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Enhancers: emerging roles in cell fate specification

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Enhancers are regulatory DNA elements that dictate the spatial and temporal patterns of gene expression during development. Recent evidence suggests that the distinct chromatin features of enhancer regions provide the permissive landscape required for the differential access of diverse signalling molecules that drive cell-specific gene expression programmes. The epigenetic patterning of enhancers occurs before cell fate decisions, suggesting that the epigenetic information required for subsequent differentiation processes is embedded within the enhancer element. Lineage studies indicate that the patterning of enhancers might be regulated by the intricate interplay between DNA methylation status, the binding of specific transcription factors to enhancers and existing histone modifications. In this review, we present insights into the mechanisms of enhancer function, which might ultimately facilitate cell reprogramming strategies for use in regenerative medicine.

Keywords: epigenetics; histones; chromatin; enhancer; differentiation

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See the Glossary for abbreviations used in this article.

Introduction

For multicellular organisms to develop, stem cells must differentiate into a broad range of specialized cells, all containing the same DNA. This remarkable feat is made possible by the presence of distinct regulatory DNA elements throughout the genome—enhancers—that control the spatial and temporal expression patterns of specific sets of genes [1]. The transcription of eukaryotic genes is an intricate process that requires many protein complexes to interact precisely with these DNA elements [2]. Enhancers belong to a class of regulatory sequences that can activate transcription independently of their location, distance or orientation with respect to the promoters of the genes they control [3].The traditional view of enhancers is that they are clusters of DNA sequences able to recruit combinations of transcription factors that then interact with components of the Mediator complex or TFIID. By looping out the intervening sequences, the complexes assembled at the enhancer can facilitate RNA polymerase II (RNAPII) recruitment to the promoter [4,5]. More recently, enhancer-bound transcription factors have been shown to recruit histone-modifying enzymes, or ATP-dependent chromatin-remodelling complexes, to alter chromatin structure and increase the accessibility of the DNA to other proteins at the promoter to facilitate transcription initiation or elongation [6–8].

Over the past few years, significant advances in the understanding of enhancer structure and function have been made possible by genome-wide mapping of different histone modifications, transcription factors and other chromatin features [9]. The emerging theme from these studies is that pre-patterning of enhancers with specific histone modifications might occur before cell fate decisions. The unique chromatin features at enhancers provide the epigenetic information required for switching on distinct differentiation programmes during development. In addition, it seems that transcription of non-coding RNAs from enhancer sequences and the recruitment of cohesin proteins might play an active role in stabilizing long-range enhancer–promoter interactions during transcription initiation [10–15]. These findings are beginning to explain the mechanisms by which enhancers might activate transcription in a distance and orientation-independent manner with exquisite precision. However, they also raise intriguing questions of whether enhancers can be functionally categorized on the basis of different epigenetic marks and the nature of the molecules that regulate the deposition of these marks at specific enhancers during development (see Sidebar A).

Recent studies in different developmental model systems are starting to address the issue of how epigenetic pre-patterning at specific enhancers can regulate cell fate decisions. Data suggest that enhancers can be functionally distinguished by the combinatorial patterns of histone modifications present at these sequences. These epigenetic patterns result from the intricate interplay between specific DNA-binding factors, unique DNA sequences and the methylation state of DNA. The acquired histone modification marks at enhancers in turn provide a permissive landscape for the binding of other transcription factors in response to differentiation signals. Since enhancers play a pivotal role in regulating the developmental competence of cells, understanding the mechanisms by which pre-patterning of enhancers occurs might have a positive impact on strategies for the manipulation of induced pluripotent stem cells (iPSCs) in regenerative medicine.

Histone modifications define specificity

Genome-wide mapping of epigenetically marked nucleosomes and transcriptional regulators, coupled with functional assays in cells and transgenic animals, have provided new insights into the chromatin landscape of enhancers [9]. The mapping of several thousand p300 binding sites in various mouse tissues has accurately identified novel enhancers that exhibit tissue-specific activity in transgenic mouse assays [16,17]. By using distal binding sites for the histone

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Glossary

ac	acetylation
AP1	activator protein 1
ATAC	ADA (adenosine deaminase) two-A containing complex
CTCF	CCCTC-binding factor
HAT	histone acetyltransferase
LSD1	lysine-specific demethylase 1
me	methylation
NuRD	nucleosome remodelling and histone deacetylase
SAGA	Spt-Ada-Gcn acetyltransferase
TF	transcription factor
TFIID	transcription factor II D

acetyltransferase p300 as a criterion, predicted putative enhancers were initially shown to be highly enriched for histone H3 mono- and dimethylated at Lys4 (H3K4me1, H3K4me2) and acetylated at Lys27 (H3K27ac), but depleted for histone H3 trimethylated at Lys4 (H3K4me3) and histone H3 [18]. These results indicate that enhancers are enriched in regions with low nucleosome occupancy—termed 'nucleosome-depleted regions'. In addition, the absence of H3 suggests that lysine residues of histone variant H3.3 might be the substrate for methylation. Consistent with this idea, many of these enhancer sites are associated with nucleosomes containing the histone variants H3.3 and H2A.Z [19–23], which are highly dynamic and sensitive to varying salt concentrations (Fig 1; [24–26]). However, it is increasingly clear that other histone modifications might occur at putative enhancers in different cell types [21,22]. For instance, a survey of 39 histone modification in human $CD4^+$ T cells indicates that 20% of putative enhancers are associated with at least six types of histone modification, including H2A.Z, H3K4me1/2/3, H3K9me1 and H3K18ac [22]. Some well-characterized enhancers, such as the CD28 response element (CD28RE) and the CNS22 enhancer, are associated with more than 10 different histone modifications [22].

