.
- [104] M. Tachibana, J. Ueda, M. Fukuda, N. Takeda, T. Ohta, H. Iwanari, et al., Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9, *Genes Dev.* 19 (2005) 815–826.

- [105] A.H. Peters, D. O'Carroll, H. Scherthan, K. Mechtler, S. Sauer, C. Schofer, et al., Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability, *Cell* 107 (2001) 323–337.
- [106] B. Lehnertz, Y. Ueda, A.A. Derijck, U. Braunschweig, L. Perez-Burgos, S. Kubicek, et al., Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin, *Curr. Biol.* 13 (2003) 1192–1200.
- [107] H. Wang, W. An, R. Cao, L. Xia, H. Erdjument-Bromage, B. Chatton, et al., mAM facilitates conversion by ESET of dimethyl to trimethyl lysine 9 of histone H3 to cause transcriptional repression, *Mol. Cell* 12 (2003) 475–487.
- [108] N. Saksouk, E. Simboeck, J. Dejardin, Constitutive heterochromatin formation and transcription in mammals, *Epigenet. Chromatin* 8 (2015) 3.
- [109] A.H. Peters, S. Kubicek, K. Mechtler, R.J. O'Sullivan, A.A. Derijck, L. Perez-Burgos, et al., Partitioning and plasticity of repressive histone methylation states in mammalian chromatin, *Mol. Cell* 12 (2003) 1577–1589.
- [110] J.A. Park, A.J. Kim, Y. Kang, Y.J. Jung, H.K. Kim, K.C. Kim, Deacetylation and methylation at histone H3 lysine 9 (H3K9) coordinate chromosome condensation during cell cycle progression, *Mol. Cell* 31 (2011) 343–349.
- [111] S. Kobayakawa, K. Miike, M. Nakao, K. Abe, Dynamic changes in the epigenomic state and nuclear organization of differentiating mouse embryonic stem cells, *Genes Cells* 12 (2007) 447–460.
- [112] E. Meshorer, D. Yellajoshula, E. George, P.J. Scambler, D.T. Brown, T. Misteli, Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells, *Dev. Cell* 10 (2006) 105–116.
- [113] N. Saksouk, T.K. Barth, C. Ziegler-Birling, N. Olova, A. Nowak, E. Rey, et al., Redundant mechanisms to form silent chromatin at pericentromeric regions rely on BEND3 and DNA methylation, *Mol. Cell* 56 (2014) 580–594.
- [114] A. Tsumura, T. Hayakawa, Y. Kumaki, S. Takebayashi, M. Sakaue, C. Matsuoka, et al., Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b, *Genes Cells* 11 (2006) 805–814.
- [115] M. Walter, A. Teissandier, R. Perez-Palacios, Bourc'his D. An epigenetic switch ensures transposon repression upon dynamic loss of DNA methylation in embryonic stem cells, *Elife* 5 (2016), e11418.
- [116] A. Bulut-Karslioglu, V. Perrera, M. Scaranaro, I.A. de la Rosa-Velazquez, S. van de Nobelen, N. Shukeir, et al., A transcription factor-based mechanism for mouse heterochromatin formation, *Nat. Struct. Mol. Biol.* 19 (2012) 1023–1030.
- [117] S.P. Sripathy, J. Stevens, D.C. Schultz, The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression, *Mol. Cell. Biol.* 26 (2006) 8623–8638.
- [118] C.L. Novo, C. Tang, K. Ahmed, U. Djuric, E. Fussner, N.P. Mullin, et al., The pluripotency factor Nanog regulates pericentromeric heterochromatin organization in mouse embryonic stem cells, *Genes Dev.* 30 (2016) 1101–1115.
- [119] C. Stocking, C.A. Kozak, Murine endogenous retroviruses, *Cell. Mol. Life Sci.* 65 (2008) 3383–3398.
- [120] I.A. Maksakova, P.J. Thompson, P. Goyal, S.J. Jones, P.B. Singh, M.M. Karimi, et al., Distinct roles of KAP1, HP1 and G9a/GLP in silencing of the two-cell-specific retrotransposon MERVL in mouse ES cells, *Epigenet. Chromatin* 6 (2013) 15.
- [121] T. Matsui, D. Leung, H. Miyashita, I.A. Maksakova, H. Miyachi, H. Kimura, et al., Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET, *Nature* 464 (2010) 927–931.
- [122] M.M. Karimi, P. Goyal, I.A. Maksakova, M. Bilenky, D. Leung, J.X. Tang, et al., DNA methylation and SETDB1/H3K9me3 regulate predominantly distinct sets of genes, retroelements, and chimeric transcripts in mESCs, *Cell Stem Cell* 8 (2011) 676–687.
