Open chromatin in pluripotency and reprogramming

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Abstract | Pluripotent stem cells can be derived from embryos or induced from adult cells by reprogramming. They are unique among stem cells in that they can give rise to all cell types of the body. Recent findings indicate that a particularly 'open' chromatin state contributes to maintenance of pluripotency. Two principles are emerging: specific factors maintain a globally open chromatin state that is accessible for transcriptional activation; and other chromatin regulators contribute locally to the silencing of lineage-specific genes until differentiation is triggered. These same principles may apply during reacquisition of an open chromatin state upon reprogramming to pluripotency, and during de-differentiation in cancer.

Embryonic stem (ES) cells are the prototypical pluripotent stem cell[1–3]: they have the capacity to generate differentiated progeny from all three embryonic germ layers (endoderm, mesoderm and ectoderm), as well as the germline[4]. ES cells also have a very high self-renewing capacity and can be expanded essentially indefinitely in culture. In contrast to ES cells, adult stem cells such as neural stem cells[5] or haematopoietic stem cells[6] have a more restricted differentiation capacity: they usually generate cells of the tissue in which they reside and are, therefore, called multipotent.

In recent years, there has been an increased interest in pluripotent stem cells because of their promise as models for the study of development and disease *in vitro* (for examples, see REFS 7,8). However, the derivation of ES cells from early embryos raises technical and ethical limitations to their use in research and the clinic. Pluripotent stem cells can also be derived from both the fetal and adult germlines[9–11], and by somatic cell reprogramming. Three major routes have been described for somatic cell reprogramming to pluripotency: nuclear transfer from a somatic cell to an enucleated oocyte; fusion of a somatic cell with an ES cell; and induction of pluripotency in somatic cells by overexpression of key transcription factors (BOX 1). All of these reprogramming methods are likely to remain useful and informative in the years ahead. The relative advantages and disadvantages of each reprogramming method have been reviewed elsewhere[12] and are not discussed here.

Major excitement has surrounded the process by which pluripotency is induced in somatic cells in the 4 years since it was described[13], because of its technical simplicity and broad applicability. Through ectopic expression of genes that are over-represented in ES cells, a set of four transcription factors (OCT4 (also known as POU5F1), Sry-box containing gene 2 (SOX2), myelocytomatosis oncogene (MYC) and Krüppel-like factor 4 (KLF4)) was shown to reprogramme differentiated mouse cells (both embryonic and adult somatic cells) into induced pluripotent stem (iPS) cells that are very similar to ES cells. The surprising ability of only four factors to induce such a dramatic change in cell fate initiated a whole new field of research. Importantly, human cells[14–17] can also be converted into iPS cells using either the same four factors as in mouse cells or a different combination of factors: OCT4, SOX2, LIN28 and NANOG[17]. Therefore, somatic cell reprogramming, in particular the induction of pluripotency, greatly expands the options for basic research and potential clinical applications of pluripotent stem cells. Understanding the molecular regulation of pluripotency is fundamentally important and will facilitate the safe and efficient application of pluripotent stem cells in the clinic.

The pluripotent stem cell state is under the control of a transcriptional circuitry that includes the reprogramming factors mentioned above (reviewed in REF. 12). Recent studies indicate that this transcriptional programme is implemented in the context of an 'open' chromatin state, and it has been proposed that this state allows transcriptional programmes to switch rapidly upon induction of differentiation[18]. This may be particularly important in pluripotent stem cells, where a broad spectrum of differentiation options needs to be available.

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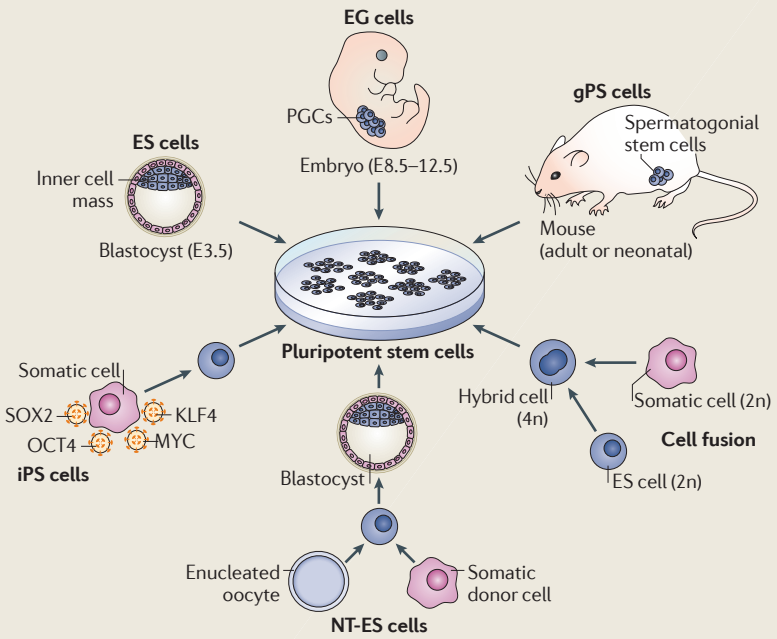
Box 1 | **Pluripotent stem cells can be derived from several sources**



There are three sources of pluripotent stem cells *in vivo* (see the figure, top half). Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst, before embryo implantation[1–3]. Embryonic germ (EG) cells are derived from primordial germ cells (PGCs) during mid-gestation (embryonic days 8.5–12.5 in the mouse)[9,10] and germline-derived pluripotent stem (gPS) cells are derived from spermatogonial stem cells of neonatal and adult testes[11].

In addition, three major routes for somatic cell reprogramming to pluripotency have been described[12] (see the figure, bottom half): fusion between a somatic cell and an ES cell giving rise to reprogrammed hybrid cells; the generation of nuclear transfer embryonic stem (NT-ES) cells, produced by reprogramming of a somatic nucleus by an enucleated oocyte, which is then cultured to the blastocyst stage to allow derivation of ES cells; and the production of induced pluripotent stem (iPS) cells, derived by somatic cell overexpression of reprogramming transcription factors, most commonly OCT4 (also known as POU5F1), Sry-box containing gene 2 (SOX2), myelocytomatosis oncogene (MYC) and Krüppel-like factor 4 (KLF4)[13].

Here, we discuss how chromatin organization is regulated in pluripotent stem cells. We begin by giving a historical perspective of how the concept of open chromatin has evolved and how it has been associated with pluripotency. We then review recent insights into the action of chromatin-remodelling factors that maintain a globally open chromatin state in pluripotent stem cells. Finally, we discuss the implications of these insights for our understanding of cellular reprogramming, and point out recent parallels found between open chromatin and cancer.

Open chromatin and pluripotency

Defining open chromatin. The term chromatin was coined by Walther Flemming in 1882, after he developed novel histological staining methods that enabled him to observe a unique fibrous structure in the nucleus. This structure was readily stained and was therefore named chromatin ('stainable material')[19,20]. Almost 50 years later, in 1928, the distinction between heterochromatin and euchromatin was made by Emil Heitz. He distinguished these two chromatin components based on differential compaction in interphase nuclei[21]: heterochromatin represented the more densely stained, compacted areas, whereas euchromatin represented the sparsely stained chromatin.