The observation that enhancers are marked by distinct patterns of histone modifications has led to the question of what consequences these modifications have on the regulatory activity of the enhancer. In human and mouse embryonic stem cells (ESCs), it seems that enhancers of actively transcribed genes are marked by the presence of H3K4me1 and H3K27ac (Fig 1A; [27–29]). However, enhancers of developmental genes poised for future activation contain repressive histone H3 trimethylated at Lys27 (H3K27me3) instead of the H3K27ac mark (Fig 1B; [27]). Similarly, in human primary haematopoietic stem cells or progenitor cells (HSCs/HPCs), enhancers of several genes involved in subsequent differentiation are enriched for H3K9me1 and H3K27me1 modifications, in addition to the H3K4me1 mark (Fig 1C; [30]). These observations suggest that enhancers might be 'pre-patterned' by H3K4me1/2 marks before their target genes are turned on, and changes in the histone modification patterns of enhancers correlate with their regulatory activity. Consistent with this idea, the distinct epigenetic marks at enhancers correlate strongly with cell-type-specific gene expression, whereas the chromatin features at promoter regions remain largely invariant across diverse cell types [31].

Histone modifications characteristic of specific enhancers continue to be found as new cell types and new methods are used to identify additional histone modifications. For example, the use of an integrated mass-spectrometry-based proteomics approach has led to the identification of 67 new histone modifications [32]. Genome-wide analysis with a pan-lysine crotonylation (Kcr) antibody has indicated that histone Kcr is highly enriched in the promoter and potential enhancers of testis-specific genes in postmeiotic male germ cells [32]. Furthermore, it was recently shown that active mesodermal enhancers in *Drosophila* are highly enriched for H3K27ac and H3K79me3 (Fig 1D; [33]).

Taking into account data from a variety of experimental systems, it is possible to postulate a model in which at least some enhancer sequences carry epigenetic information that changes complexity and records the differentiation history of cells during development. The basic characteristics of enhancers are probably defined by the presence of H3K4me1/2 and H3.3/H2A.Z. Additional modifications might act to alter the response of a subset of enhancers and restrict their potential to activate genes.

Sidebar A | In need of answers

(i) What are the chromatin features of different types of enhancer?
(ii) How do enhancers restrict competence of developing cells?
(iii) How does 'pre-patterning' of enhancers with specific histone modifications occur?
(iv) What are the practical implications of 'chromatin pre-patterning' on the establishment of iPS cells?

Chromatin signatures signal gene induction

One of the enigmas in developmental biology is what underlies the selective occupancy of transcription factors in different cell types in response to differentiation cues. It has been widely accepted that transcription factors preferentially bind to 'open' chromatin, based on the strong correlation between DNase I hypersensitive sites (DHS) and gene regulatory sequences [34,35]. This view was corroborated by recent genome-wide studies in which many transcription factors were found to bind preferentially to their DNA recognition sites located within nucleosome depleted regions. Conversely, putative transcription factor recognition sites occupied by nucleosomes are often not accessible for binding [21,24,36,37].

It is conceivable that the presence of unstable H3.3/H2A.Z variants containing nucleosomes at many H3K4me1/2 enhancers might provide the permissive landscape for the binding of transcription factors. Indeed, it was shown recently that the transcriptional competency of the *MYOD1* gene is dependent on the presence of nucleosome-depleted regions at its H3K4me1-marked enhancer [23]. Binding of the androgen receptor to cognate enhancers is also facilitated by the dynamic nature of H2A.Z-containing nucleosomes [19]. Recently, it was demonstrated that distinct sets of glucocorticoid receptor (GR) recognition sites are hypersensitive to DNase I in different cell types. Upon hormone induction, 95% of genomic binding of the GR is targeted to pre-existing foci of accessible chromatin marked by DNase I hypersensitivity [38]. This result suggests that predetermined nucleosome-depleted regions at enhancers underlie the cell-type-specific response to glucocorticoids.