- [123] H.M. Rowe, J. Jakobsson, D. Mesnard, J. Rougemont, S. Reynard, T. Aktas, et al., KAP1 controls endogenous retroviruses in embryonic stem cells, *Nature* 463 (2010) 237–240.
- [124] G. Falco, S.L. Lee, I. Stanghellini, U.C. Bassey, T. Hamatani, M.S. Ko, Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells, *Dev. Biol.* 307 (2007) 539–550.
- [125] M. Zalzman, G. Falco, L.V. Sharova, A. Nishiyama, M. Thomas, S.L. Lee, et al., Zscan4 regulates telomere elongation and genomic stability in ES cells, *Nature* 464 (2010) 858–863.
- [126] T.S. Macfarlan, W.D. Gifford, S. Driscoll, K. Lettieri, H.M. Rowe, D. Bonanomi, et al., Embryonic stem cell potency fluctuates with endogenous retrovirus activity, *Nature* 487 (2012) 57–63.
- [127] T. Amano, T. Hirata, G. Falco, M. Monti, L.V. Sharova, M. Amano, et al., Zscan4 restores the developmental potency of embryonic stem cells, *Nat. Commun.* 4 (2013) 1966.
- [128] T. Akiyama, L. Xin, M. Oda, A.A. Sharov, M. Amano, Y. Piao, et al., Transient bursts of Zscan4 expression are accompanied by the rapid derepression of heterochromatin in mouse embryonic stem cells, *DNA Res.* 22 (2015) 307–318.
- [129] C. Mozzetta, J. Pontis, L. Fritsch, P. Robin, M. Portoso, C. Proux, et al., The histone H3 lysine 9 methyltransferases G9a and GLP regulate polycomb repressive complex 2-mediated gene silencing, *Mol. Cell* 53 (2014) 277–289.
- [130] J.E. Dodge, Y.K. Kang, H. Beppu, H. Lei, E. Li, Histone H3-K9 methyltransferase ESET is essential for early development, *Mol. Cell. Biol.* 24 (2004) 2478–2486.
- [131] P. Yuan, J. Han, G. Guo, Y.L. Orlov, M. Huss, Y.H. Loh, et al., Eset partners with Oct4 to restrict extraembryonic trophoblast lineage potential in embryonic stem cells, *Genes Dev.* 23 (2009) 2507–2520.
- [132] Z.D. Smith, A. Meissner, DNA methylation: roles in mammalian development, *Nat. Rev. Genet.* 14 (2013) 204–220.
- [133] M.B. Stadler, R. Murr, L. Burger, R. Ivanek, F. Lienert, A. Scholer, et al., DNA-binding factors shape the mouse methylome at distal regulatory regions, *Nature* 480 (2011) 490–495.
- [134] P.G. Constantinides, P.A. Jones, W. Gevers, Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment, *Nature* 267 (1977) 364–366.
- [135] T.S. Mikkelsen, J. Hanna, X. Zhang, M. Ku, M. Wernig, P. Schorderet, et al., Dissecting direct reprogramming through integrative genomic analysis, *Nature* 454 (2008) 49–55.
- [136] E. Li, T.H. Bestor, R. Jaenisch, Targeted mutation of the DNA methyltransferase gene results in embryonic lethality, *Cell* 69 (1992) 915–926.

- [137] M. Okano, D.W. Bell, D.A. Haber, E. Li, DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development, *Cell* 99 (1999) 247–257.
- [138] L. Jackson-Grusby, C. Beard, R. Possemato, M. Tudor, D. Fambrough, G. Csankovszki, et al., Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation, *Nat. Genet.* 27 (2001) 31–39.
- [139] H.G. Leitch, K.R. McEwen, A. Turp, V. Encheva, T. Carroll, N. Grabole, et al., Naive pluripotency is associated with global DNA hypomethylation, *Nat. Struct. Mol. Biol.* 20 (2013) 311–316.
- [140] J.A. Hackett, S. Dietmann, K. Murakami, T.A. Down, H.G. Leitch, M.A. Surani, Synergistic mechanisms of DNA demethylation during transition to ground-state pluripotency, *Stem Cell Rep.* 1 (2013) 518–531.
- [141] E. Habibi, A.B. Brinkman, J. Arand, L.I. Kroeze, H.H. Kerstens, F. Matarese, et al., Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells, *Cell Stem Cell* 13 (2013) 360–369.
- [142] G. Ficz, T.A. Hore, F. Santos, H.J. Lee, W. Dean, J. Arand, et al., FGF signaling inhibition in ESCs drives rapid genome-wide demethylation to the epigenetic ground state of pluripotency, *Cell Stem Cell* 13 (2013) 351–359.
