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Programming the genome in embryonic and somatic stem cells

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Abstract

In opposition to terminally differentiated cells, stem cells can self-renew and give rise to multiple cell types. Embryonic stem cells retain the ability of the inner cell mass of blastocysts to differentiate into all cell types of the body and have acquired in culture unlimited self-renewal capacity. Somatic stem cells are found in many adult tissues, have an extensive but finite lifespan and can differentiate into a more restricted array of cell types. A growing body of evidence indicates that multi-lineage differentiation ability of stem cells can be defined by the potential for expression of lineage-specification genes. Gene expression, or as emphasized here, potential for gene expression, is largely controlled by epigenetic modifications of DNA and chromatin on genomic regulatory and coding regions. These modifications modulate chromatin organization not only on specific genes but also at the level of the whole nucleus; they can also affect timing of DNA replication. This review highlights how mechanisms by which genes are poised for transcription in undifferentiated stem cells are being uncovered through primarily the mapping of DNA methylation, histone modifications and transcription factor binding throughout the genome. The combinatorial association of epigenetic marks on developmentally regulated and lineage-specifying genes in undifferentiated cells seems to define a pluripotent state.

Keywords: epigenetics • chromatin • DNA methylation • differentiation • embryonic stem cell • mesenchymal stem cell

Introduction

Whereas terminally differentiated cells do not divide and are developmentally programmed to carry out a specific function, stem cells have the intrinsic ability to self-renew and to give rise to multiple cell types.

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Embryonic stem cells (ESCs), *in vitro* derivatives of the inner cell mass of blastocysts, retain the ability of the inner cell mass to differentiate into all cell types of the body and acquire unlimited self-renewal capacity. For these reasons, human ESCs (hESCs) have received considerable attention since their derivation nearly a decade ago [1] because of their perceived use in regenerative medicine. Multiple extracellular factors are required for the establishment and maintenance of pluripotency in ESCs [2, 3]. These factors stimulate signal transduction cascades, such as the leukaemia inhibitory factor signalling pathway (in the mouse, not in human beings), the bone morphogenic protein-4 cascade that feeds into the leukaemia inhibitory factor pathway to enhance self-renewal and pluripotency, and the canonical Wnt signalling pathway that maintains the pluripotent phenotype by sustaining expression of pluripotency factors. These signalling pathways have been reviewed in detail elsewhere recently [2] and are beyond the scope of this review. Multi-lineage differentiation ability of ESCs is defined by the potential for expression of lineage-specification genes. Mechanisms by which these genes are poised for transcription are being unravelled through the identification and mapping of chromatin-associated proteins on gene regulatory regions.

Somatic stem cells have in recent years also been identified in many adult organs and are presumably responsible for maintaining tissue homeostasis. In particular, stromal stem cells found in a variety of mesenchymal tissues are also being scrutinized due to their potential use in autologous cell replacement therapy [4, 5]. In contrast to ESCs, mesenchymal stem cells (MSCs) seem to be restricted to forming preferentially mesodermal cell types, such as adipocytes, myocytes, osteocytes and chondrocytes. However, rare subsets of MSCs identified in bone marrow seem to have the ability to form cells types of all three germ layers (endoderm, mesoderm, ectoderm) and have challenged the restrictive differentiation potential of somatic stem cells [6]. A convenient and recently explored source of MSCs is adipose tissue-derived stem cells (ASCs) purified from liposuction material [7, 8]. Like bone marrow-derived MSCs, ASCs can differentiate into mesodermal cell types; however, recent findings suggest a limited differentiation ability even within mesodermal lineages [9, 10]. So although MSCs retain the ability of express various lineage-specific genes upon differentiation, this potential is clearly more restricted than in ESCs.

Increasing evidence suggests that the *potential* for gene expression in stem cells is regulated by epigenetic processes that confer a specific chromatin configuration on gene regulatory and coding regions. Epigenetic mechanisms refer to modifications on DNA and chromatin that do not affect DNA sequence, and that are heritable. The best characterized epigenetic DNA modification is cytosine methylation, in general associated with gene silencing. Epigenetic modifications of chromatin include post-translational alteration of histones including phosphorylation, acetylation, methylation, ubiquitination and SUMOylation, and dynamic replacement of core histone by histone variants, such as, for example, the deposition of histone 3.3 on transcriptionally active promoters [11, 12]. Additionally, in combination with epigenetic changes, positioning of transcriptional activators, transcriptional repressors, other adenosine triphosphate (ATP)-dependent chromatin remodelling enzymes, and small interfering RNAs on target genes also contribute to regulating gene expression. This review highlights our current view of the epigenetic landscape of undifferentiated and differentiated ESCs and somatic stem cells, and how this picture seems to provide molecular grounds for gene activation potential. Research perspectives aiming at defining pluripotency and further enhancing the differentiation potential in somatic stem cells are also outlined.

Epigenetic makeup of embryonic stem cells: keeping chromatin loose

DNA methylation and gene expression

Methylation of DNA consists in the addition of a methyl group to the 5 position of a cytosine in a cytosine-phosphate-guanine (CpG) dinucleotide (p signifies that the C and the G are connected by a phosphodiester bond) (Fig. 1A). CpG methylation is symmetrical—it occurs on both DNA strands (Fig. 1B)—and targets isolated CpGs, clustered CpGs or clustered CpGs within a CpG island. A CpG island is defined as a sequence in which the observed/expected C frequency is greater than 0.6 with a GC dinucleotide

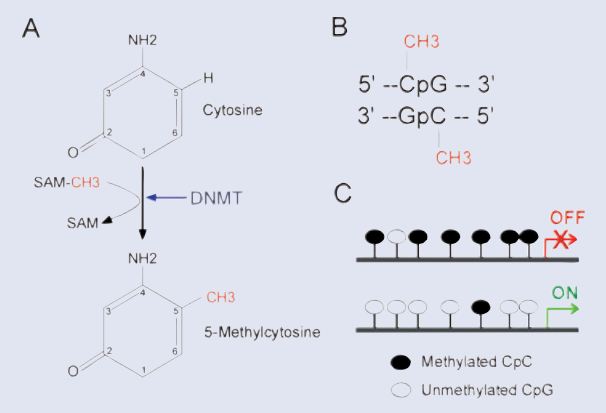

Fig. 1 CpG methylation. (**A**) Mechanism of DNA methylation. (**B**) CpG methylation is symmetrical and occurs on both DNA strands. (**C**) Simplified textbook view of the relationship between DNA methylation and gene expression.

content greater than 50%. According to Gardiner-Garden and Frommer [13], the expected number of CpG dimers in a given 200 bp window is calculated as the number of C's in the window multiplied by the number of G's in the window, divided by window length. This 200 bp window is moving across the sequence of interest at 1 bp intervals. CpG islands are often found in the 5′ regulatory regions of vertebrate housekeeping genes. CpG islands are often protected from methylation, enabling constitutive expression of these genes. CpG islands in the promoter of tumour suppressor genes, for instance, are unmethylated in normal cells, whereas a hallmark of cancer is *de novo* methylation of these CpG islands, resulting in repression of tumour suppressor genes and triggering of an uncontrolled cell cycle. DNA methylation of tumour suppressor genes constitutes the basis of a number of anti-cancer therapies relying on the inhibition of DNA methyl transferases [14].