Given its ability to bind directly to nucleosomes, it seems that the pioneer transcription factor foxhead box protein A1 (FoxA1) might have evolved a slightly different strategy to trigger cell-specific responses, as it has been shown to bind directly to nucleosomal DNA [39]. In MCF7 cells, enhancers that contain oestrogen-receptor -binding sites are marked by H3K4me1/2; whereas in LNCaP cells, enhancers that contain androgen receptor binding sites are marked

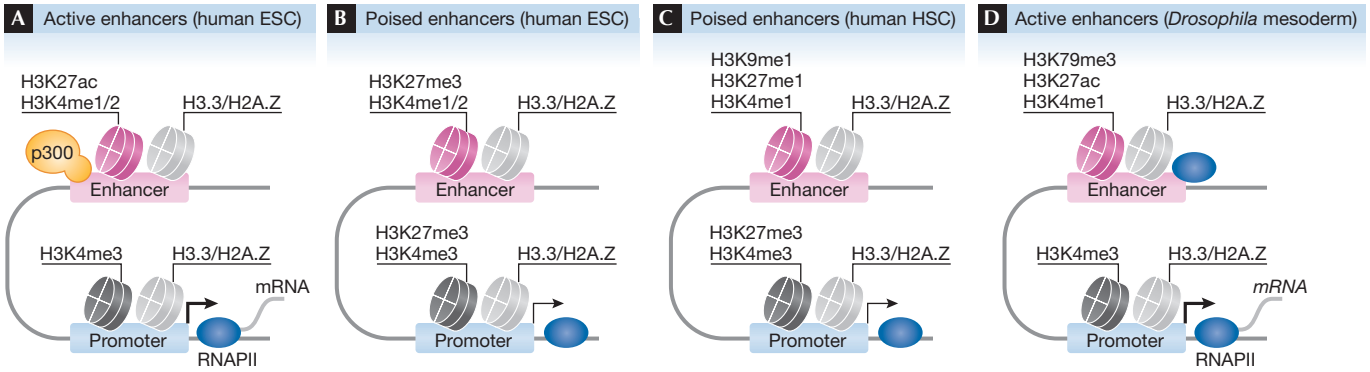


Fig 1 | Enhancer function correlates with distinct histone modification patterns in different cell types. Many enhancers are characterized by the presence of H3K4me1/2, H3.3/H2A.Z and the absence of an H3K4me3 mark. (**A**) In human ESCs, enhancers of actively transcribed genes are marked with H3K27ac. However, enhancers associated with poised genes contain (**B**) H3K27me3 in human ESCs or (**C**) H3K9me1 and H3K27me1 in human haematopoietic stem cells. (**D**) In *Drosophila* mesodermal cells, enhancers of actively transcribed genes are marked with H3K27ac and H3K79me3. Dark grey nucleosomes contain canonical histones. Light grey nucleosomes are highly dynamic and contain histone variants. Pink nucleosomes contain modified histones that are associated with many enhancers. ESC, embryonic stem cell; SC, stem cell.

by H3K4me1/2. The differential binding of FoxA1 to selected H3K4me1/2-marked enhancers is followed by recruitment of either an oestrogen or androgen receptor to synergistically activate oestrogen- and androgen-responsive programmes, respectively (Fig 2A; [40]). In addition, preferential recruitment of protein acetyltransferase TIP60 to H3K4me1-marked enhancers was also shown to be necessary for oestrogen-induced transcription [41].

These results suggest that enhancers are epigenetically modified before gene induction, and H3K4me1/2 or H3.3/H2A.Z marks at enhancers facilitate rapid gene activation through efficient recruitment of transcription factors and other chromatin-remodelling enzymes. Consistent with this, H3K4me2 marks are enriched at enhancers of a subset of developmentally poised haematopoietic genes in a multipotent haematopoietic cell line [42], supporting the idea that the cell-specific distribution of epigenetic marks at enhancers might be the underlying basis for context-dependent signalling output during differentiation.

TFs, DNA methylation and enhancer patterning

Observations suggesting that pre-existing histone modifications at many enhancers dictate context-dependent gene activation during cell differentiation raise the key question of how enhancer sequences acquire specific patterns before cell fate decisions. Although the enzymes involved in various histone modifications and H3.3/H2A.Z deposition are well characterized [43–45], their spatial and temporal engagement with DNA regulatory elements remains poorly understood. In the zebrafish embryo, H3K4me3 and H3K27me3 marks are present at promoters of both active and inactive genes in the absence of sequence-specific transcriptional activators or RNAPII [46,47], suggesting that histone modifications at regulatory elements precede gene activation. Recent lineage analyses of chromatin signatures, transcription factor occupancy and the status of DNA methylation in several developmental model systems are beginning to shed light on how the patterning of enhancers might be influenced by the intricate interplay between the recruitment of chromatin factors and the DNA methylation status of the underlying sequences.