- [143] F. von Meyenn, M. Iurlaro, E. Habibi, N.Q. Liu, A. Salehzadeh-Yazdi, F. Santos, et al., Impairment of DNA methylation maintenance is the main cause of global demethylation in naive embryonic stem cells, *Mol. Cell* 62 (2016) 983.
- [144] H. Wu, Y. Zhang, Reversing DNA methylation: mechanisms, genomics, and biological functions, *Cell* 156 (2014) 45–68.
- [145] V. Valinluck, H.H. Tsai, D.K. Rogstad, A. Burdzy, A. Bird, L.C. Sowers, Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2), *Nucleic Acids Res.* 32 (2004) 4100–4108.
- [146] S.G. Jin, S. Kadam, G.P. Pfeifer, Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine, *Nucleic Acids Res.* 38 (2010), e125.
- [147] M.M. Dawlaty, A. Breiling, T. Le, M.I. Barrasa, G. Raddatz, Q. Gao, et al., Loss of Tet enzymes compromises proper differentiation of embryonic stem cells, *Dev. Cell* 29 (2014) 102–111.
- [148] H.Q. Dai, B.A. Wang, L. Yang, J.J. Chen, G.C. Zhu, M.L. Sun, et al., TET-mediated DNA demethylation controls gastrulation by regulating Lefty-Nodal signalling, *Nature* 538 (2016) 528–532.
- [149] I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, et al., Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells, *Cell* 113 (2003) 643–655.
- [150] J. Krejci, R. Uhlířova, G. Galiova, S. Kozubek, J. Smigova, E. Bartova, Genome-wide reduction in H3K9 acetylation during human embryonic stem cell differentiation, *J. Cell. Physiol.* 219 (2009) 677–687.
- [151] S. Efroni, R. Duttagupta, J. Cheng, H. Dehghani, D.J. Hoepfner, C. Dash, et al., Global transcription in pluripotent embryonic stem cells, *Cell Stem Cell* 2 (2008) 437–447.
- [152] K. Ahmed, H. Dehghani, P. Rugg-Gunn, E. Fussner, J. Rossant, D.P. Bazett-Jones, Global chromatin architecture reflects pluripotency and lineage commitment in the early mouse embryo, *PLoS One* 5 (2010), e10531.
- [153] I. Hiratani, T. Ryba, M. Itoh, J. Rathjen, M. Kulik, B. Papp, et al., Genome-wide dynamics of replication timing revealed by in vitro models of mouse embryogenesis, *Genome Res.* 20 (2010) 155–169.
- [154] E. Fussner, U. Djuric, M. Strauss, A. Hotta, C. Perez-Iratxeta, F. Lanner, et al., Constitutive heterochromatin reorganization during somatic cell reprogramming, *EMBO J.* 30 (2011) 1778–1789.
- [155] J.J. Zyllicz, S. Dietmann, U. Gunesdogan, J.A. Hackett, D. Cougot, C. Lee, et al., Chromatin dynamics and the role of G9a in gene regulation and enhancer silencing during early mouse development, *Elife* 4 (2015), e09571.
- [156] B. Wen, H. Wu, Y. Shinkai, R.A. Irizarry, A.P. Feinberg, Large histone H3 lysine 9 dimethylated chromatin blocks distinguish differentiated from embryonic stem cells, *Nat. Genet.* 41 (2009) 246–250.
- [157] E. Bartova, J. Krejci, A. Hamcarova, S. Kozubek, Differentiation of human embryonic stem cells induces condensation of chromosome territories and formation of heterochromatin protein 1 foci, *Differentiation* 76 (2008) 24–32.
- [158] D. Constantinescu, H.L. Gray, P.J. Sammak, G.P. Schatten, A.B. Csoka, A. Lamin, C expression is a marker of mouse and human embryonic stem cell differentiation, *Stem Cells* 24 (2006) 177–185.
- [159] D. Bhattacharya, S. Talwar, A. Mazumder, G.V. Shivashankar, Spatio-temporal plasticity in chromatin organization in mouse cell differentiation and during *Drosophila* embryogenesis, *Biophys. J.* 96 (2009) 3832–3839.
- [160] C.R. Clapier, B.R. Cairns, The biology of chromatin remodeling complexes, *Annu. Rev. Biochem.* 78 (2009) 273–304.
- [161] D.C. Hargreaves, G.R. Crabtree, ATP-dependent chromatin remodeling: genetics, genomics and mechanisms, *Cell Res.* 21 (2011) 396–420.
- [162] J.K. Kim, S.O. Huh, H. Choi, K.S. Lee, D. Shin, C. Lee, et al., Srg3, a mouse homolog of yeast SWI3, is essential for early embryogenesis and involved in brain development, *Mol. Cell. Biol.* 21 (2001) 7787–7795.