CpG methylation is catalysed by DNA methyltransferases (DNMTs). The maintenance DNA methyltransferase DNMT1 specifically recognizes hemi-methylated DNA after replication and methylates the daughter strand, ensuring fidelity in the methylation profile after replication [15]. In contrast to DNMT1, DNMT3a and DNMT3b are implicated in *de novo* DNA methylation that takes place during embryonic development and cell differentiation [16], as a means of shutting down genes whose activity is no longer required as cells differentiate (*e.g.* that of pluripotency-associated genes). The fourth DNMT, DNMT2, has to date no clear ascribed function in DNA methylation [17–21], but has been shown to have cytoplasmic transfer RNA methyltransferase activity [22, 23].

DNA methylation is a hallmark of long-term gene silencing (Fig. 1C). The methyl groups create target sites for methyl-binding proteins which induce transcriptional repression by recruiting co-repressors, such as histone deacetylases [24]. So DNA methylation largely contributes to gene silencing [25, 26] and as such it is essential for development [27–30], X chromosome inactivation [31] and genomic imprinting [32-35]. The relationship between DNA methylation and gene expression is complex [36] and recent evidence based on genome-wide CpG methylation profiling highlights promoter CpG content as a component of this complexity [37] (see below). *In vitro* differentiation of ESCs and embryonal carcinoma (EC) cells also correlates with changes in DNA methylation notably on the promoter of developmentally regulated genes expressed in pluripotent ESCs, such as the transcription factors *OCT4* and *NANOG* [38–40]. However to date, only sporadic indications of CpG methylation changes have been reported during differentiation of MSCs or precursor cells [9, 10, 41, 42].

CpG methylation profiles in mouse ESCs

Limited evidence suggests that the DNA methylation signature of ESCs is distinct from that of differentiated somatic cells; however, whether this reflects differences in gene expression or the true pluripotent nature of ESCs is unclear. Restriction enzyme digestion-mediated analyses of global DNA methylation show that mouse ESC genomes are less methylated than those of differentiated somatic cells [43, 44]. Notably, XX chromosome-bearing mouse ESCs are further hypomethylated relative to XY ESCs. Hypomethylation affects both repetitive and unique sequences including differentially methylated regions which regulate expression of paternally imprinted loci [44]. Increased hypomethylation of XX ESCs has been attributed to the presence of two active X chromosomes (active X is hypomethylated relative to inactive X) and to reduced levels of DNMT3a and 3b. However, in DNMT-deficient [*Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$]

mouse ESCs, only 0.6% of CpGs are demethylated [43] so the extent to which DNMT3a and 3b contribute to global DNA methylation in mouse ESCs remains uncertain. DNMT1 deficiency, in contrast, reduces global methylation levels from 65% to 20%, a condition which blocks differentiation potential [43]. Unfortunately, no indication currently exists on the methylation status of regulatory regions of lineage-specific genes in mouse ESCs, which could account for their potential for expression upon differentiation.

CpG methylation patterns in human ESCs

DNA methylation analyses of hESCs have been promoted by *in vitro* fertilization data on the unexpectedly high incidence of imprinting and other epigenetic abnormalities in embryos [45], suggesting that hESCs may also display variation in their epigenetic makeup. A restriction analysis-based methylation profiling of over 1,500 CpG sites from 371 genes in 14 hESC lines [46] revealed an average of 35% methylation, a value substantially lower than that reported for mouse ES cells [44]. hESC methylation profiles were segregated from those of normal and cancer cell lines, normal tissue and somatic stem cells, reflecting an epigenetic distance between hESCs and other cell types [46]. Interestingly, less than 50 CpGs within 40 genes contributed to this difference. Another 25 CpG sites from 23 genes distinguished hESCs from normal differentiated cells and somatic stem cells; these 25 sites were found to represent markers of developmental potential [46]. Other genes differentially methylated in hESCs relative to somatic cells are markers of pluripotency, such as *OCT4* and *NANOG*, which are unmethylated in undifferentiated hESCs [47], while being partially methylated in human MSCs in which they are not expressed (ST and PC, unpublished data). Thus, on the basis of these analyses, it appears that the methylation pattern of a relatively small number of developmentally controlled genes may constitute an epigenetic mark unique to hESCs.

The requirement of large scale *in vitro* expansion of hESCs for any potential therapeutic use raises the question of epigenetic stability of hESCs in long-term culture. The consensus from published reports is that extended culture of hESCs can alter DNA methylation patterns. Restriction landmark genome scanning analysis of ~2,000 loci has identified epigenetic variations between hESC lines at least in loci functionally important for differentiation [48]. Most changes occur shortly after hESC derivation and are heritable, whereas some alterations are maintained even after *in vitro* differentiation. This study is supported by a similar methylation drift at a small number of promoters examined in late passage cultures of additional hESC lines [46, 49]. In contrast, however, stable methylation profiles were reported by bisulfite genomic sequencing in a limited number of imprinted loci in four different hESC lines [50]. So epigenetic variation occurs during extended culture of hESCs, but the timing and degree of this epigenetic drift are likely to be cell line-dependent.

An intriguing feature of DNA methylation changes reported in hESCs by Allegrucci and colleagues is heritability upon long-term expansion, raising the hypothesis that long-term culture may elicit a (re)programming of the hESC epigenome [48]. In contrast, we found that human ASCs undergo stochastic methylation changes upon culture [9, 10] (see below). So the hypothesis of programmed CpG methylation changes during culture may not necessarily hold for cell types other than hESCs. Several reports on random methylation events in human cell cultures supports this view [51–53].

Another issue is whether unscheduled CpG methylation occurs upon *in vitro* differentiation of hESCs. Analysis of over 4,600 CpG islands revealed that 65 (1.4%) undergo unexpected hypermethylation upon neurogenic differentiation of hESCs, in regulatory regions of genes involved in metabolism, signal transduction and differentiation [54]. Although distinct from tumour suppressor CpG island methylation, this hypermethylation leads to the down-regulation of the affected genes, and as such has been suggested to have implications in the development of metabolic diseases [54]. Thus, the risk of aberrant CpG island methylation upon hESC differentiation should be considered when optimizing differentiation protocols, in particular for therapeutic purposes.