On the basis of predominantly histological evidence, many stem and progenitor cells, from neoblast cells in planaria[22] to haematopoietic stem cells in mammals[23], have been classically described as having a typical open chromatin conformation that is mostly devoid of heterochromatin. In such studies, histological analysis of the nucleus was sufficient to suggest a significant difference in chromatin structure between these progenitor cells and their differentiated progeny.

Open chromatin in pluripotent stem cells. The idea of open chromatin is supported by more than histological examinations and, in the past several years, the chromatin state of pluripotent stem cells has attracted considerable attention owing to its distinct features[24]. Indeed, chromatin in pluripotent stem cells is increasingly being recognized as open when compared with chromatin in somatic cells, implying that its overall structure is less condensed and that the ratio between euchromatin and heterochromatin is higher than in differentiating cells.

The first line of evidence came from visualizing chromatin in ES cells using electron microscopy: heterochromatin was prevalent in differentiated cells but much less so in undifferentiated ES cells[25]. Similarly, electron spectroscopic imaging (ESI) demonstrated that the majority of chromatin in ES cells is homogeneously spread and largely devoid of compact heterochromatin blocks, whereas in differentiated cells chromatin appeared heterogeneous with distinct blocks of compaction[26]. Importantly, this pattern of chromatin organization was recently found *in vivo*: cells in the inner cell mass (ICM) of the mouse blastocyst at day 3.5, which are the source of ES cells, share the same open chromatin conformation as ES cells[27]. ICM cells have highly dispersed chromatin, with a significantly lower number of condensed clusters relative to lineage-committed cells. Analysis of global chromatin compaction using nucleases such as DNase I and micrococcal nuclease (MNase) also indicates that chromatin becomes less accessible, and thus less sensitive to nuclease digestion, upon differentiation of ES cells to embryoid bodies (EBs) (A.A. and E.M., unpublished observations, and K. Ura, personal communication) or induction of differentiation with retinoic acid[28].

The relatively low abundance of heterochromatin also supports the idea of chromatin being in an open conformation. Western blot and immunofluorescence analyses of histone post-translational modifications (PTMs), such as histone H3 tri-methylation on Lys9 (H3K9me3), that are enriched in heterochromatin (BOX 2), suggest that ES cells have considerably less heterochromatin than differentiated cells[29]. Subsequently, ChIP–chip assays for H3K9me2, which forms 'large organized chromatin K9 modifications' (LOCKs), showed that these domains spread considerably during differentiation[30]. Furthermore, ChIP–seq analyses showed that H3K9me3 and H3K27me3 expand from around 4% genome coverage in ES cells to 12% and 16%, respectively, in

Endoderm
The innermost of the three germ layers that are formed during embryonic development. Prominent examples of endodermal tissues include the epithelia of the gastrointestinal and respiratory tracts, thyroid, liver and pancreas, as well as of the auditory and urinary systems.

Mesoderm
The middle of the three germ layers that are formed during embryonic development. Prominent examples of mesodermal tissues include bone, cartilage, blood, muscle, heart, connective tissue and kidney.

Box 2 | **Chromatin and epigenetic patterns**

Chromatin is a complex assembly of DNA, histone proteins and other non-histone protein components. Histone proteins form chromatin building blocks, the nucleosomes, around which DNA is wrapped. Each nucleosome consists of an octamer of the canonical core histones H2A, H2B, H3 and H4 and, between two nucleosomes, the histone H1 acts as a linker. Alterations to the chromatin structure that do not affect the genomic sequence are defined as epigenetic modifications. These epigenetic patterns include methylation of DNA, post-translational modifications (PTMs) of histones (also called histone marks) and histone variants that are incorporated into nucleosomes.

The amino-terminal tails of histones are subject to various PTMs with either activating or inhibiting effects on transcription, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, poly-ADP ribosylation and proline isomerization. The most commonly studied are: methylation, in which histone methyltransferases (HMTs) add a methyl group and histone demethylases (HDMs) remove this group; and acetylation, in which the addition and removal of an acetyl group is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Typically, the tri-methylation of Lys 4 in H3 (H3K4me3), together with histone acetylation, signals binding of RNA polymerase II and transcriptional activation. H3K27me and H3K9me3 signal a repressive transcriptional state, although through recruitment of distinct silencing factors. Chromatin-remodelling complexes also often include regulators of PTMs and may mediate incorporation of histone variants (such as H3.3 and H2AZ or macroH2A), which can be associated with either inactive or active chromatin[58].

Modification of the DNA itself is also important. Cytosine DNA methylation on CpG islands is mediated by DNA methyltransferases (DNMTs) and is usually repressive. DNA methylation is typically a more stable and inheritable epigenetic pattern that can persist for several cell generations. However, DNA methylation can be lost passively by a lack of methylation after replication, and there also appear to be factors that can actively de-methylate DNA[58]. See FIG. 2 for schematic details of these histone and DNA modifications.

differentiated cells[31]. On the other hand, histone acetylation, a general mark of open chromatin, has been shown to be increased in undifferentiated human ES cells, particularly at the H3K9 residue[32].

There is also indirect evidence that supports the concept of a preferentially open chromatin state in pluripotent stem cells. In ES cells, fluorescence recovery after photobleaching experiments have indicated that chromatin contains a fraction of loosely bound architectural chromatin proteins, such as core[33] and linker histones and heterochromatin protein 1 (HP1)[29]; this fraction is not observed in differentiating cells[29,33]. In addition, the ES cell genome is transcriptionally hyperactive: it transcribes normally silenced repetitive elements as well as coding and non-coding regions, resulting in increased levels of total RNA and mRNA[26] (FIG. 1). One way to counteract this pervasive transcription in ES cells may be by proteasome-mediated degradation of pre-initiation transcription assemblies that form at specific regulatory genes primed for transcription[34].

Taken together, these data indicate that chromatin in ES cells is globally decondensed compared with differentiated cells, and that a smaller fraction of the genome in ES cells is organized as repressive heterochromatin.

Control of the chromatin landscape

Chromatin in ES cells is characterized by a distinct set of features, and a better knowledge of the enzymes that modify this structure has provided insights into the control of chromatin state. Genome-wide mapping of core histone PTMs, or histone marks, has been of great use in defining the epigenetic patterns (BOX 2) that may regulate pluripotency[30,31,35,36]. In addition, several chromatin-modifying enzymes, such as DNA methyltransferases (DNMTs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone acetyltransferases (HATs), histone deacetylases (HDACs) and chromatin-remodelling proteins, have recently been shown to have important roles in ES cells, and these are described below. Interplay between chromatin regulation and the transcriptional network that governs pluripotency[37] is also critical and has been reviewed elsewhere[38].