- [163] C. Sumi-Ichinose, H. Ichinose, D. Metzger, P. Chambon, SNF2beta-BRG1 is essential for the viability of F9 murine embryonal carcinoma cells, *Mol. Cell. Biol.* 17 (1997) 5976–5986.
- [164] S. Bultman, T. Gebuhr, D. Yee, C. La Mantia, J. Nicholson, A. Gilliam, et al., A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes, *Mol. Cell* 6 (2000) 1287–1295.
- [165] A. Klochendler-Yeivin, L. Fiette, J. Barra, C. Muchardt, C. Babinet, M. Yaniv, The murine SNF5/INI1 chromatin remodeling factor is essential for embryonic development and tumor suppression, *EMBO Rep.* 1 (2000) 500–506.
- [166] A. Shilatifard, The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis, *Annu. Rev. Biochem.* 81 (2012) 65–95.
- [167] C.V. Andreu-Vieyra, R. Chen, J.E. Agno, S. Glaser, K. Anastassiadis, A.F. Stewart, et al., MLL2 is required in oocytes for bulk histone 3 lysine 4 trimethylation and transcriptional silencing, *PLoS Biol.* 8 (8) (2010), e1000453.
- [168] S. Glaser, S. Lubitz, K.L. Loveland, K. Ohbo, L. Robb, F. Schwenk, et al., The histone 3 lysine 4 methyltransferase, Mll2, is only required briefly in development and spermatogenesis, *Epigenet. Chromatin* 2 (2009) 5.
- [169] S. Glaser, J. Schaft, S. Lubitz, K. Vintersten, F. van der Hoeven, K.R. Tuffeland, et al., Multiple epigenetic maintenance

- factors implicated by the loss of Mll2 in mouse development, *Development* 133 (2006) 1423–1432.
- [170] A.S. Bledau, K. Schmidt, K. Neumann, U. Hill, G. Ciotta, A. Gupta, et al., The H3K4 methyltransferase Setd1a is first required at the epiblast stage, whereas Setd1b becomes essential after gastrulation, *Development* 141 (2014) 1022–1035.
- [171] P. Ernst, M. Mabon, A.J. Davidson, L.I. Zon, S.J. Korsmeyer, An Mll-dependent Hox program drives hematopoietic progenitor expansion, *Curr. Biol.* 14 (2004) 2063–2069.
- [172] J.E. Lee, C. Wang, S. Xu, Y.W. Cho, L. Wang, X. Feng, et al., H3K4 mono- and di-methyltransferase MLL4 is required for enhancer activation during cell differentiation, *Elife* 2 (2013), e01503.
- [173] D. Hu, A.S. Garruss, X. Gao, M.A. Morgan, M. Cook, E.R. Smith, et al., The Mll2 branch of the COMPASS family regulates bivalent promoters in mouse embryonic stem cells, *Nat. Struct. Mol. Biol.* 20 (2013) 1093–1097.
- [174] S. Denissov, H. Hofemeister, H. Marks, A. Kranz, G. Ciotta, S. Singh, et al., Mll2 is required for H3K4 trimethylation on bivalent promoters in embryonic stem cells, whereas Mll1 is redundant, *Development* 141 (2014) 526–537.
- [175] S. Lubitz, S. Glaser, J. Schaft, A.F. Stewart, K. Anastassiadis, Increased apoptosis and skewed differentiation in mouse embryonic stem cells lacking the histone methyltransferase Mll2, *Mol. Biol. Cell* 18 (2007) 2356–2366.
- [176] L. Fang, J. Zhang, H. Zhang, X. Yang, X. Jin, L. Zhang, et al., H3K4 methyltransferase Set1a is a key Oct4 coactivator essential for generation of Oct4 positive inner cell mass, *Stem Cells* 34 (2016) 565–580.
- [177] J. Wysocka, T. Swigut, T.A. Milne, Y. Dou, X. Zhang, A.L. Burlingame, et al., WDR5 associates with histone H3 methylated at K4 and is essential for H3 K4 methylation and vertebrate development, *Cell* 121 (2005) 859–872.
- [178] Y.S. Ang, S.Y. Tsai, D.F. Lee, J. Monk, J. Su, K. Ratnakumar, et al., Wdr5 mediates self-renewal and reprogramming via the embryonic stem cell core transcriptional network, *Cell* 145 (2011) 183–197.
- [179] M. Wan, J. Liang, Y. Xiong, F. Shi, Y. Zhang, W. Lu, et al., The trithorax group protein Ash2l is essential for pluripotency and maintaining open chromatin in embryonic stem cells, *J. Biol. Chem.* 288 (2013) 5039–5048.