With the exception of a handful of genes, methylation patterns in the human EC cell line NTERA2 are globally similar to those of ESCs [46]. Our own work also illustrates the hypomethylated state of the *OCT4* promoter and enhancer regions, the *NANOG* upstream region [40] and the *SOX2* promoter (ST and PC, unpublished data) in undifferentiated human EC cells, similarly to hESCs [47]. EC cells are in

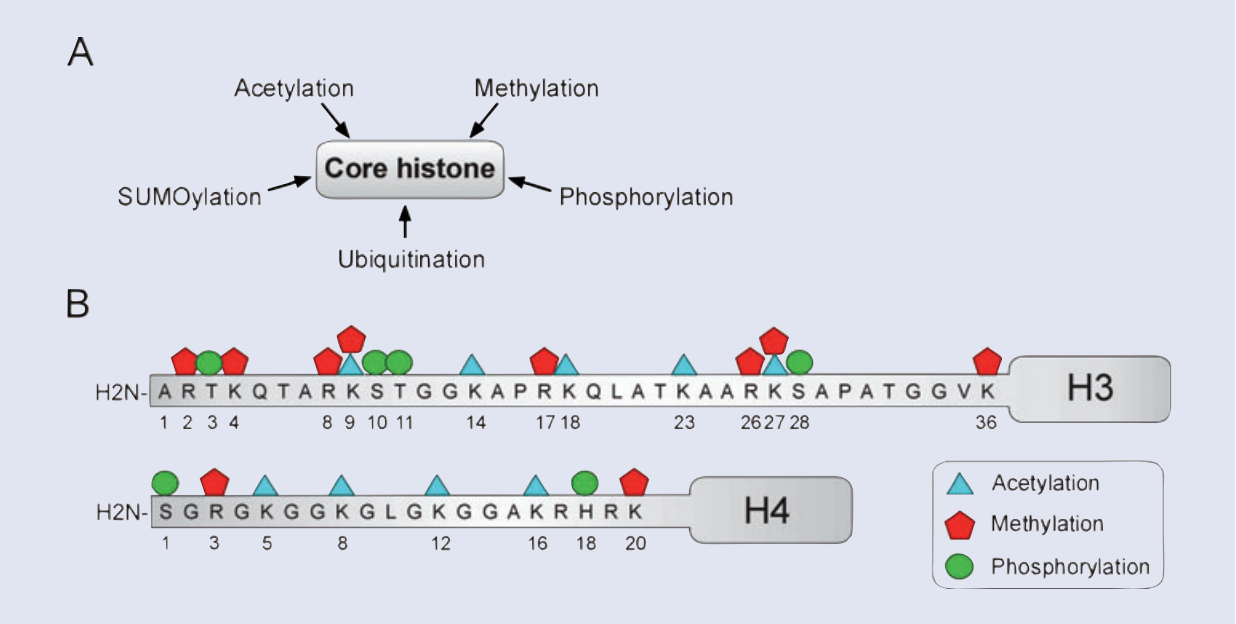

Fig. 2 Post-translational histone modifications. (**A**) Core histones can be methylated, acetylated, phosphorylated, ubiquitinated or SUMOylated, to modulate gene expression. (**B**) Known modifications on the amino-terminal tails of core histones H3 and H4.

effect ESCs that have adapted to tumour growth, so despite previous findings [46], methylation differences on, for example, cancer-associated genes might be anticipated.

In spite of these recent advances, what is currently missing is a high-resolution genome-wide DNA methylation profiling across regulatory and coding regions in ESC lines. Methyl-DNA immunoprecipitation (MeDIP) assays coupled to genomic array hybridization are particularly well suited for whole-genome and promoter investigations [37, 55]. Such data can be superimposed onto transcription factor binding [37, 55] and histone modification maps to elaborate a multi-layered epigenetic profile characteristic of pluripotent cells. Clearly, novel results are anticipated in the area of DNA methylation in ESCs.

Both active and inactive histone modification marks on developmentally regulated genes in ESCs suggest transcriptional activation potential

The eukaryotic genome is packaged and stabilized by interactions of DNA with proteins into a chromatin structure. The core element of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around two subunits of each of histone H2A, H2B, H3 and H4. Nucleosomes are spaced by the linker histone H1. The amino-terminal tails of histones are post-translationally modified to confer physical properties that affect their interactions with DNA on gene regulatory sequences. Histone modifications not only influence chromatin packaging but are also 'read' by adaptor molecules, chromatin modifying enzymes, transcription factors and transcriptional repressors, and thereby contribute to the regulation of transcription [56–59]. Epigenetic histone modifications have been best characterized so far for histone H3 and H4 and include combinatorial phosphorylation, ubiquitination, SUMOylation, acetylation and methylation (Fig. 2A and B). In particular, di- and trimethylation of H3 lysine 9 (H3K9m2/m3) and trimethylation of H3K27 (H3K27m3) elicit the formation of repressive heterochromatin through the recruitment of heterochromatin protein 1 (HP1) [60] and polycomb group (PcG) proteins, respectively [61–63]. However, whereas H3K9m3 marks constitutive heterochromatin [64], H3K27m3 characterizes facultative heterochromatin, or chromatin domains harbouring transcriptionally repressed genes that

can be activated upon *ad hoc* stimulation [65, 66]. In contrast, acetylation of histone tails loosens their interaction with DNA and creates a chromatin conformation suitable for targeting of transcriptional activators. Thus, acetylation on H3K9 (H3K9ac) and H4K16 (H4K16ac), together with di- or trimethylation of H3K4 (H3K4m2/3), are exclusively found in euchromatin, often in association with transcriptionally active genes [67–69]. In addition to altering histone-DNA interactions, H3K4m3 and H3K9ac mediate the recruitment and tethering of transcriptional activators [70, 71]. Mapping of the positioning of histone modifications throughout the genome or on given promoters has been enabled by chromatin immunoprecipitation (ChIP) assays, whereby a specific histone modification is immunoprecipitated and associated DNA sequences are identified by polymerase chain reaction (PCR) or by labelling and hybridization onto genomic arrays (reviewed in [72]).

Dynamic rearrangement of chromatin is essential for organizing heritable transcriptional programs in the context of lineage-specification [73]. Many structural chromatin proteins, such as heterochromatin protein 1 (HP1) and histones have been shown to bind more loosely to chromatin of ESCs than of differentiated or somatic cells [74]. These proteins are also hyperdynamic in ESCs relative to differentiated cells. Fluorescence recovery after photobleaching studies have shown that all three isoforms of HP1 fused with green fluorescence protein (GFP) exchange faster in heterochromatic foci of undifferentiated mouse ESCs than after differentiation [74] (Fig. 3A). Likewise, exchange rates of fluorescently tagged histones H1, H2B and H3 are significantly higher in pluripotent ESCs than in differentiated counterparts. These studies unravel the existence of a greater fraction of loosely bound HP1 as well as core and linker histones in ESCs (Fig. 3B). The hyperdynamic nature of chromatin-associated proteins in pluripotent ESCs reflects some plasticity in chromatin organization and thereby provides a basis for pluripotency. The concept of hyperdynamic chromatin in ESCs is line with an attractive yet highly speculative 'histone modification pulsing' model whereby developmentally regulated genes would be marked by transient histone modifications in pluripotent cells to enable the appropriate response upon differentiation [75]. This model remains to be validated.