DNA methylation. DNA methylation is a key mechanism of epigenetic regulation in eukaryotes [48]. In mammals, DNA methyltransferases establish and maintain methylation of cytosine residues in DNA within CpG dinucleotides. CpG islands (CGIs) are short genomic regions highly enriched in CpG dinucleotides. Interestingly, CpGs located within CGIs tend to be unmethylated compared with other sites across the genome. The mapping of epigenetic changes that occur during haematopoietic development has revealed a complex interdependence between DNA sequence, histone modifications and developmental gene function [42]. Most haematopoietic lineage-specific genes lack CGIs. Importantly, enhancers of these genes are unmodified (H3K4me2–/me3–) in ESCs. These enhancers acquire H3K4me2 marks only upon their commitment to multipotent haematopoietic stem cells, poising their genes for future expression during terminal differentiation. The association of known haematopoietic transcription factors—for example, PU.1—with these poised enhancers suggests that PU.1 might be the key player involved in establishing the H3K4me2 modification upon ESCs differentiation. However, CGI-containing genes are largely composed of either poised developmental regulators (H3K4me2+/me3–) or constitutively active housekeeping genes (H3K4me2+/me3+) in ESCs (Fig 2B; [42]). The correlation between histone modification and CGI status suggests that DNA methylation might influence the H3K4me state of enhancers. Consistent with this hypothesis, a recent comparative analysis of $CD4^+$ conventional T cells and regulatory T cells revealed more than 100 differentially methylated regions (DMRs) located at promoter-distal sites of lineage-specific genes that exhibit enhancer activity in reporter gene assays [49]. Furthermore, there is coenrichment of cell-type-specific DNA hypomethylation sites with increased H3K4 methylation within the same cell type (Fig 2C). Similarly, in ESCs and differentiated IMR90 lung fibroblasts, cell-specific histone modifications at enhancers are inversely related to the level of DNA methylation [50]. Although the functional dependency between histone modifications and DNA methylation at the enhancers was not addressed, these results suggest that lineage-specific enrichment of H3K4me1 at enhancers might be in part regulated by a low level of DNA methylation. It has been shown that binding of CXXC finger protein 1 (Cfp1) to non-methylated CGIs at promoter regions recruits the H3K4 methyltransferase Setd1 to deposit H3K4me3 marks [51]. Therefore, it is plausible that other CGI-associated proteins might be responsible for depositing H3K4me1/2 marks at enhancers.

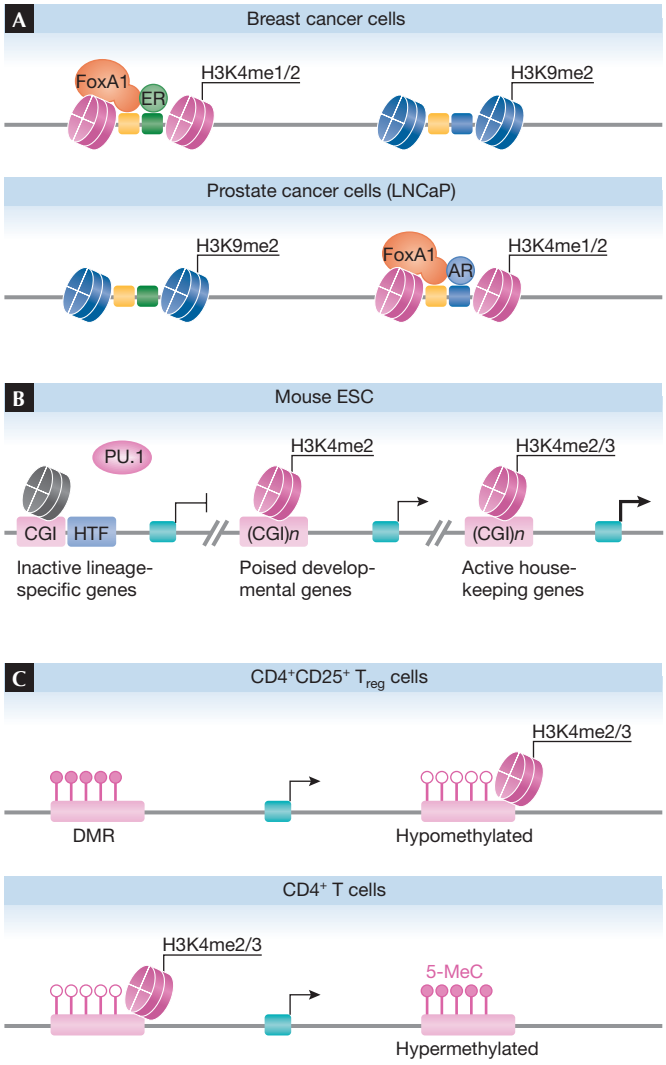


Fig 2 | Context-dependent transcriptional output is regulated by cell-specific epigenetic features at enhancers. (**A**) The presence of distinct H3K4me2-marked enhancers allows differential binding of FoxA1, which in turn recruits either ER or AR to turn on specific transcriptional programmes. (**B**) Enhancers of different genes are associated with distinct epigenetic marks in ESCs. Enhancers of inactive lineage-specific genes are located in regions with low CGIs and are associated with binding sites for lineage-specific transcription factors. PU.1 might be required for the deposition of the active H3K4me2 mark at these enhancers. Enhancers of poised developmental genes and active housekeeping genes contain H3K4me2 and H3K4me2/3, respectively. Both types of enhancer are located in regions with high CGI [(CGI)*n*]. (**C**) The distribution of DMRs is correlated with the presence of active H3K4me2/3 marks at distal regulatory elements in various types of T cells. Methylated and unmethylated CpG sites are denoted by filled and open red sticks, respectively. AR, androgen receptor; CGI, CpG island; DMR, differentially methylated region; ER, oestrogen receptor; ESC, embryonic stem cell; HTF, haematopoietic transcription factor.