- [180] J.Z. Stoller, L. Huang, C.C. Tan, F. Huang, D.D. Zhou, J. Yang, et al., Ash2l interacts with Tbx1 and is required during early embryogenesis, *Exp. Biol. Med. (Maywood)* 235 (2010) 569–576.
- [181] D.L. Carlone, D.G. Skalnik, CpG binding protein is crucial for early embryonic development, *Mol. Cell. Biol.* 21 (2001) 7601–7606.
- [182] T. Clouaire, S. Webb, P. Skene, R. Illingworth, A. Kerr, R. Andrews, et al., Cfp1 integrates both CpG content and gene activity for accurate H3K4me3 deposition in embryonic stem cells, *Genes Dev.* 26 (2012) 1714–1728.
- [183] P. Sudarsanam, F. Winston, The Swi/Snf family nucleosome-remodeling complexes and transcriptional control, *Trends Genet.* 16 (2000) 345–351.
- [184] C.W. Roberts, S.H. Orkin, The SWI/SNF complex—chromatin and cancer, *Nat. Rev. Cancer* 4 (2004) 133–142.
- [185] W. Wang, J. Cote, Y. Xue, S. Zhou, P.A. Khavari, S.R. Biggar, et al., Purification and biochemical heterogeneity of the mammalian SWI–SNF complex, *EMBO J.* 15 (1996) 5370–5382.
- [186] J.C. Reyes, J. Barra, C. Muchardt, A. Camus, C. Babinet, M. Yaniv, Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha), *EMBO J.* 17 (1998) 6979–6991.
- [187] S.J. Bultman, T.C. Gebuhr, H. Pan, P. Svoboda, R.M. Schultz, T. Magnuson, Maternal BRG1 regulates zygotic genome activation in the mouse, *Genes Dev.* 20 (2006) 1744–1754.
- [188] C.J. Guidi, A.T. Sands, B.P. Zambrowicz, T.K. Turner, D.A. Demers, W. Webster, et al., Disruption of Ini1 leads to peri-implantation lethality and tumorigenesis in mice, *Mol. Cell. Biol.* 21 (2001) 3598–3603.
- [189] X. Gao, P. Tate, P. Hu, R. Tjian, W.C. Skarnes, Z. Wang, ES cell pluripotency and germ-layer formation require the SWI/SNF chromatin remodeling component BAF250a, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 6656–6661.
- [190] J. Lessard, W. Ji, J.A. Ranish, M. Wan, M.M. Winslow, B.T. Staahl, et al., An essential switch in subunit composition of a chromatin remodeling complex during neural development, *Neuron* 55 (2007) 201–215.
- [191] L. Ho, J.L. Ronan, J. Wu, B.T. Staahl, L. Chen, A. Kuo, et al., An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 5181–5186.
- [192] Z. Yan, Z. Wang, L. Sharova, A.A. Sharov, C. Ling, Y. Piao, et al., BAF250B-associated SWI/SNF chromatin-remodeling complex is required to maintain undifferentiated mouse embryonic stem cells, *Stem Cells* 26 (2008) 1155–1165.
- [193] B.L. Kidder, S. Palmer, J.G.S.W.I. Knott, SNF-Brg1 regulates self-renewal and occupies core pluripotency-related genes in embryonic stem cells, *Stem Cells* 27 (2009) 317–328.
- [194] L. Ho, R. Jothi, J.L. Ronan, K. Cui, K. Zhao, G.R. Crabtree, An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 5187–5191.
- [195] L. Ho, E.L. Miller, J.L. Ronan, W.Q. Ho, R. Jothi, G.R. Crabtree, esBAF facilitates pluripotency by conditioning the genome for LIF/STAT3 signalling and by regulating polycomb function, *Nat. Cell Biol.* 13 (2011) 903–913.
- [196] M. de Dieuleveult, K. Yen, I. Hmitou, A. Depaux, F. Boussovar, D. Bou Dargham, et al., Genome-wide nucleosome specificity and function of chromatin remodellers in ES cells, *Nature* 530 (2016) 113–116.
- [197] I. Lei, J. West, Z. Yan, X. Gao, P. Fang, J.H. Dennis, et al., BAF250a protein regulates nucleosome occupancy and histone modifications in priming embryonic stem cell differentiation, *J. Biol. Chem.* 290 (2015) 19,343–19,352.
- [198] S. Awad, A.H. Hassan, The Swi2/Snf2 bromodomain is important for the full binding and remodeling activity of the SWI/SNF complex on H3- and H4-acetylated nucleosomes, *Ann. N. Y. Acad. Sci.* 1138 (2008) 366–375.