Chromatin poised for differentiation. ES cells have a globally open chromatin structure with abundant levels of epigenetic marks that are indicative of active transcription, such as histone H3K4me3 and acetylation of histones H3 and H4 (REFS 29,32,39). However, there must be countering mechanisms that silence developmental regulatory genes and prevent premature differentiation. It is thought that these developmental regulators are silenced but poised for activation by the presence of both the activating mark (H3K4me3) and the repressive mark (H3K27me3)[35,36,39]. These 'bivalent' domains, although not strictly specific to ES cells, may lead to the rapid activation of lineage-specific genes through loss of H3K27me3 when differentiation is induced.

The repressive H3K27 methylation mark is regulated by the polycomb group (PcG) proteins. PcG proteins include the polycomb repressive complex 2 (PRC2), which is involved in the addition of the histone mark, and PRC1, which recognizes this mark. Genome-wide analyses of several PcG proteins in human and mouse ES cells revealed their local enrichment in silenced developmental regulatory genes[40,41]. Moreover, the target genes of PcG proteins tend to be co-occupied by the transcription factors OCT4, SOX2 and NANOG, which are critical regulators of the pluripotent state. However, PcG proteins are not essential for ES cell self-renewal: in the absence of PcG proteins such as embryonic ectodermal development (EED)[40,42], Suppressor of zeste 12 homologue (SUZ12)[41] and Enhancer of zeste homologue 2 (EZH2)[43], ES cells can still be propagated in the undifferentiated state. However, these PcG-deficient ES cells cannot silence several lineage-specific markers and have differentiation defects. PcG proteins are recruited to target DNA by the cofactor JARID2 (jumonji/ARID domain-containing 2)[44]. JARID2 also seems to inhibit the enzymatic methyltransferase activity of PRC2, and may therefore regulate both targeting and fine-tuning of PRC2 activity in ES cells and during differentiation[44–47].

Heterochromatin regulation in ES cells. Another histone mark that is commonly associated with gene repression is methylation at H3K9, which increases with differentiation of ES cells. One enzyme that is responsible for H3K9 methylation is the HMT G9a (also known as EHMT2). Interestingly, G9a is required for the silencing of OCT4 upon differentiation[48]. G9a binds directly to the promoter of OCT4 and leads to H3K9 methylation,

Ectoderm
The outermost of the three germ layers that are formed during embryonic development. Prominent examples of ectodermal tissues include the nervous system, hair, skin, nails and eyes, as well as the various derivatives of the neural crest, including bones of the head and peripheral nerves.

Heterochromatin
Highly compacted chromatin that is transcriptionally inactive. Includes structural regions of the chromosome, such as centromeres, that lack genes ('constitutive' heterochromatin) and regions in which genes are silenced in a given cell type ('facultative' heterochromatin).

Euchromatin
A form of chromatin that is relatively decondensed and often transcriptionally active during interphase.

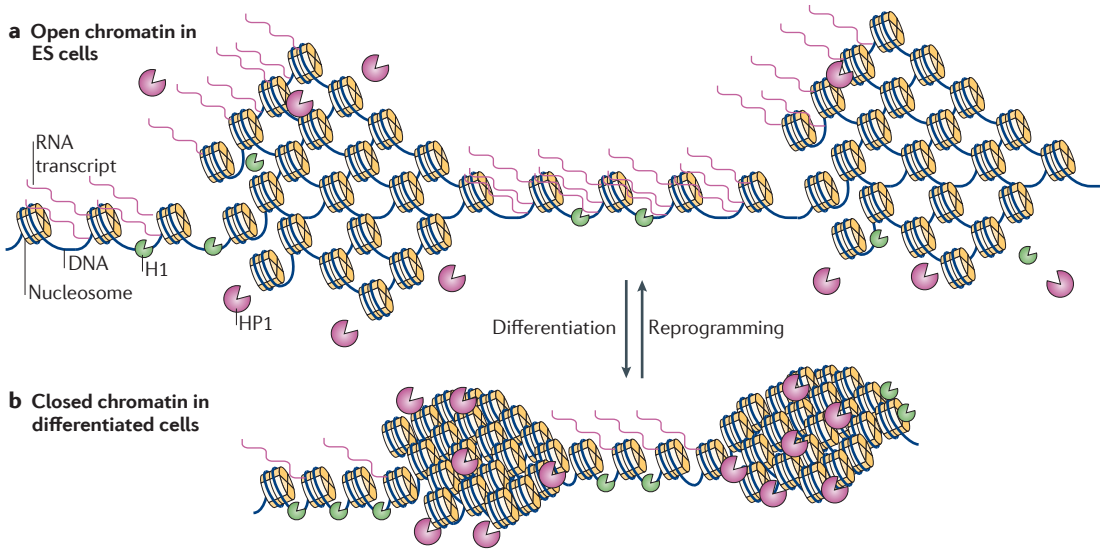


Figure 1 | **Chromatin in pluripotent stem cells versus differentiated cells.** The structure of chromatin differs between undifferentiated embryonic stem (ES) cells (**a**) and differentiated cells (**b**) in several ways. Chromatin structure becomes more condensed upon differentiation and more open upon reprogramming. In ES cells, chromatin is globally decondensed; there are fewer heterochromatin foci and they are larger and more dispersed compared with those of differentiated cells. Architectural chromatin proteins, represented here by the histone H1 and heterochromatin protein 1 (HP1), are loosely bound to chromatin in ES cells and are bound more tightly to chromatin in differentiated cells. In ES cells, chromatin, including heterochromatin, is transcriptionally hyperactive, shown here by high levels of RNA transcripts.

Electron spectroscopic imaging
(ESI). Energy-filtered transmission electron microscopy, in which the image is formed only by electrons transmitted within a certain energy window. It allows direct quantitative imaging of elements within the specimen.

Embryoid body
(EB). A cellular aggregate that is produced when ES cells are induced to differentiate in non-adherent conditions that mimic the early stages of embryogenesis.

ChIP–chip
Chromatin immunoprecipitation (ChIP) followed by microarray. ChIP is a method that allows isolation of DNA sequences that are bound to a protein of interest using specific antibodies. DNA isolated by ChIP is denatured and hybridized to a tiling array, which typically includes probes covering the entire genome. Paired probes indicate that the protein of interest was bound to that particular region of DNA.

ChIP–seq
Chromatin immunoprecipitation (ChIP) followed by sequencing. Refers to high-throughput sequencing of ChIP-isolated DNA, and provides genome-wide information of the DNA binding sites of the protein of interest.

Heterochromatin protein 1
(HP1). A heterochromatin-binding protein that recognizes and binds to histone H3 tri-methylated on Lys9. It includes three isoforms (α, β and γ), which are encoded by three different genes (*CBX5*, *CBX1* and *CBX3*, respectively).