Pluripotent ESC factors and lineage-specific TFs. Evidence accumulated during the past few years indicates that the chromatin state of enhancers is intricately orchestrated by stepwise recruitment of multiple transcription factors during development. In ESCs, the transcription factors Oct4, Sox2 and Nanog are required to maintain the pluripotent state [52]. Interestingly, apart from maintaining ESC identity, Oct4 and Sox2 also participate in germ-layer fate selection. Oct4 suppresses neural ectodermal differentiation and promotes mesendodermal differentiation, whereas Sox2 acts in an opposite manner. Differentiation signals continuously and asymmetrically modulate Oct4 and Sox2 protein levels, altering their binding pattern in the genome, and leading to cell fate decisions [53].

How do Oct4 or Sox2 affect the epigenetic pattern of enhancers? Studies in B-cell and neural lineage development support a 'factor relay model' whereby ESC factors establish active epigenetic marks at tissue-specific elements before being replaced by cell-type-specific factors as cells differentiate [54]. For instance, Sox2 contributes to the establishment of the H3K4me2 mark in the enhancers of several B-cell-differentiation genes in ESCs. The replacement of Sox2 by the lineage-specific transcription factor Sox4 at these enhancers leads to specific gene expression as ESCs differentiate into pro-B cells [54]. Similarly, Sox2 preselects for neural-lineage-specific genes in ESCs destined to be bound and activated by Sox3 in neural precursor cells (NPCs; [55]). In addition to histone modification, the presence of lineage/master transcription factors at distinct enhancers might regulate the DNA occupancy of signalling molecules to mount cell-type-specific responses [56,57]. For example, binding of the transcription factors Smad2/3 to their DNA recognition sequences is governed by Oct4 in ESCs, PU.1 in pro-B cells and Myod1 in myotubes.

The establishment of epigenetic marks at various enhancers by other transcription factors has been shown in several other model systems. Geminin was initially characterized as a nuclear protein that could regulate the expansion of the neural plate in early *Xenopus* embryos and inhibit DNA replication origin licensing [58,59]. It was recently demonstrated that Geminin recruitment of the Polycomb-group protein Ezh2 is necessary to restrain mesodermal and endodermal lineage commitment in the early *Xenopus* embryo (Fig 3A; [60]). However, during development of the ectoderm, Geminin promotes the neural fate acquisition of mouse ESCs by maintaining the chromatin of lineage-specific genes in an accessible and hyperacetylated state (Fig 3B; [61]). These studies indicate that a transcription factor might have contrasting roles in regulating chromatin features at various enhancers.

Cell-type-specific occupancy of enhancers by p300, SAGA and ATAC HAT complexes regulates distinct transcriptional programmes [31,62]. How HATs are recruited to acetylate H3K27 at specific enhancers is not fully understood. During liver development, recruitment of p300 to regulatory elements of hepatic genes by the bone morphogenetic protein signal molecule Smad4 is required to enhance the production of liver progenitors (Fig 3C; [63]), suggesting that the cell-specific distribution of HATs at certain enhancers might be regulated by specific signalling molecules.

Interplay between TF binding and DNA methylation. Studies on the mechanisms by which the GR activates gene expression suggest an interesting interplay between the roles of DNA methylation and transcription factor binding in the establishment of epigenetic signatures at enhancers (Fig 4; [64]). In GR-responsive cells, GR can bind to pre-programmed DHS (accessible) chromatin sites, or at non-DHS *de novo* binding sites. Pre-programmed DHS sites are enriched for CpG whereas *de novo* binding sites are located in regions with low CpG density. Interestingly, CpG demethylation at pre-programmed GR-binding sites correlates with cell-type-specific DHS sites.