- [199] L. Ho, G.R. Crabtree, Chromatin remodelling during development, *Nature* 463 (2010) 474–484.
- [200] S.K. Hota, B.G. Bruneau, ATP-dependent chromatin remodeling during mammalian development, *Development* 143 (2016) 2882–2897.
- [201] T. Stopka, A.I. Skoultschi, The ISWI ATPase Snf2h is required for early mouse development, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 14,097–14,102.
- [202] C.G. Marfella, A.N. Imbalzano, The Chd family of chromatin remodelers, *Mutat. Res.* 618 (2007) 30–40.
- [203] A. Gaspar-Maia, A. Alajem, F. Polesso, R. Sridharan, M.J. Mason, A. Heidersbach, et al., Chd1 regulates open chromatin and pluripotency of embryonic stem cells, *Nature* 460 (2009) 863–868.
- [204] M.P. Schnetz, L. Handoko, B. Akhtar-Zaidi, C.F. Bartels, C.F. Pereira, A.G. Fisher, et al., CHD7 targets active gene enhancer

- elements to modulate ES cell-specific gene expression, *PLoS Genet.* 6 (2010), e1001023.
- [205] K. Kaji, I.M. Caballero, R. MacLeod, J. Nichols, V.A. Wilson, B. Hendrich, The NuRD component Mbd3 is required for pluripotency of embryonic stem cells, *Nat. Cell Biol.* 8 (2006) 285–292.
- [206] K. Kaji, J. Nichols, B. Hendrich, Mbd3, a component of the NuRD co-repressor complex, is required for development of pluripotent cells, *Development* 134 (2007) 1123–1132.
- [207] B. Hendrich, J. Guy, B. Ramsahoye, V.A. Wilson, A. Bird, Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development, *Genes Dev.* 15 (2001) 710.
- [208] Y. Rais, A. Zviran, S. Geula, O. Gafni, E. Chomsky, S. Viukov, et al., Deterministic direct reprogramming of somatic cells to pluripotency, *Nature* 502 (2013) 65–70.
- [209] N. Reynolds, P. Latos, A. Hynes-Allen, R. Loos, D. Leaford, A. O'Shaughnessy, et al., NuRD suppresses pluripotency gene expression to promote transcriptional heterogeneity and lineage commitment, *Cell Stem Cell* 10 (2012) 583–594.
- [210] N. Reynolds, M. Salmon-Divon, H. Dvinge, A. Hynes-Allen, G. Balasooriya, D. Leaford, et al., NuRD-mediated deacetylation of H3K27 facilitates recruitment of polycomb repressive complex 2 to direct gene repression, *EMBO J.* 31 (2012) 593–605.
- [211] C. Gorrini, M. Squatrito, C. Luise, N. Syed, D. Perna, L. Wark, et al., Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response, *Nature* 448 (2007) 1063–1067.
- [212] Z. Herceg, W. Hulla, D. Gell, C. Cuenin, M. Leonart, S. Jackson, et al., Disruption of Trp53 causes early embryonic lethality and defects in cell cycle progression, *Nat. Genet.* 29 (2001) 206–211.
- [213] Y. Hu, J.B. Fisher, S. Koprowski, D. McAllister, M.S. Kim, J. Lough, Homozygous disruption of the Tip60 gene causes early embryonic lethality, *Dev. Dyn.* 238 (2009) 2912–2921.
- [214] T.G. Fazzio, J.T. Huff, B. Panning, An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity, *Cell* 134 (2008) 162–174.
- [215] M. Squatrito, C. Gorrini, B. Amati, Tip60 in DNA damage response and growth control: many tricks in one HAT, *Trends Cell Biol.* 16 (2006) 433–442.
- [216] S.K. Pradhan, T. Su, L. Yen, K. Jacquet, C. Huang, J. Cote, et al., EP400 deposits H3.3 into promoters and enhancers during gene activation, *Mol. Cell* 61 (2016) 27–38.
- [217] L. Wang, Y. Du, J.M. Ward, T. Shimbo, B. Lackford, X. Zheng, et al., INO80 facilitates pluripotency gene activation in embryonic stem cell self-renewal, reprogramming, and blastocyst development, *Cell Stem Cell* 14 (2014) 575–591.
- [218] L. Bintu, J. Yong, Y.E. Antebi, K. McCue, Y. Kazuki, N. Uno, et al., Dynamics of epigenetic regulation at the single-cell level, *Science* 351 (2016) 720–724.
- [219] R.S. Illingworth, J.J. Holzspies, F.V. Roske, W.A. Bickmore, J.M. Brickman, Polycomb enables primitive endoderm lineage priming in embryonic stem cells, *Elife* 5 (2016).