In support of the view that ESC chromatin is in a loose configuration, recent mapping of histone modifications has shown that lineage-specific genes, which are either silent or active in differentiated somatic cells, are in a potentially active state in pluripotent ESCs. Genome-wide and locus-specific ChIP analyses reveal that repressed but potentially active promoters are associated with so-called 'bivalent' histone modifications characterized by H3K4m3, a mark of active genes, and H3K27m3, which associates with inactive genes [65, 66] (Fig. 4A). Azuara *et al.* [65] have shown that several transcription factors essential for lineage specification are not expressed in mouse ESCs but are marked on their promoter by H3K4m3, H3K27m3, as well as by H3K9ac. Unscheduled expression of these genes is induced in ESCs deficient for embryonic ectoderm development (Eed) protein, a component of the polycomb repressor complex PRC2 (see below), which harbours H3K27 methyltransferase activity [76], demonstrating the essential role of trimethylation of H3K27 in maintaining a transcriptional brake in a context of transcriptionally permissive chromatin. At the genome-wide level, these 'bivalent domains' consist of large regions of H3K27 trimethylation embedding smaller areas of H3K4 trimethylation [66]. Consistent with the Azuara *et al.* data [65], these domains include transcription factor encoding genes that are repressed or expressed at low levels. Intriguingly, the correlation between histone methylation marks and genomic sequence in ESCs raises the hypothesis that DNA sequence may prime the epigenetic landscape in pluripotent cells [66]. Nevertheless, not all lineage-control genes in ESCs are associated with bivalent histone modifications; rather, they are marked by H3K4m3 only or do not display H3K4m3 or H3K27m3 [66]. The critical role of these genes in lineage determination suggests that they are also in a transcriptionally poised state and await, through yet unknown epigenetic mechanisms, permission for transcription.

A regulatory role of histone H1 in gene expression in embryonic stem cells?

The linker histone H1 spaces nucleosomes on the chromatin fibre and associates with the nucleosome at the point of DNA entry presumably to prevent unwinding of the DNA at the nucleosome entry point. As referred to earlier, association of H1 with DNA is dynamic [74]. It has long been taken for granted that

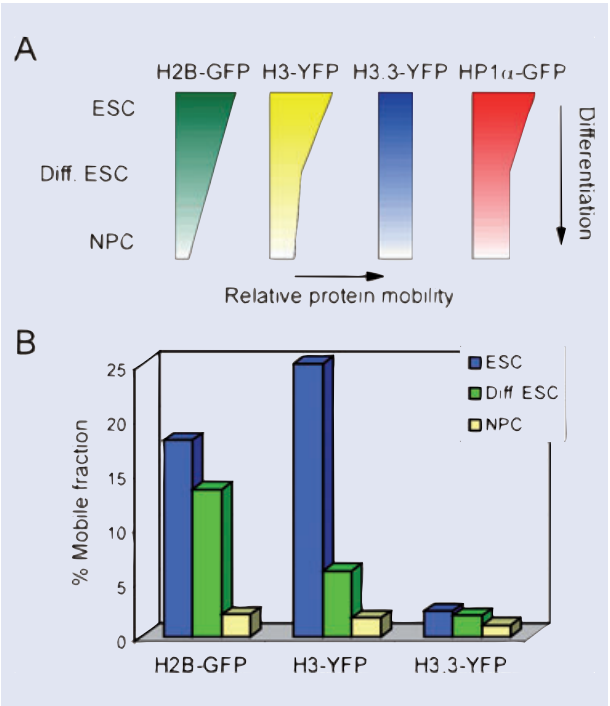

Fig. 3 Pluripotent ESCs display greater histone mobility than differentiated cells. (**A**) Relative mobility of H2B-green fluorescent protein (GFP), H3-yellow fluorescent protein (YFP) and of the histone variant H3.3-YFP was determined by fluorescent recovery after photobleaching in undifferentiated mouse ESCs, in ESCs cultured for 24 hrs without leukaemia inhibitory factor (Diff. ESCs) and in neuronal progenitor cells (NPC). Relative protein mobility (or protein recovery rate after photobleaching) is indicated by the width of the coloured bar. Mobility of HP1-GFP is also reduced upon differentiation of ESCs. (**B**) Proportion of the mobile fraction of H2B-GFP, H3-YFP and H3.3-YFP in ESCs, differentiated ESCs and neuronal progenitor cells (NPC). Figure was drawn from data presented in [74].

each nucleosome is spaced by one H1 molecule. However, it turns out that the stoichiometry of linker-to-core histones can greatly vary between cell types [77]. In particular, mouse ESCs harbour only one linker histone per two nucleosomes [78], which may also contributes to the loosening of chromatin structure. Forced reduced expression of the three H1 variants in mouse embryos, an embryonic lethal phenotype, has global effects on chromatin such as shortened nucleosome repeat length and reduced H4K12 acetylation [79]. Surprisingly though, these alterations only moderately influence gene expression: 0.5% of the genes are up- or down-regulated in H1-depleted ESCs, suggesting that the effect is restricted to specific genes. Consistent with this finding, H1 has been proposed to modulate the positioning of key nucleosomes in promoter regions, a pre-requisite for proper targeting of transcriptional regulators. Notably, the imprinted *h19* locus displays sub-stoichiometric amounts of H1 even in wild type ESCs [79], suggesting that global reduction of histone H1 plays a regulatory function, rather than structural role, on gene expression [80].

H1 positioning at critical nucleosomes is also likely to modulate transcription by controlling DNA methylation. Indeed, whereas global DNA methylation levels are normal in H1-depleted ESCs, specific CpGs have been found to be hypomethylated within the imprinting control regions of the *H19-Igf2* and *Gtl2-Ik1* loci [79]. An attractive possibility, then, is that a minimal amount of histone H1 is necessary to establish gene-specific DNA methylation patterns.

Polycomb group proteins impose a transcriptional brake on lineage-priming genes

PcGs are transcriptional repressors [81, 82] found within two distinct and conserved PRCs (PRC1 and PCR2) working co-operatively [83]. Involvement of PRCs in pluripotency has been suggested by the requirement of PcG proteins for the patterning of gene expression during development, for establishing pluripotent ESCs and for maintaining somatic stem cell cultures (reviewed in [84]).