Proteasome
A large multisubunit protein complex that degrades proteins. Undesired proteins are labelled for degradation by the addition of a chain of the small protein ubiquitin; a process that is mediated by a family of enzymes called ubiquitin ligases.

which is followed by recruitment of DNMTs to signal a more definite repressive state. G9a may have a dual role of methylating H3K9 (as a known HMT) and recruiting DNMTs — an example of how several layers of regulation accomplish proper silencing of a particular gene[49]. Therefore, the increase in heterochromatin that occurs upon ES cell differentiation may directly contribute to the silencing of regulators of self-renewal and pluripotency. G9a is also required for the establishment of domains of H3K9me2 (LOCKs) in differentiated cells[30], suggesting a more global role for G9a in differentiation-induced heterochromatinization.

The low level of H3K9 methylation in undifferentiated ES cells is maintained by the histone H3K9 HDMs JMJD1A (jumonji domain-containing 1A; also known as KDM3A) and JMJD2C (also known as KDM4C). These regulate global levels of the repressive marks H3K9me2 and H3K9me3, respectively, and maintain the ES cell state by directly demethylating H3K9 at the promoter regions of core ES cell factors, allowing their expression[50]. Interestingly, the genes encoding JMJD1A and JMJD2C are regulated by OCT4, representing a positive feedback-loop that integrates the action of transcription factors and histone modifiers to maintain the undifferentiated ES cell state.

A different layer of epigenetic regulation in ES cells is the DNA methylation of CpG islands. DNMTs are responsible for this repressive mark, which is correlated with specific histone marks[51]: methylated CpG islands are present mainly at promoter regions of repressed genes, usually correlated with unmethylated H3K4 and H3K9me3, and represent around 30% of genes in ES cells[52]. However, cross-referencing genomic regions with methylation patterns and binding of OCT4, NANOG, SOX2 and PcG revealed little overlap[52]. Moreover, ES cells show a significant enrichment of methylation outside CpG islands, a feature that seems to be unique to these cells[53]. These observations suggest that DNA methylation may represent a unique epigenetic layer that complements other mechanisms of gene repression and contributes to tight regulation of the transcriptional programmes that are activated upon differentiation.

Chromatin remodelling in ES cells

The addition or removal of histone marks or DNA methylation is only one way in which the chromatin state can affect the transcriptional programme and thus pluripotency in stem cells. The structure of chromatin itself, and the positions of nucleosomes, can be altered both globally and at the level of specific genetic loci by chromatin-remodelling proteins that alter the histone–DNA contacts using the energy of ATP hydrolysis[54]. The disruption of the histone–DNA contact itself is poorly understood, but the consequences are that DNA becomes exposed to regulatory proteins, and nucleosomes and the histones become more actively mobile[55].

Chromatin-remodelling proteins can be divided into four families: SWI/SNF (switch/sucrose nonfermentable), CHD (chromodomain helicase DNA-binding), ISWI (imitation switch) and INO80 (inositol-requiring 80). Chromatin remodellers usually form a complex that contains a catalytic subunit with a SWI2/SNF2 ATPase domain, a subunit that recognizes chromatin, and additional regulatory subunits that mediate interactions with other proteins and with chromatin itself[56]. At least one member of each of these four families is essential

for mouse embryogenesis (TABLE 1), demonstrating the central role that chromatin remodellers have in development. Recent studies have begun to shed light on the specific roles that chromatin remodellers have in ES cells.

SWI/SNF family. The SWI/SNF family is composed of two major complexes: BRG- or BRM-associated factor (BAF) and polybromo BAF (PBAF)[57] (TABLE 1). There is some heterogeneity in the composition of the BAF and PBAF complexes in different cell types and tissues[58]. ES cells have a specialized subunit composition termed esBAF, which is dynamically regulated during differentiation[59], and it is not yet clear whether two distinct complexes (esBAF and esPBAF) exist in ES cells or whether the different subunits combine to form a single esBAF.

BRG1 (also known as SMARCA4) is the catalytic subunit of the esBAF complex. It is downregulated upon differentiation and seems to be gradually replaced by a different catalytic subunit, BRM[59,60]. *Brg1*-null mice die at the peri-implantation stage[61], and knockdown experiments in ES cells resulted in aberrant morphology, decreased proliferation rate and reduced differentiation capacity[26,59,62,63]. Furthermore, genome-wide ChIP–chip and ChIP–seq experiments revealed enrichment of BRG1 at promoter regions of genes that are also occupied by the pluripotency regulators OCT4, SOX2 and NANOG[63,64]. Intriguingly, BRG1 inhibition in ES cells leads to upregulation of both developmental genes and ES cell-specific genes. These results suggest that BRG1 may not only contribute to the repression of developmental genes but may also fine-tune the expression level of ES cell-specific genes, such as *Oct4* and *Sox2* (REFS 63,64).

An additional member of the BAF complex that has a role in ES cells is BAF250 (also known as ARID1), which includes two related subunits, BAF250A and BAF250B. BAF250A incorporation into the BAF complex is most prominent in undifferentiated ES cells, whereas BAF250B is mostly incorporated after differentiation[59]. *Baf250a*-deficient mouse ES cells fail to maintain the expression of stem cell markers and instead activate genes with known roles in early development and organogenesis[65]. Furthermore, *Baf250a*$^{-/-}$ ES cells are prone to differentiation but they seem to lose the ability to form cells of the mesodermal lineage, which is in agreement with the absence of detectable mesoderm in early mouse *Baf250a*$^{-/-}$ embryos[65]. Unlike *Baf250a*$^{-/-}$ ES cells, *Baf250b*$^{-/-}$ ES cells give rise to all three germ layers[66], but disruption of *Baf250b* results in reduced self-renewal ability and accelerated ES cell differentiation[66].

There are mixed reports as to the role of BAF155 (also known as SMARCC1) in ES cells. It is highly expressed in ES cells[59,28] and its reduction leads to aberrant colony morphology[62] and decreased OCT4 expression[64] in undifferentiated ES cells. However, in differentiating ES cells, loss of BAF155 results in perturbed chromatin condensation and increased OCT4 expression[28]. Based on these results, it can be speculated that the stoichiometry of different BAF subunits, and not their actual levels, determines their function, perhaps reconciling these studies.

CHD family. Four subunits from the CHD family of chromatin-remodelling enzymes — CHD1, CHD3, CHD4 and CHD7 — are implicated in ES cell identity and function, although their mechanisms of action differ. CHD1 and CHD7 have not yet been clearly associated with a known complex (TABLE 1), but the latter binds multiple subunits of the PBAF complex in neural crest cells derived from human ES cells. In these neural crest cells[67] and mouse ES cells[68], CHD7 was enriched at enhancer regions, together with H3K4me1, suggesting that CHD7 may maintain transcriptional competence in both undifferentiated and differentiating ES cells.

CHD1 binds globally to active euchromatin and colocalizes with RNA polymerase II (RNAPII) in ES cells[69]. ES cells in which CHD1 has been depleted by RNA interference accumulate high levels of heterochromatin and, although they can be propagated in the undifferentiated state, they cannot differentiate normally. These results indicate that CHD1 establishes a balance between euchromatin and heterochromatin in ES cells, which may be critical for the maintenance of pluripotency.