- [220] H.G. Leitch, M.A. Surani, P. Hajkova, DNA (de)methylation: the passive route to naivety? *Trends Genet.* 32 (2016) 592–595.
- [221] N. Festuccia, R. Osomo, V. Wilson, I. Chambers, The role of pluripotency gene regulatory network components in mediating transitions between pluripotent cell states, *Curr. Opin. Genet. Dev.* 23 (2013) 504–511.
- [222] T. Boroviak, R. Loos, P. Bertone, A. Smith, J. Nichols, The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification, *Nat. Cell Biol.* 16 (2014) 516–528.
- [223] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell* 126 (2006) 663–676.
- [224] D. Egli, G. Birkhoff, K. Eggan, Mediators of reprogramming: transcription factors and transitions through mitosis, *Nat. Rev. Mol. Cell Biol.* 9 (2008) 505–516.
- [225] C. Alabert, T.K. Barth, N. Reveron-Gomez, S. Sidoli, A. Schmidt, O.N. Jensen, et al., Two distinct modes for propagation of histone PTMs across the cell cycle, *Genes Dev.* 29 (2015) 585–590.
- [226] H.S. Lee, S.A. Lee, S.K. Hur, J.W. Seo, J. Kwon, Stabilization and targeting of INO80 to replication forks by BAP1 during normal DNA synthesis, *Nat. Commun.* 5 (2014) 5128.
- [227] S.M. Cohen, P.D. Chastain II, G.B. Rosson, B.S. Groh, B.E. Weissman, D.G. Kaufman, et al., BRG1 co-localizes with DNA replication factors and is required for efficient replication fork progression, *Nucleic Acids Res.* 38 (2010) 6906–6919.
- [228] H. Julienne, B. Audit, A. Armeodo, Embryonic stem cell specific “master” replication origins at the heart of the loss of pluripotency, *PLoS Comput. Biol.* 11 (2015), e1003969.
- [229] X.Q. Ge, J. Han, E.C. Cheng, S. Yamaguchi, N. Shima, J.L. Thomas, et al., Embryonic stem cells license a high level of dormant origins to protect the genome against replication stress, *Stem Cell Rep.* 5 (2015) 185–194.
- [230] A. Gagliardi, N.P. Mullin, Z. Ying Tan, D. Colby, A.I. Kousa, F. Halbritter, et al., A direct physical interaction between Nanog and Sox2 regulates embryonic stem cell self-renewal, *EMBO J.* 32 (2013) 2231–2247.
- [231] M.H. Kagey, J.J. Newman, S. Bilodeau, Y. Zhan, D.A. Orlando, N.L. van Berkum, et al., Mediator and cohesin connect gene expression and chromatin architecture, *Nature* 467 (2010) 430–435.
- [232] J. Yan, M. Enge, T. Whittington, K. Dave, J. Liu, I. Sur, et al., Transcription factor binding in human cells occurs in dense clusters formed around cohesin anchor sites, *Cell* 154 (2013) 801–813.
- [233] E.F. Michelotti, S. Sanford, D. Levens, Marking of active genes on mitotic chromosomes, *Nature* 388 (1997) 895–899.
- [234] C.C. Hsiung, C.S. Morrissey, M. Udugama, C.L. Frank, C.A. Keller, S. Baek, et al., Genome accessibility is widely preserved and locally modulated during mitosis, *Genome Res.* 25 (2015) 213–225.
- [235] F. Verdeguer, S. Le Corre, E. Fischer, C. Callens, S. Garbay, A. Doyen, et al., A mitotic transcriptional switch in polycystic kidney disease, *Nat. Med.* 16 (2010) 106–110.
- [236] J.M. Caravaca, G. Donahue, J.S. Becker, X. He, C. Vinson, K.S. Zaret, Bookmarking by specific and nonspecific binding of FoxA1 pioneer factor to mitotic chromosomes, *Genes Dev.* 27 (2013) 251–260.
- [237] D.W. Young, M.Q. Hassan, J. Pratap, M. Galindo, S.K. Zaidi, S.H. Lee, et al., Mitotic occupancy and lineage-specific transcriptional control of rRNA genes by Runx2, *Nature* 445 (2007) 442–446.
- [238] R. Zhao, T. Nakamura, Y. Fu, Z. Lazar, D.L. Spector, Gene bookmarking accelerates the kinetics of post-mitotic transcriptional re-activation, *Nat. Cell Biol.* 13 (2011) 1295–1304.
- [239] G. Martello, T. Sugimoto, E. Diamanti, A. Joshi, R. Hannah, S. Ohtsuka, et al., Esrrb is a pivotal target of the Gsk3/Tcf3 axis regulating embryonic stem cell self-renewal, *Cell Stem Cell* 11 (2012) 491–504.