In undifferentiated ESCs, PcGs preferentially (but not exclusively) occupy genes that are activated upon differentiation, consistent with the view that these genes are poised for transcription [85–87] (Fig. 4B). Histone methyltransferase activity of Eed and enhancer of zeste homologue 2 (Ezh2; another PRC2 component) is responsible for trimethylation of H3K27 on these target genes [61, 62]. In addition, trimethylation of H3K4 is mediated by Trithorax group (Trx) proteins [82]. Thus, the known interplay between PcG and Trx proteins is also likely to establish bivalent histone modifications on developmentally regulated genes in pluripotent cells. PcGs, however, are also dynamic and not always associated with transcriptionally repressed genes. For genes activated upon

differentiation, PcGs are displaced from promoters [87]. Furthermore, genes that are repressed during differentiation have also paradoxically been found to be already occupied by PcG proteins in undifferentiated cells, while in a state of activity. These findings suggest that PRCs constitute a 'pre-programmed memory system' established during embryogenesis [87]. This program would mark certain genes for transcriptional repression upon differentiation, while other genes would be primed for activation (Fig. 4B). It will be interesting to determine whether genes poised for transcriptional activation or repression by PcG proteins are marked by distinct histone modifications (*e.g.* different levels of the active H3K9ac mark) or by a specific CpG methylation status. An increasing body of evidence, therefore, suggests that unique combinations of CpG methylation, histone modifications, PcG occupancy and nucleosome positioning [88–91] on developmentally regulated gene promoters, in a context of hyperdynamic chromatin, define a pluripotent genomic organization in ESCs.

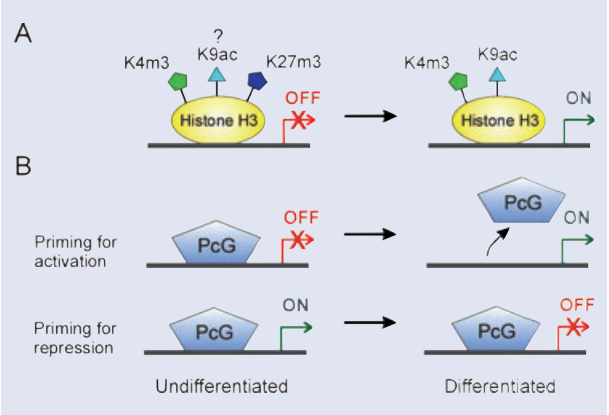

Fig. 4 Regulation of lineage-specific gene expression by histone H3K27 methylation and PcGs. (**A**) In undifferentiated cells, repressed lineage-specific genes are marked by trimethylation of K4 and K27 (the bivalent marks) and acetylation of H3K9. These marks are believed to prime genes for activation. Upon differentiation, demethylation of H3K27 results in transcriptional activation of the gene. (**B**) In undifferentiated cells, repressed lineage-specific genes can be primed for activation by occupancy of PcGs on the promoter; differentiation coincides with removal of the PcG complex and activation of the gene. However, genes expressed in undifferentiated cells can also be primed for transcriptional repression by PcG complexes on the promoter. PcG complexes (PRCs) are therefore suggested to form a 'pre-programmed memory system' established during embryogenesis [87].

The epigenetic makeup of mesenchymal stem cells reflects restricted differentiation potential

The interplay between epigenetic modifications and potential for gene activation in ESCs is being unravelled, however, the picture remains largely incomplete when it comes to somatic stem cells. This section highlights recent published and unpublished findings on the relationship between DNA methylation of lineage-specification genes, gene expression and potential for cell differentiation in MSCs, with a focus on ASCs from which most epigenetic analyses have been reported. A concept emerging from these studies is that a CpG methylation pattern pre-programs ASCs for differentiation into adipocytes preferentially over other cell types.

CpG methylation patterns on lineage-specific promoters in adipose stem cells

Adipose tissue harbours an abundant source of MSCs [7, 8, 92, 93]. Human ASCs with a $CD34^{+}CD105^{+}CD45^{-}CD31^{-}$ phenotype have been isolated with high purity (~99%) from the stromal vascular fraction of liposuction material [7]. ASCs display a gene expression profile and surface antigen phenotype similar to bone marrow-derived MSCs [7, 94–96], highlighting a common mesodermal ancestor. ASCs exhibit primarily mesodermal differentiation abilities *in vitro* and can promote neuronal functions, osteogenic repair and reconstitution of the immune system *in vivo* (reviewed in [41, 92]). ASCs also can differentiate toward the endothelial cell lineage *in vitro* and contribute to the re-vascularization of ischaemic tissue; however, whether their contribution is direct or indirect remains debated [9, 97]. Transcriptional profiling of freshly isolated, uncultured ASCs reveals expression of genes extending across the three germ layers, suggestive of a differentiation potential toward non-mesodermal lineages [7]. Yet, whether ASCs form functional tissues of these lineages *in vivo* is still unclear.

Recent studies have begun to unravel the CpG methylation profile of a number of tissue-specific genes in human ASCs (Fig. 5A and B). Bisulphite genomic sequencing of four adipogenic promoters (leptin [*LEP*], peroxisome proliferator activated receptor gamma 2 [*PPARG2*], fatty acid-binding protein 4 [*FABP4*] and lipoprotein lipase [*LPL*]) reveals several DNA methylation features in freshly isolated, uncultured ASCs [10]. First, these promoters are globally hypomethylated, with 5–30% methylated CpGs. Second, CpG methylation profiles are mosaic between ASC donors and within donors. Mosaic methylation is consistent with that observed in stem cells isolated from single intestinal crypts [98–100]. Mosaicism is believed to result from stochastic methylation which accumulates independently in different cells as a result of exposure to environmental, aging and health factors [14, 100–103], together with a propensity of certain CpGs to be hypermethylated [52, 104]. Indeed, each locus examined in ASCs displays CpGs that are preferentially susceptible to methylation [10].

In contrast to adipogenic promoters, however, myogenic or endothelial cell regulatory regions display significantly more methylation [9, 10] (Fig. 5B). The myogenic promoter myogenin (*MYOG*) is completely methylated in freshly isolated ASCs. *MYOG* is also completely methylated in endothelial cells as expected from this cell type (A.C. Boquest, A.L. Sørensen and PC, unpublished data). In addition, regulatory regions of the *CD31* (also called platelet endothelial cell adhesion molecule-1 or *PECAM1*) and *CD144* (also called vascular endothelium cadherin or *CDH5*) genes are also extensively methylated in ASCs but not in endothelial progenitor or differentiated cells [9]. Housekeeping genes such as *GAPDH* and *LMNB1* are unmethylated, as expected from their constitutive expression. Thus, current results illustrate the hypomethylation of adipogenic genes in freshly isolated ASCs, while non-adipogenic lineage-specific genes are methylated. This raises the view of an epigenetic programming of ASCs for preferred adipogenic differentiation, imposed by a DNA methylation pattern at key promoters.