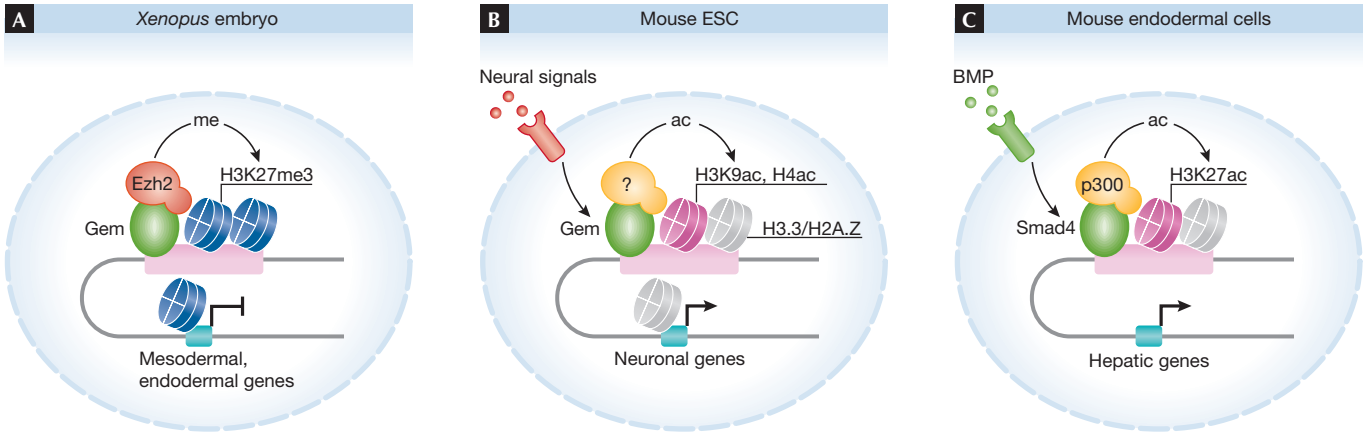


Fig 3 | The deposition of specific histone modifications at enhancers is regulated by different nuclear factors. (**A**) In Xenopus embryos, Geminin recruitment of the Polycomb-group protein Ezh2 is necessary to restrain mesodermal and endodermal lineage commitment by maintaining the repressive H3K27me3 mark at enhancers. (**B**) During development of the ectoderm, Geminin promotes neural fate acquisition of mouse ESCs by maintaining chromatin of lineage-specific genes in an accessible (H3.3/H2A.Z-containing nucleosomes) and hyperacetylated state (pink-nucleosomes). (**C**) During liver development, the binding of the BMP signalling molecule SMAD4 at enhancers recruits p300, which in turn deposits H3K27ac marks and leads to the activation of hepatic genes. ac, acetylation; BMP, bone morphogenetic protein; ESC, embryonic stem cell; Gem, Geminin; me, methylation.

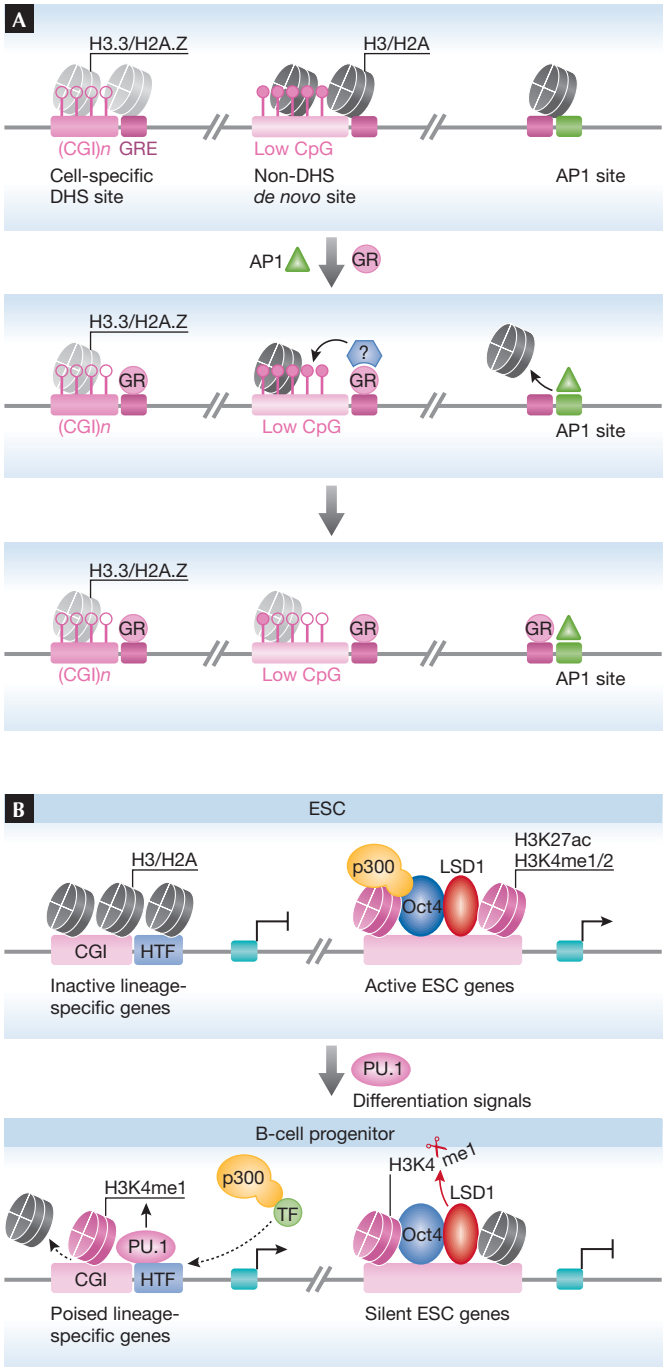
However, the functional dependency between DNA methylation status and DHS sites at the pre-programmed GR sites is unclear. It was recently found that nucleosome positioning seems to influence DNA methylation patterns and that DNA methyltransferases preferentially target nucleosome-bound DNA [65]. This suggests that the presence of pre-existing DHS sites—that is, nucleosome-depleted regions—might precede the removal of DNA methylation. In addition, GR binding can reduce DNA methylation at the *de novo* binding sites [64], suggesting that pioneer transcription factors such as GR can create regions of unmethylated DNA at enhancer elements (Fig 4A; [64]). Consistent with this idea, FoxA1 can bind to both highly methylated CpG sites as well as cell-specific hypomethylated enhancers during the neural differentiation of P19 cells. FoxA1 binding in turn leads to DNA demethylation and deposition of the H3K4me2 modification at enhancers [66]. Similarly, the forkhead family member FoxD3 is essential for maintaining the unmethylated CpG mark at the enhancer of the lineage-specific gene *Alb1* in ESC cells [67]. Taken together, these results suggest that the initial epigenetic status of enhancers might be determined by the cross-talk between pioneer transcription factors and CpG methylation state.