CHD3 and CHD4 constitute the catalytic subunit of the nucleosome-remodelling (NuRD) complex (TABLE 1), which has been implicated in regulation of ES cells. For example, ES cells lacking the NuRD subunit methyl-CpG-binding domain 3 (MBD3) retain their OCT4 expression when induced to differentiate, and show aberrant differentiation potential[70,71]. MBD3-knockdown ES cells also express trophectodermal markers, which are not usually detected in ES cells. Deletion of another subunit, encoded by *Hdac1*, also results in aberrant differentiation of mouse ES cells, leading to spontaneous generation of mesodermal and ectodermal lineages at the expense of endoderm[72]. Importantly, knockout of *Hdac1* (but not *Hdac2*) leads to mouse embryonic lethality[73–76]. NuRD therefore seems to have a dual role in silencing differentiation genes in ES cells as well as ES cell-specific genes during differentiation. Finally, NuRD subunits MBD3 and metastasis-associated 2 (MTA2) interact with the SWI/SNF component BRG1 specifically in ES cells but not in differentiating cells[59], implying that there may be crosstalk between chromatin-remodelling complexes in pluripotent cells.

ISWI family. The ISWI family of remodellers can form three distinct complexes — nucleosome-remodelling factor (NURF), chromatin accessibility complex (CHRAC) and ATP-utilizing chromatin assembly and remodelling factor (ACF) — of which, the NURF complex seems to have the most prominent role in ES cells. Bromodomain PHD finger transcription factor (BPTF), a member of the NURF complex, is required for ES cell differentiation both *in vivo* and *in vitro*. *Bptf*-knockout ES cells cannot form teratomas, and *Bptf*-knockout EBs exhibit severely defective expression of all three germ layer markers. In line with this, *Bptf*-knockout mouse embryos are defective in the establishment of the anterior–posterior axis during the earliest stages of development and are embryonic lethal at day 8.5 (REF. 77) (TABLE 1).

CpG island
A genomic region which contains a high content of cytosine (C) and guanine (G) dinucleotides (the 'p' refers to the phosphodiester bond linking the two bases). CpG islands are found in many mammalian promoters, and unlike scattered CpGs throughout the genome, which are usually hypermethylated, promoter CpG islands are normally hypomethylated.

Helicase
A protein that can unwind DNA or RNA.

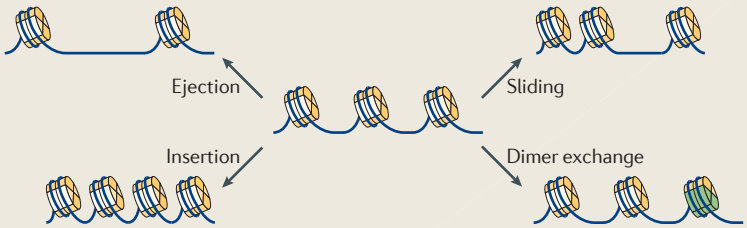
Teratoma
A confined tumour, originating from pluripotent cells, that includes tissues of the three germ layers, endoderm, mesoderm and ectoderm.

Table 1 | **Chromatin remodellers in ES cells**

Complex	Protein subunits	Effect on ES cells			Embryonic lethality
		Morphology	Proliferation	Differentiation	
SWI/SNF family					
BAF	β-actin	N/A	N/A	N/A	E9.5 (REF. 135)
	BAF47	N/A	N/A	N/A	Peri-implantation[134]
	BAF53A, BAF57, BAF60A	N/A	N/A	N/A	N/A
	BAF155	Yes[62]	Yes[59]	N/A	Post-implantation[133]
	BAF250A	Yes[62,65]	Yes[65]	Yes[65]	E6.5 (REF. 65)
	BAF250B	No[66]	Yes[66]	Yes[66]	N/A
	BRG1*	Yes[59,62,63]	Yes[26,59]	Yes[26,59,63]	Peri-implantation[61]
PBAF	β-actin	N/A	N/A	N/A	E9.5 (REF. 135)
	BAF47	N/A	N/A	N/A	Peri-implantation[134]
	BAF53A, BAF57, BAF60A, BAF180, BAF200	N/A	N/A	N/A	N/A
	BAF155	Yes[62]	Yes[59]	N/A	Post-implantation[133]
	BRG1*	Yes[59,62,63]	Yes[26,59]	Yes[26,59,63]	Peri-implantation[61]
CHD family					
N/A	CHD1*	No[69]	Yes[69]	Yes[69]	N/A
N/A	CHD2*	N/A	N/A	N/A	Perinatal[136]
N/A	CHD7*	N/A	N/A	N/A	E10.5 (REF. 137)
N/A	CHD8*	N/A	N/A	N/A	E8.5 (REF. 138)
NuRD	CHD3*, CHD4*, GATAD2B, MTA1, MTA2, MTA3, RBBP4, RBBP7	N/A	N/A	N/A	N/A
	GATAD2A	N/A	N/A	N/A	E10 (REF. 139)
	HDAC1	N/A	N/A	Yes[72]	E9.5 (REF. 74)
	HDAC2	N/A	No[72]	No[72]	Perinatal[76]
	MBD3	Yes[71]	Yes[70,71]	Yes[70,71]	N/A
ISWI family					
NURF	BPTF	N/A	Yes[77]	Yes[77]	E8.5 (REF. 77)
	RBBP4, RBBP7, SNF2L*	N/A	N/A	N/A	N/A
INO80 family					
TIP60	β-actin	N/A	N/A	N/A	E9.5 (REF. 135)
	BAF53A, BRD8, EPC1, EPC-like, MEAF6, MRGBP, MRGX, VPS72	N/A	N/A	N/A	N/A
	DMAP1	Yes[62]	Yes[62]	Yes[62]	N/A
	MRG15	N/A	N/A	N/A	E14.5 (REF. 141)
	p400*	Yes[62]	Yes[62]	Yes[62]	N/A
	RUVBL1, RUVBL2	Yes[62]	Yes[62]	N/A	N/A
	TIP60	Yes[62]	Yes[62]	Yes[62]	~E3.5 (REF. 140)
	TRRAP	Yes[62]	Yes[62]	N/A	Peri-implantation[142]
	YEATS4	Yes[62]	Yes[62]	N/A	N/A

BAF, BRG- or BRM-associated factor; BPTF, bromodomain PHD finger transcription factor; BRD8, bromodomain-containing 8; CHD, chromodomain helicase DNA-binding; DMAP1, DNA methyltransferase 1-associated 1; E, embryonic day; EPC, enhancer of polycomb; ES, embryonic stem; GATAD, GATA zinc finger domain-containing; HDAC, histone deacetylase; INO80, inositol-requiring 80; ISWI, imitation switch; MBD3, methyl-CpG-binding domain 3; MEAF6, MYST/ESA1-associated factor 6; MRG, MORF-related gene; MRGBP, MRG-binding protein; MTA, metastasis-associated; N/A, data not available; NuRD, nucleosome-remodelling; NURF, nucleosome-remodelling factor; PBAF, polybromo BAF; RBBP, retinoblastoma-binding protein; RUVBL1, RuvB-like 1; SWI/SNF, switch/sucrose nonfermentable; TIP60, TAT-interacting protein of 60 kDa (also known as KAT5); TRRAP, transformation/transcription domain-associated protein; VPS72, vacuolar protein sorting-associated 72. *Catalytically active.