Clonal culture of human ASCs does not significantly alter methylation of adipogenic and non-adipogenic promoters. Few CpGs in the *LEP*, *FABP4* and *LPL* promoters become methylated in culture, while even fewer are demethylated. However, increased mosaicism in CpG methylation occurs between cell clones compared to that detected between individual ASC donors [10], although culture of ASCs to senescence does not enhance mosaicism (AN and PC, unpublished data). In contrast to a previous report on CpG methylation in hESCs [48], we have no evidence of heritable methylation changes in cultured ASCs, suggesting randomness. In addition to presumed defects in DNMT1 function, it is possible that different cells in the initial ASC population display mosaic CpG methylation. Moreover, asymmetric cell division, a characteristic of pluripotent stem cells, is expected to generate a different epigenetic pattern in each daughter cell within a clonal population. Collectively, these studies suggest that hypomethylation of adipogenic promoters, in contrast to other lineage-specific promoters, constitutes an epigenetic signature of human ASCs. A possibility, then, is that MSCs are pre-programmed by DNA methylation of lineage-specific genes to preferentially differentiate into the cell type(s) of the tissues in which they reside. We are currently testing this hypothesis.

The hypomethylated state of adipogenic promoters in undifferentiated ASCs raises the question of how DNA methylation correlates with transcription. Interestingly, all genes examined in the above study were expressed in freshly isolated (uncultured) ASCs, but not all clonal ASC cultures expressed all adipogenic genes examined despite their hypomethylated state [10]. Conversely, hypermethylation does not preclude expression, as exemplified by transcription of the methylated *CD31* and *CD144* loci in ASCs [9] (see also below). Thus, gene expression in ASCs does not correlate with a specific methylation pattern in any of the genes examined thus far. This observation is not restricted to pluripotent cells [36, 105].

CpG content affects the relationship between promoter DNA methylation and transcriptional activity

Recent genome-wide DNA methylation profiling shows that the relationship between promoter DNA methylation and promoter activity depends on the CpG content of the promoter [37]. Promoters with low CpG content display no significant correlation between activity (determined by RNA polymerase II occupancy) and abundance of methylated CpGs,

arguing that transcriptionally active low CpG promoters (LCPs) are not necessarily un- or hypomethylated [37]. Indeed, it seems that most low CpG promoters are methylated whether they are active or not. On the contrary, activity of intermediate CpG promoters (ICPs) and high CpG promoters (HCPs) was found to inversely correlate with the extent of methylation [37]. In these categories, the proportion of transcriptionally active promoters decreases as DNA methylation increases, arguing that methylation of ICPs and HCPs is incompatible with transcription. Further analysis, however, shows that inactive ICPs and HCPs differ in their DNA methylation status: most inactive HCPs are unmethylated, whereas a high proportion of inactive ICPs are methylated. So collectively, the work of Weber and colleagues [37] elegantly argues that inactive HCPs globally remain unmethylated, inactive ICPs are often methylated, whereas LCPs are frequently methylated regardless of their activation status.

Can we then account for the lack of relationship between CpG methylation promoter activity in ASCs [9, 10]? The hypomethylated state of the *LEP* promoter in undifferentiated cells, irrespective of its activation state, would be consistent with the findings of Weber *et al.* [37], as CpG distribution in a CpG island across the *LEP* promoter most likely places it in the HCP category. Indeed, most CpG island promoters remain unmethylated even in cell types in which the gene is not expressed [106]. In addition, the methylation percentage of the *FABP4* promoter (likely an LCP) is higher than that of the other promoters examined, and this percentage remains unaltered upon differentiation-induced up-regulation of the promoter. The *PPARG2* promoter, in contrast, may be an exception to the LCP class, because it remains hypomethylated regardless of expression level. Further, the *LPL* promoter may belong to the ICP category despite its constitutive hypomethylated state irrespective of activity. Lastly, the *CD31* promoter is expected to belong to the ICP category on the basis of its CpG content [9] and in agreement with the Weber contentions [37], it is hypermethylated in the state of weak activity in undifferentiated ASCs, whereas activation in endothelial precursor or differentiated cells correlates with CpG demethylation [9]. It will be interesting to carry out genome-wide promoter CpG methylation studies in different MSC populations to determine whether the relationship between CpG content, methylation state and transcriptional status identified by Weber and colleagues [37] also applies to embryonic and somatic stem cells.

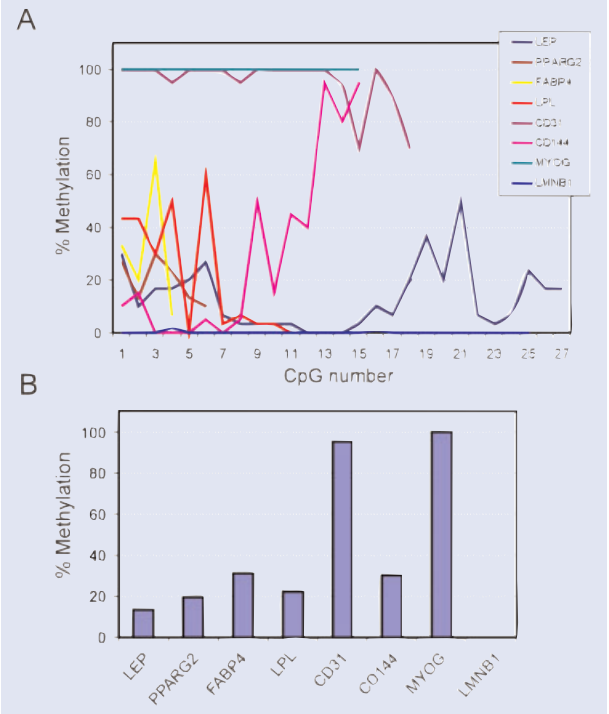

Fig. 5 CpG methylation profile in the promoter region of lineage-specific and housekeeping genes in undifferentiated human ASCs. Genes indicative of the adipogenic lineage (*LEP, PPARG2, FABP4, LPL*), endothelial cell lineage (*CD331, CD144*) and myogenic lineage (*MYOG*) are represented. Lamin B1 (*LMMB1*) is a constitutively expressed gene. (**A**) The graph shows the average percentage of methylation of each cytosine in CpG dinucleotides in these promoter regions as determined by bisulphite genomic sequencing [9, 10]. (**B**) Mean percentage of methylation across the promoter regions examined. Note the greater percentage of methylation in *CD31* and *MYOG* relative to adipogenic promoters ($P<0.001$; t-tests). The *CD144* promoter appears relatively hypomethylated due to unmethylation of the 5′ half of the region examined, while the 3′ half is fully methylated in undifferentiated ASCs [9]. The *LMNB1* promoter is unmethylated.

Bivalent histone modifications on potentially active genes?