Apart from signal-dependent transcription factors, recent data suggest that general transcription factors, transcriptional co-regulators and other histone-modifying enzymes might also play a role in establishing epigenetic marks at enhancers. For instance, in a mammary epithelial cell line, the binding of AP1 is necessary to potentiate chromatin accessibility and subsequent recruitment of GR (Fig 4A; [68]). During B-cell development, binding of the transcription factor PU.1 initiates nucleosome remodelling and H3K4me1 modification at enhancers, facilitating the recruitment of additional transcription factors that drive cell-specific programmes (Fig 4B; [69]). In double-positive T cells, general transcription factors and RNAPII are recruited to tissue-specific enhancers, leading to transcription of both polyadenylated and non-polyadenylated RNA species [70]. How these factors affect the histone modification state at these enhancers remains to be determined. It is noteworthy that the timely removal of active H3K4me1/2 and H3K27ac marks from selected enhancers is equally crucial for proper differentiation to take place. Indeed, it was demonstrated recently that the presence of the histone demethylase LSD1–NuRD complexes at Oct4-regulated active enhancers in ESCs is necessary for the removal of the H3K4me1 mark as ESCs differentiate. Concomitantly, inhibition of LSD1 leads to incomplete silencing of ESC genes and improper cellular differentiation (Fig 4B; [71]).

In summary, the current evidence supports a model in which the establishment of epigenetic signatures at enhancers might be regulated by the intricate interplay between the status of DNA methylation, histone modifications and other chromatin factors. The pattern at a subset of enhancers of a particular cell type might be a transient end-result of this interplay, where any of the three components is likely to be a catalyst of the patterning process. The DNA-recognition specificity of *Drosophila* Hox proteins is determined by their interaction with the dimeric cofactor Extradenticle-Homothorax (Exd) [72]. Thus, it is possible that the presence of specific cofactors in ESCs might dictate the genomic distribution of transcription factors, which in turn define the epigenetic patterns of enhancers. Interestingly, in breast cancer cells, the CTCF insulator protein has been shown to regulate the cell-specific distribution of FoxA1, which in turn affects epigenetic patterning of enhancers [73]. As CTCF regulates patterns of gene expression through higher-order chromatin organization [74], this finding highlights an unappreciated influence of nuclear architecture on epigenetic patterning of enhancers. By mediating intra- and inter-chromosomal interactions [75], CTCF might then bring co-regulated enhancers into distinct nuclear foci in a cell-specific manner, providing the spatial proximity for *en masse* patterning of enhancers by unique chromatin-modifying enzymes.

Enhancer patterning and stem cell biology

Enhancers necessary for the transcriptional activation of lineage-specific genes during ESC differentiation are epigenetically pre-patterned through specific histone modifications. As cellular



◀ **Fig 4** | Interplay between DNA methylation status, transcription factor binding and histone modification in the patterning of enhancers. (**A**) Three types of GRE have been described. Pre-programmed DHSs constitute the majority of GR occupancy upon hormone induction and are highly enriched for CGIs [(CGI) *n*]. The presence of cell-specific DHSs is correlated with DNA hypomethylation (open red stick). *De novo* binding sites are located in regions with low CpG density. Binding of GR at these sites can lead to DNA demethylation by unknown mechanisms (depicted by the binding of the unknown blue factor to GR). The third type of GRE is associated with binding sites for AP1. Recruitment of AP1 is necessary to potentiate chromatin accessibility and subsequent binding of GR. Accessible chromatin is denoted by the H3.3/H2A.Z-containing nucleosomes (light grey), whereas stable and inaccessible chromatin is denoted by H3/ H2A-containing nucleosomes (dark grey). (**B**) Many lineage-specific genes, located in regions with low density of CGIs, have inactive enhancers. During differentiation, binding of transcription factor PU.1 to HTF recognition sites initiates nucleosome remodelling and H3K4me1 modifications. This pre-patterning event allows recruitment of additional specific TFs and co-activators (p300) to these enhancers. In ESCs, active Oct4 enhancers, marked by H3K4me1/2 and H2K27ac histone modifications, are occupied by LSD1–NuRD complexes. The histone demethylase activity of LSD1 is inhibited, probably by the high level of Oct4 and p300. During differentiation, the decrease in Oct4 binding and loss of p300 allow LSD1 to demethylate H3K4me1 (red arrow), thereby decommissioning the active enhancer. AP1, activator protein 1; CGI, CpG island; DHS, DNase I hypersensitive site; ESC, embryonic stem cell; GR, glucocorticoid receptor; GRE, GR enhancer element; HTF, haematopoietic transcription factor; LSD1, lysine-specific demethylase 1; NuRD, nucleosome remodelling and histone deacetylase; TF, transcription factor.