- [240] S.S. Teves, L. An, A.S. Hansen, L. Xie, X. Darzacq, R.A. Tjian, Dynamic mode of mitotic bookmarking by transcription factors, *Elife* 5 (2016), e22280.
- [241] R.P. Halley-Stott, J. Jullien, V. Pasque, J. Gurdon, Mitosis gives a brief window of opportunity for a change in gene transcription, *PLoS Biol.* 12 (2014), e1001914.
- [242] Z. Shao, C. Yao, A. Khodadadi-Jamayran, W. Xu, T.M. Townes, M.R. Crowley, et al., Reprogramming by de-bookmarking the somatic transcriptional program through targeting of BET Bromodomains, *Cell Rep.* 16 (2016) 3138–3145.
- [243] C. Faust, A. Schumacher, B. Holdener, T. Magnuson, The eed mutation disrupts anterior mesoderm production in mice, *Development* 121 (1995) 273–285.
- [244] M. de Napoles, J.E. Mermoud, R. Wakao, Y.A. Tang, M. Endoh, R. Appanah, et al., Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation, *Dev. Cell* 7 (2004) 663–676.
- [245] M. del Mar Lorente, C. Marcos-Gutierrez, C. Perez, J. Schoorlemmer, A. Ramirez, T. Magin, et al., Loss- and gain-of-function mutations show a polycomb group function for Ring1A in mice, *Development* 127 (2000) 5093–5100.
- [246] D.L. Carlone, J.H. Lee, S.R. Young, E. Dobrota, J.S. Butler, J. Ruiz, et al., Reduced genomic cytosine methylation and defective cellular differentiation in embryonic stem cells lacking CpG binding protein, *Mol. Cell. Biol.* 25 (2005) 4881–4891.
- [247] A. Bertero, P. Madrigal, A. Galli, N.C. Hubner, I. Moreno, D. Burks, et al., Activin/nodal signaling and NANOG orchestrate human embryonic stem cell fate decisions by controlling the H3K4me3 chromatin mark, *Genes Dev.* 29 (2015) 702–717.
- [248] S.L. Smith-Roe, S.J. Bultman, Combined gene dosage requirement for SWI/SNF catalytic subunits during early mammalian development, *Mamm. Genome* 24 (2013) 21–29.
- [249] C. Schaniel, Y.S. Ang, K. Ratnakumar, C. Cormier, T. James, E. Bernstein, et al., Smarcc1/Baf155 couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells, *Stem Cells* 27 (2009) 2979–2991.
- [250] M.D. Kaeser, A. Aslanian, M.Q. Dong, J.R. Yates III, B.M. Emerson, BRD7, a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells, *J. Biol. Chem.* 283 (2008) 32,254–32,263.
- [251] M. Guzman-Ayala, M. Sachs, F.M. Koh, C. Onodera, A. Bulut-Karslioglu, C.J. Lin, et al., Chd1 is essential for the high transcriptional output and rapid growth of the mouse epiblast, *Development* 142 (2015) 118–127.
- [252] S. Suzuki, Y. Nozawa, S. Tsukamoto, T. Kaneko, I. Manabe, H. Imai, et al., CHD1 acts via the Hmgpi pathway to regulate mouse early embryogenesis, *Development* 142 (2015) 2375–2384.
- [253] C.G. Marfella, Y. Ohkawa, A.H. Coles, D.S. Garlick, S.N. Jones, A.N. Imbalzano, Mutation of the SNF2 family member Chd2 affects mouse development and survival, *J. Cell. Physiol.* 209 (2006) 162–171.
- [254] L. Siggins, L. Cordeddu, M. Ronnerblad, A. Lennartsson, K. Ekwall, Transcription-coupled recruitment of human CHD1 and CHD2 influences chromatin accessibility and histone H3 and H3.3 occupancy at active chromatin regions, *Epigenet. Chromatin* 8 (2015) 4.
- [255] D. Zhu, J. Fang, Y. Li, J. Zhang, Mbd3, a component of NuRD/Mi-2 complex, helps maintain pluripotency of mouse embryonic stem cells by repressing trophoctoderm differentiation, *PLoS One* 4 (2009), e7684.
- [256] A. O'Shaughnessy-Kirwan, J. Signolet, I. Costello, S. Gharbi, B. Hendrich, Constraint of gene expression by the chromatin remodelling protein CHD4 facilitates lineage specification, *Development* 142 (2015) 2586–2597.
- [257] J. Landry, A.A. Sharov, Y. Piao, L.V. Sharova, H. Xiao, E. Southon, et al., Essential role of chromatin remodeling protein Bptf in early mouse embryos and embryonic stem cells, *PLoS Genet.* 4 (2008), e1000241.