Virtually nothing is known on the histone modification pattern of somatic stem cells and of MSCs in particular. Analyses have to date been restricted to normal

differentiated cultured cells, cancer cell lines and murine ESCs. The availability of ChIP assays suitable for chromatin from small cell numbers (in the hundreds) [39, 107], however, opens avenues for investigating limiting cell samples, such as embryonic cells or hESCs [107]. Preliminary observations from our laboratory point to, as in ESCs, the presence of the activating H3K4m3 mark (together with acetylated H3K9) and of the repressive H3K27m3 modification on adipogenic promoters of undifferentiated ASCs (AN and PC, unpublished data) (Fig. 6, MSCs). So together with the hypomethylated state of these promoters [10], these presumably bivalent histone marks (co-occupancy on the same nucleosome remains to be demonstrated) reinforce the view of an adipogenic promoter pre-programmed for activation upon adipogenic stimulation. Upon differentiation, gene activation is accompanied by a reduction in trimethylated H3K27 (AN and PC, unpublished data), possibly as a result of PRC2 removal or active demethylation of H3K27 (Fig. 6). Inactivation of the promoter, in contrast, would lead to deacetylation and trimethylation of H3K9, removal of demethylation of H3K4 and maintenance of trimethylated H3K27.

Linking DNA methylation to histone modifications, chromatin packaging and (re)organization of the nuclear compartment

DNA methylation has long been implicated in the organization of the nuclear compartment, particularly in regions of constitutive heterochromatin (see [108] for an overview of the evidence). A recent study shed light on the nature of the relationship between global DNA methylation levels and chromatin organization [108] (Fig. 7A). Indeed, *Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$ mouse ESCs lacking DNA methylation have been shown to exhibit enhanced clustering of pericentric heterochromatin and major changes in chromatin structure [108]. More specifically, levels of dimethylated H3K9 are reduced (H3K9m3 level remains surprisingly unaltered) while levels of acetylated H3K9, H4K5 and H4K16 increase, both globally and on major satellite repeats, suggesting a reorganization of heterochromatin in these cells. Mobility of the linker histones H1 and H5 is also reduced. In contrast, absence of DNA methylation does not seem to affect compaction of bulk and heterochromatin, on the basis of nuclease digestion, nucleosome spacing and chromatin fractionation [108] (Fig. 7A). Interestingly, genes reactivated by elimination of DNMT1 in mouse ESCs become enriched in acetylated H3K9 and H3K14, acetylated H4 and trimethylated H3K4, while those not reactivated by removal of DNA methylation show no hyperacetylation [109] (Fig. 7B). Thus, some methylated genes in ESCs are subject to additional repressive mechanisms affecting histone H3 acetylation. These studies illustrate how DNA methylation affects global chromatin packaging and subsequently, organization of the nucleus, but in a manner that does not involve chromatin compaction. Despite these global changes, however, different classes of genes respond differently to the absence of DNA methylation.

Timing of DNA replication has been shown to be influenced by the state of chromatin (active vs. inactive), albeit not always by transcription *per se* [65, 110]. Replication timing has been introduced as an additional epigenetic component [111], although whether it qualifies as an 'epigenetic' component on the basis of the definition of epigenetics remains questionable (replication timing is *per se* not a modification of DNA or chromatin). Interestingly, in mouse ESCs, a number of genes not necessarily expressed but which may be important later during differentiation have been shown to replicate early in S phase [65]. Genes that are not needed, however, replicate later in S phase. Indeed, genes encoding key neuronal-specific transcription factors replicate early in undifferentiated ESCs, but late in haematopoietic stem cells in which these genes are not required [65]. Therefore, lineage-specification genes are able to undergo modifications in chromatin organization and switch from early to late replication timing in the course of differentiation.

Early replication timing has been linked to enriched histone acetylation [112, 113], but how replication timing functionally relates to DNA methylation remains to be explored. Recent evidence indicates that genes whose expression is dependent on DNA demethylation in ESCs consistently replicate early in S phase, while half of those genes not reactivated by DNA demethylation replicate late [109]. Nonetheless, the overall replication timing pattern

does not seem to be dependent on CpG methylation [114] and methylation is not necessarily affected by replication timing profile, suggesting that replication timing and DNA methylation profiles are independently established [109].

Perspectives: towards remodelling the stem cell epigenome?

The advent of genome-wide technologies has provided a wealth of information on mechanisms regulating gene expression in the context of development, differentiation, cell cycle and disease. These studies have also started to unravel the epigenetic landscape of ESCs and somatic stem cells, providing a molecular frame for the pluripotent state. Such approaches have in our opinion been welcome because defining pluripotency on mere gene expression profiling in hESCs has proven deceptive [115].

Several aspects of stem cell function remain nevertheless to be investigated. For one, we are most likely looking the tip of the iceberg when it comes to understanding epigenetic programming of stem cells. Mapping of novel histone modifications and of novel transcriptional regulators [116] together with improved bioinformatics tools will enhance the resolution of the current stem cell epigenetic map.

Secondly, a largely unexplored area is *in vivo* epigenetics [9, 10]. The fate of ESCs after transplantation into animal models is being extensively examined, but the extent of contribution of MSCs to various tissues remains debated. Our preliminary analyses of DNA methylation changes in ASCs after *in vitro* differentiation suggest that the cells retain an undifferentiated ASC epigenetic program despite phenotypic changes [41]. In the event MSCs do directly contribute to host tissue *in vivo*, a hypothesis is that the target tissue provides a beneficial environment for stem cell function. Intuitively, the *in vivo* milieu may be more conducive to epigenetic commitment of MSCs than the Petri dish.

Thirdly, broader application of imaging techniques to stem cell chromatin dynamics, gene expression and epigenetics [73, 74, 117–120] is also likely to contribute to our understanding of genome organization in stem cells. Ultimately, compilation of nucleus-wide four-dimensional imaging data and genome-wide biochemical and genetic data sets promises to provide an integrated representation of genome organization in relation to function in stem cells.

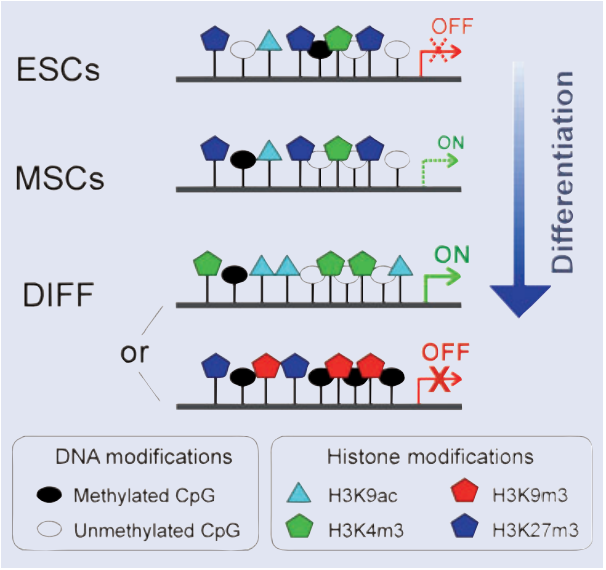

Fig. 6 Epigenetic landscape of genes associated with lineage specification as function of differentiation. ESCs, undifferentiated embryonic stem cells; MSCs, undifferentiated mesenchymal stem cells; DIFF, differentiated somatic cells. Two scenarios are presented for lineage-specific genes in differentiated cells, depending on whether the gene is activated or up-regulated (ON), or turned off (OFF). Note that relationship between promoter DNA methylation and promoter activity depends on CpG content of the promoter [37]; thus, an expressed gene in differentiated cells is not necessarily unmethylated (see text for details).