differentiation is accompanied by changes to the epigenetic patterns of enhancers [30,42,76], reprogramming of somatic cells to iPSCs would necessitate the reversion of these changes. Pre-patterning of enhancers in ESCs, which are obtained from the inner cell mass at the blastocyst stage, is likely to occur during the early stages of embryonic development. The information for pre-patterning of enhancers in ESCs might be inherited from epigenetic signatures carried in the mature egg and sperm. Alternatively, it might be reset during spermatogenesis and oogenesis, and re-established after fertilization in the zygote. Evidence from genome-wide analyses of chromatin features in sperm suggests that both events might take place at different loci. The comparison of sperm and ESCs has revealed the presence of both overlapping and unique distributions of histone modifications and DNA methylation patterns across different genomic regions [77,78]. For instance, genes involved in developmental processes have similar epigenetic patterns (H3K4me3/H3K27me3 and hypomethylated DNA), whereas genes involved in specific functions—for example, spermatogenesis and *HOX* clusters—exhibit significant variations. A similar observation was made in zebrafish sperm, in which genes activated after mid-blastula transition are pre-patterned by specific histone modifications [79]. However, the low levels of histone modifications detected in pre-mid-blastula transition, immediately after fertilization, indicate that the epigenetic marks present in the sperm might be highly dynamic or unstable [46,47]. Elucidating the mechanisms that regulate the establishment of epigenetic patterns after fertilization will be important to understand how enhancer function is determined in ESCs.

Insights into the remodelling of epigenetic marks at enhancers during normal development might be applicable to the understanding of the mechanisms underlying the establishment of iPSCs. These cells can be obtained through the reprogramming of somatic cells by ectopic expression of defined transcription factors [80]. iPSCs are useful in a wide range of applications, including autologous cell therapy, disease modelling and as substrates for drug screening. Although chemical compounds that alter DNA methylation or chromatin modifications have been used to improve the reprogramming of various cells [81], recent comparison of iPSCs and ESCs revealed that the former retained significant somatic epigenetic patterns in the form of DNA methylation and histone modifications [82,83]. Similar observations were made in the analysis of the H3K4me1 mark in iPSC enhancers [28]. It therefore remains a challenge to understand how

different technical methodologies affect the quality of iPSCs in terms of transcriptional signatures, epigenetic status and genomic integrity.

Our understanding of enhancer features and functions in different developmental models perhaps favours the use of two alternative approaches in generating cells useful for regenerative medicine. The first approach would be transdifferentiation, in which differentiated cells are converted directly into a cell of interest without proceeding through a pluripotent intermediate [84]. Alternatively, cells can be reverted into their immediate progenitor by dedifferentiation. In theory, these two approaches should involve fewer transcription factors and might avoid extensive and aberrant reprogramming of the epigenome. Results from studies of enhancer function indicate that epigenetic outcomes can be highly context-dependent and dynamic. For example, the binding of Oct4 or Myod1 to the same permissive enhancers might elicit completely different epigenetic outcomes [23]. Myod1 activates its own transcription by binding first at the enhancer, which then leads to the formation of a transcription-permissive nucleosome-depleted region at its associated promoter. However, the binding of Oct4 to the enhancer converts the monovalent H3K27me3 mark at its cognate promoter into a bivalent state characteristic of stem cells [23]. This suggests that pluripotency transcription factors can coordinate the epigenetic states of enhancer–promoter pairs throughout the genome. Similarly, the binding of pioneering factor FoxA1 to hypomethylated enhancers can result in the further reduction of DNA methylation while increasing H3K4me2 modifications [66]. These studies suggest that enhancers might act as the signal-integrating sites for reprogramming the epigenome. Further insights into how individual reprogramming factors alter the epigenetic state of enhancers might help in the establishment of fully reprogrammed iPSCs or other progenitor cells.

Conclusions

Recent advances in genomic analyses have provided a wealth of knowledge on the features and functions of enhancers. Accumulated evidence indicates that enhancers impart epigenetic memory and dictate the context-dependent transcriptional outcomes through their unique chromatin features. Importantly, chromatin patterning of enhancers occurs before gene activation. Through differential recruitment of transcription factors and other chromatin-modifying enzymes, enhancers might determine cell fate choices and competency during development. Therefore, it is important to understand how chromatin signatures at different enhancers might be edited and maintained during differentiation. To better understand the epigenetic changes associated with disease progression, it will also be useful to identify the chromatin features of enhancers associated with various signalling pathways—for example, Notch, TGF-β, Hedgehog, Hippo and Wnt. It is obvious that the epigenetic states of enhancers are highly dynamic and regulated by various mechanisms during development. It will be interesting to find out whether the epigenetic signatures of enhancers are also regulated by other strategies including the RNAi machinery/non-coding RNA, post-translational modifications of specific transcription factors and nuclear organization.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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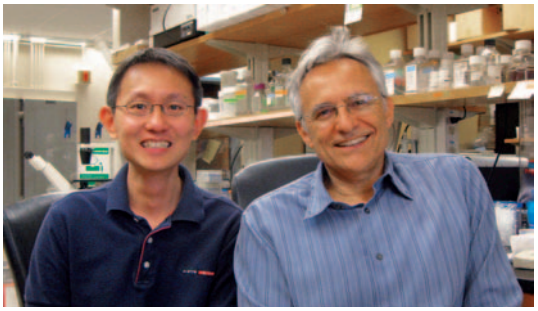

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