Box 3 | **The actions of chromatin-remodelling factors**



Chromatin remodellers are ATP-dependent machines that act to alter the local structure of chromatin by repositioning (or 'sliding'), ejecting or incorporating nucleosomes. During DNA replication, for example, a group of chromatin remodellers act to insert nucleosomes into the newly forming chromatin fibre (see the figure, bottom left), but other groups of remodellers are active throughout the cell cycle to modify the local structure of chromatin, thereby regulating gene expression. For example, chromatin-remodelling factors such as SWI/SNF (switch/sucrose nonfermentable) and CHD (chromodomain helicase DNA-binding) family proteins can trigger ejection of a nucleosome (top left). Other chromatin-remodelling factors, such as ISWI (imitation switch) family proteins, can slide a nucleosome (top right). The INO80 (inositol-requiring 80) family proteins exchange histone dimers (bottom right), which can introduce histone variants or modified histones, and have a local impact on chromatin activity[56].

INO80 family. The INO80 family members can form three distinct complexes, INO80, SNF2-related CBP activator protein (SRCAP) and TAT-interacting protein of 60 kDa (TIP60; also known as KAT5)–p400, but only the last has been shown to be important in ES cells so far. The TIP60–p400 complex facilitates transcription by combining nucleosome remodelling with histone acetylase activity. ES cells depleted in different subunits of the TIP60–p400 complex show strikingly similar phenotypes, including altered colony morphology, decreased proliferation rates, reduced pluripotency and overall reduced viability[62], which seem to be a phenotype specific to ES cells[78]. TIP60–p400 probably acts to maintain the undifferentiated state of ES cells by binding to the H3K4me3 mark, an interaction that is facilitated by NANOG. In addition, TIP60–p400 promotes histone H4 acetylation at both active and repressed genes[62], which is also likely to support the stem cell state.

Together, these studies highlight the importance of chromatin-remodelling complexes for integrating the transcriptional programme for pluripotency with epigenetic information and for silencing this pluripotency programme upon differentiation. In addition, chromatin remodelling may potentially have a broader role in the global maintenance of the open chromatin state of ES cells.

Maintaining open chromatin in ES cells

In addition to being affected by enriched active histone marks, open chromatin may also be actively maintained in ES cells by the above-mentioned ATP-dependent chromatin-remodelling enzymes, for example, through the disassembly of nucleosomes and/or the 'unwinding' of higher-order chromatin structures (BOX 3). Interestingly, the expression of many of these chromatin-remodelling enzymes is significantly enriched in ES cells, including the esBAF complex and CHD members[26]. It is possible that integrating high levels of active histone marks with the high expression of particular chromatin remodellers globally orchestrates an open chromatin state.

The chromatin remodeller CHD1 may repress formation of heterochromatin in ES cells[69]. However, the mechanisms that orchestrate this opening of chromatin, tilting the balance between euchromatin and heterochromatin towards the former, remain unknown (FIG. 2). Such global 'anti-silencing' mechanisms have been studied in other species, such as budding and fission yeast, and such studies may help us understand the principles that govern this battle between heterochromatin and euchromatin. In yeast, silent information regulator (SIR) proteins bind preferentially to telomeric regions and promote the formation of heterochromatin. Two redundant mechanisms prevent the spreading of SIR proteins and heterochromatin: the incorporation of the histone variant H2AZ and the methylation of H3K4, mediated by the methyltransferase SET domain-containing 1 (Set1). Thus, incorporation of specific histone variants or a modification of canonical histones prevents binding of SIR proteins[79]. Another important anti-silencing mechanism is histone hyperacetylation, which also prevents SIR proteins from binding[80]. The local silencing mediated by the SIR family protein Sir3 requires a complex interaction between the HAT Sas2, the HMTs disrupter of telomere silencing 1 (Dot1) and Set1, and the HDM Jhd2 (REF. 81), which determine the dynamic balance of silencing versus activation by directing a competing addition and removal of methyl groups at H3K4 and H3K79. Therefore, not only can different types of histone modifications (acetylation or methylation) interact to regulate silencing but also there is a dynamic balance between the opposing actions of histone-modifying enzymes to regulate formation of euchromatin or heterochromatin.

Extrapolating on the telomere studies from yeast, one possible mechanism by which an open chromatin state is maintained in ES cells may be through deposition of specific histone variants. For example, H3.3 has been generally associated with active genes and is less prone to H3K9 methylation[82,83]. H3.3 is incorporated in a replication-independent manner by the chaperone HIRA[84], and typically colocalizes with regions enriched in methylation of H3K4 (REFS 85,86). This is thought to be a mechanism by which cells may maintain a transcriptional memory; for example, lineage-specific genes marked by H3.3 are still expressed after reprogramming in *Xenopus laevis*[87]. Interestingly, CHD1 is required in the *Drosophila melanogaster* oocyte for incorporation of H3.3 into sperm chromatin: CHD1-mutant oocytes cannot incorporate H3.3 into the male pronucleus, which renders the male genome incapable of contributing to development[88]. These results demonstrate the broad impact that H3.3 incorporation has for male chromatin in *D. melanogaster*. The possibility that a similar mechanism, involving H3.3 incorporation, also maintains the global open chromatin state of ES cells warrants future investigation, even though this variant is also present in telomeric regions[85].

Telomeric region
A region of repetitive DNA at the ends of chromosomes that protects the chromosomes from premature deterioration, rearrangements and chromosome fusion.

Histone hyperacetylation
A state in which many Lys residues are acetylated on many of the histones present in a given region of chromatin.