Lastly, the apparent restricted differentiation potential of MSCs currently limits their application to regenerative medicine. Qualities of the ideal stem cell in a clinical setting are expected to be extensive (unlimited?) ability to be expanded in culture without genetic and epigenetic abnormalities, ability to form functional cell types *in vitro* and *in vivo*, and immunocompatibility with the patient. Patient-derived somatic stem cells fulfil the latter requirement; however, they currently do not meet the first two. Attempts to alleviate limited differentiation potential of MSCs aim at enhancing differentiation plasticity through a nuclear re-programming process. Current strategies for re-programming somatic cells to pluripotency include nuclear transplantation into eggs [121–123], fusion with ESCs [124–126], treatment with extracts

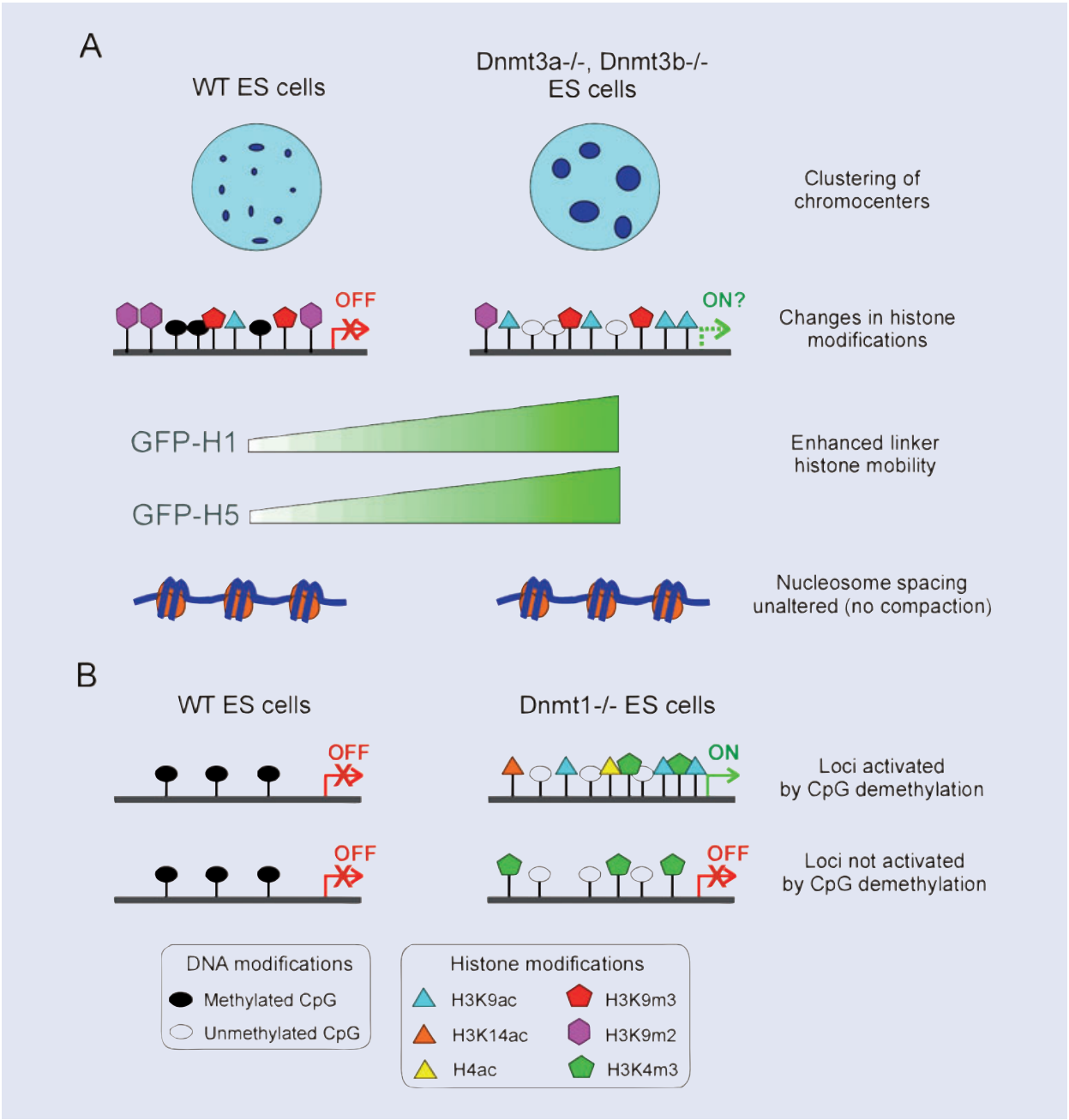

Fig. 7 Ablation of DNA methylation in mouse ESCs results in reorganization of the nuclear space. (**A**) ESCs depleted of DNMT3a and 3b (obtained from *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ embryos), compared to wild type (WT) ESCs, display a clustering of chromocentres, CpG demethylation associated with enhanced H3K9ac and reduced H3K9m2 (while H3K9m3 remains unaltered), and increased mobility of the linker histones H1 and H5. However nucleosome spacing is not altered, indicative of absence of marked chromatin compaction. (**B**) In ESCs depleted of DNMT1 (obtained from *Dnmt1*$^{-/-}$ embryos), histone modification changes differ on promoters activated by loss of CpG methylation (top panels) and on those not activated by loss of CpG methylation (bottom panels).

from eggs [127], ESCs or other pluripotent cells [40] and retroviral transduction of pluripotency-associated factors [128]. These approaches have been recently reviewed [129, 130]. Few attempts are reprogramming somatic stem cells have been reported to date, and recent results in the mouse suggest that cloning efficiency of progenitor cells, compared to terminally differentiated cells, by nuclear transfer is not improved (on the contrary) [131]. Examination of the epigenetic profile of cloned embryos and nuclear transfer-derived ESCs from somatic stem cell and differentiated cell donors may provide an indication on the origin of the developmental defects of the cloned embryos. So will somatic stem cells one day be safely reprogrammed to a pluripotent state to enable their use in therapeutic applications? More hard work will tell.

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