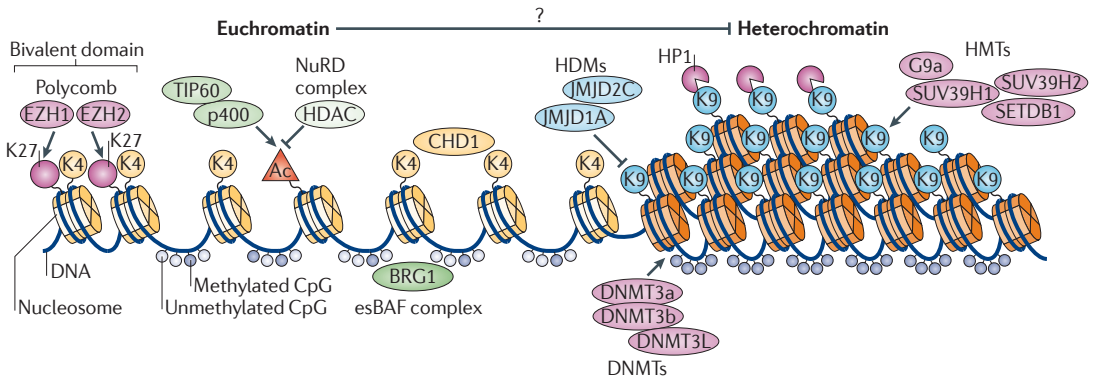


Figure 2 | **The balance between euchromatin and heterochromatin in ES cells.** Several epigenetic regulators orchestrate the open chromatin state of embryonic stem (ES) cells and set the stage for the transcriptional network. Relevant epigenetic marks include histone modifications and incorporation of different core histone variants (yellow and orange cylinders) that alter access and efficiency of the transcriptional machinery. The main histone marks, the active H3 tri-methylated on Lys 4 (H3K4me3) and the repressive H3K9me3 and H3K27me3 (represented by the circles K4, K9 and K27), are positively regulated by specific histone methyltransferases (HMTs; including G9a (also known as EHMT2), SUV39H1, SUV39H2 and SETDB1) and negatively regulated by the respective histone demethylases (HDMs; including jumonji domain-containing 2C (JMJD2C; also known as KDM4C) and JMJD1A (also known as KDM3A)). Active (K4) and repressive (K27) marks can be present in the promoter regions of developmental genes to prevent their expression while allowing rapid activation by transcription factors such as the polycomb proteins Enhancer of zeste homologue 1 (EZH1) and EZH2 (termed bivalent domains). Histone acetylation also marks active chromatin, and the acetyl group (the orange triangle, Ac) can be added through complexes such as TAT-interacting protein of 60 kDa (TIP60; also known as KAT5)–p400 and removed by histone deacetylases (HDACs), which can be part of repressive complexes such as the nucleosome-remodelling (NuRD) complex. DNA (dark blue line) methylation is typically present on CpG islands in promoter regions and heterochromatin (marked by H3K9me3 and heterochromatin protein 1 (HP1)). DNA can be hypermethylated, as a result of the action of DNMTs, such as DNMT3a–DNMT3b or DNMT3L, but in euchromatic regions DNA is generally unmethylated. Chromatin-remodelling proteins such as chromodomain helicase DNA-binding 1 (CHD1) and BRG1 in the ES cell-specific BRG- or BRM-associated factor (esBAF) complex may regulate the open chromatin state, possibly by contributing to boundary determination between euchromatin and heterochromatin. There is growing evidence that the formation of euchromatin can repress the establishment of heterochromatin nearby (as it has not been confirmed in ES cells, this is denoted by a question mark).

Alternatively, or in addition, other mechanisms may directly protect H3K4me3 from demethylation. Binding of chromatin remodellers such as CHD1 directly to H3K4me3 via its chromodomains[89] may protect against the action of demethylases and selectively cooperate with HMTs to maintain the H3K4me3 mark. For example, CHD1 interacts, through its chromodomain, with the HMT ASH2, which methylates H3K4 (REF. 90). This histone mark prevents the binding of repressive complexes such as the NuRD deacetylation complex[91,92] and the DNMT subunit DNMT3L (REF. 93). The opening of chromatin can also be complemented by histone hyperacetylation, as shown for telomeres in yeast[80]. In fact, the HAT and remodelling complex TIP60–p400 recognizes H3K4me3 and depends on this mark to bind its targets[62].

All of these mechanisms may orchestrate a complex, dynamic regulation of open versus compact chromatin in ES cells (FIG. 2). It will therefore be important to determine, in a genome-wide manner using ChIP–seq, how epigenetic marks change when regulators of open chromatin such as CHD1 are lost. Further genetic and biochemical studies, in particular epistatic analyses and dissection of protein–protein interactions, should also help define the relative contribution of these mechanisms to the chromatin state and pluripotency of ES cells.

Lessons from reprogramming somatic cells

The process of generating iPS cells reverts somatic cells back to a pluripotent stem cell state that is very similar to that of ES cells and may provide an alternative to the use of ES cells for dissecting the relationship between open chromatin and pluripotency[94]. Although molecular landmarks that arise during the course of reprogramming have been identified, the process remains largely unsolved at the mechanistic level. Upon expression of the reprogramming factors (generally OCT4, SOX2, MYC and KLF4), alkaline phosphatase (AP) activity and expression of the cell surface marker SSEA1 (also known as FUT4) are early markers of the undifferentiated state. AP and SSEA1 can be detected as early as 3 and 9 days, respectively, after the onset of reprogramming in mouse cells. Endogenous expression of OCT4 and NANOG can be detected only after about 10 days post-induction, and the four exogenous factors, generally delivered by viral constructs, need to be expressed during all of that period. However, cells only fully reprogramme upon silencing of the viral vectors[95]. The main question that arises is: what are the immediate downstream effects of the reprogramming factors that trigger induction of pluripotency? OCT4 and SOX2 are part of an autoregulatory loop that maintains pluripotency in ES cells[96], and MYC binds to a separate class of genes not bound by OCT4, SOX2 or KLF4 (REF. 97), in concert with self-renewal regulators

Box 4 | **Open chromatin and the undifferentiated state in cancer cells**

The acquired ability of cancer cells to divide perpetually and at the same time to support tumour growth, metastasis and invasiveness, bears resemblance to stem cell biology[117]. It is thought that this acquired immortality is obtained through the activation of stem cell-specific pathways that are essential for self-renewal, such as Wnt, sonic hedgehog (SHH) or Notch pathways[118,119]. There is also a correlation between the transcriptomes of stem cells and highly undifferentiated cancer cells from tumours with higher proliferation rates and poorer prognosis[120–124]. For example, myelocytomatosis oncogene (MYC) can reactivate an embryonic stem (ES) cell-like programme in normal and cancer cells[124]. However, MYC has several functions, and the mechanism by which MYC activates this ES cell-like programme could be independent of its canonical transcription factor activity[125]. In particular, MYC regulates large domains of euchromatin, possibly by inducing histone hyperacetylation[126,127]. It is therefore possible that there are commonalities between undifferentiated cancer cells and ES cells that include a shared transcriptional programme linked with reorganization of the chromatin to include euchromatic histone marks[128].

Some aspects of higher order chromatin conformation may have similarities between ES cells and certain undifferentiated types of cancer. For example, loss of heterochromatin markers such as heterochromatin protein 1α (HP1α)[129,130] and H3 di-methylated on Lys 9 (H3K9me2)[30] have been observed in metastatic breast cancer and lymphoid cancer cell lines, respectively. In addition, many genes marked with bivalent domains in ES cells, including those encoding tumour suppressors and pro-differentiation factors, further acquire H3K9 methylation in embryonic carcinoma cells and DNA methylation in adult cancer cells[121]. These additional repressive marks may contribute to a higher-order chromatin organization and permanent silencing of tumour suppressors and pro-differentiation factor genes in cancer cells[131]. Furthermore, the process of inducing pluripotency has similarities to cellular transformation and is facilitated by the activation of oncogenes such as MYC and the inhibition of tumour suppressors such as p53 (for reviews, see REFS 94,132). It will therefore be of interest to explore potential parallels between the regulation of the chromatin state in pluripotent stem cells and cancer cells.

such as E2F1 and zinc-finger X-chromosomal (ZFX). MYC is not essential for reprogramming[17,98,99] but it facilitates early stages of the process, possibly through its direct action on chromatin[100] or indirect action via repression of differentiation genes[101]. The ability to dissect how individual factors contribute to the generation of iPS cells would greatly benefit from methods that allow high-efficiency synchronized reprogramming, ideally, coupled with analysis at the single cell level, neither of which is as yet possible. Nevertheless, studies so far have already provided insights into chromatin-level regulation of reprogramming.

Chromatin reconfiguration during reprogramming. A large reconfiguration of the chromatin structure, from DNA methylation to histone modifications and nucleosome spacing, occurs during reprogramming. Such layers of epigenetic regulation are often used as repressive mechanisms in somatic cells to prevent unwanted gene expression from other lineages. How these epigenetic barriers to reprogramming are overcome is a key question. Several lines of evidence support the notion that the process of reprogramming involves rare stochastic epigenetic events. The reprogramming process is slow and gradual, with several intermediate states[101–103]. Reactivation of endogenous ES cell genes such as OCT4 can occur at very different time points in different iPS cell lines derived from the same clone[102]. Eventually, almost all cells are reprogrammed to pluripotency, albeit with different and often long latency periods[104]. Inhibition of the p53/p21 pathway and overexpression of LIN28 accelerate the kinetics of reprogramming by increasing the cell division rate, which may facilitate the acquisition of DNA and/or histone modifications. This reinforces the idea that reprogramming is a complex process that may use stochastic events to overcome epigenetic barriers; however, the underlying molecular mechanisms remain unknown. Interestingly, some of the same epigenetic barriers may also be overcome in cancer progression (BOX 4).

Recent insights have been gained from treating reprogramming cells with agents that affect the chromatin state. In particular, treatment with agents that promote chromatin decondensation, such as the DNMT inhibitor 5-aza-cytidine, the HDAC inhibitor valproic acid or a G9a methyltransferase chemical inhibitor, leads to increased efficiency of iPS cell generation and sometimes can substitute for a particular transcription factor[103,105–107]. It is likely that a key step in the generation of iPS cells is the re-opening of the somatic cell chromatin. Consistent with this, in a recent unbiased screen for components of ES cell extracts that facilitate reprogramming, the BAF family components BRG1 and BAF155 (REF. 108) could substitute for MYC. Moreover, they promoted the opening of chromatin during the reprogramming process, through DNA demethylation, and increased H3K4me3 in the promoter regions of important transcription factors[108]. Suppression of CHD1 also inhibits the generation of iPS cells[69]. Additional evidence comes from other reprogramming assays, such as somatic cell nuclear transfer[109]. Here again, BRG1 is an essential nuclear factor for nuclear reprogramming[110]. Furthermore, treatment with HDAC inhibitors enhances efficiency of development after nuclear transfer[111]. These results suggest that the chromatin remodellers that maintain the ES cell state, including BRG1, BAF155 and CHD1, may re-open chromatin during reprogramming and set the stage for activating the transcriptional network for pluripotency.

Transcriptional memory. A final insight into the epigenetic regulation of cell states comes from the recent observation that, although iPS cells are remarkably similar to ES cells, they may have transcriptional differences[112,113]. Mouse iPS cells appear to retain a residual

Genetic epistasis
The relationship or order in which two genes act in a pathway (that is, upstream or downstream, synergistic or antagonistic), which can be studied by analysing single and double mutants.

DamID
A method that is used to analyse binding of proteins to DNA. Genetically modified *Drosophila melanogaster* culture cell lines express a protein of interest fused with a bacterial DNA adenine methyltransferase. Local DNA methyltransferase activity indicates protein binding.

DNA methylation signature from their original somatic cells[114,115], and a similar phenomenon is observed in human iPS cells (M.R.-S. laboratory, unpublished observations). The transcriptional profile of human iPS cells becomes more similar to that of human ES cells after several passages[112], suggesting that some form of reprogramming happens with continued culturing. The functional significance of these transcriptional differences remains to be fully understood. Interestingly, in frog embryos generated by nuclear transfer of muscle cells, which express the muscle-specific gene myogenic differentiation 1 (MYOD1), expression of this gene is maintained in non-muscle lineages even after several divisions[87]. This transcriptional memory may be mediated through deposition of the histone variant H3.3 (REF. 87). This chromatin mark could establish, through an unknown mechanism, a memory of the genes that had been previously transcribed in the somatic cell.

Such epigenetic memory, potentially mediated by DNA methylation or histone variant incorporation, may contribute to differences between iPS cells and ES cells and suggests that competing epigenetic influences may affect chromatin re-opening during reprogramming. A mechanistic understanding of these epigenetic influences, which is at present lacking, should shed light not only on how iPS cells are generated but also, more broadly, on cellular transitions that occur during differentiation or transformation.

Conclusions

Significant new insights have been gained into the regulation of pluripotency and reprogramming at the chromatin level. The emerging picture is that a globally open chromatin state that is accessible for transcriptional activation is actively maintained in pluripotent stem cells. In this context that is permissive for transcription, there are additional epigenetic mechanisms that promote silencing of lineage-specific genes while leaving them poised for rapid activation. A major gap in our understanding of pluripotency is how the different layers of epigenetic regulation of the chromatin state impact one another and the transcriptional network. Clearly, much effort should now focus on integrating the various levels of epigenetic regulation in pluripotent stem cells — for example, using analyses of genetic epistasis and protein–protein interactions — and understanding how such information may be parsed out during differentiation. New approaches for defining the chromatin landscape are being established, which will allow for a better understanding of the chromatin structure and its significance for the identity of a particular cell type. For example, the use of DamID in *D. melanogaster* has identifed five different types of chromatin (instead of the classic three: euchromatin, heterochromatin and facultative heterochromatin), according to the chromatin proteins that are bound to these domains[116]. They include three types of silencing or repressive chromatin — one bound by HP1, another bound by Polycomb and a third with no apparent known repressive or active marks — which encompass more than 50% of the genome. The euchromatic regions are divided into two domains, one enriched with H3K36me3 and the other mostly bound by regulatory factors, and include most developmental genes. Studies such as this in mammalian cells will hopefully provide a more comprehensive picture of 'open' and 'closed' chromatin.

In addition, much remains to be learned about the mechanisms that regulate epigenetic reprogramming during the generation of iPS cells. We must remember that ES cells and iPS cells are cultured *in vitro*, and that the molecular mechanisms that underlie their biology evolved for processes in the context of the whole embryo that remain poorly understood and deserve further investigation. Finally, it will be important to assess the significance of the intriguing epigenetic similarities observed between pluripotent stem cells and undifferentiated cancer cells (BOX 4).

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