

Epigenetics

Judge, jury and executioner of stem cell fate

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Emerging evidence is shedding light on a large and complex network of epigenetic modifications at play in human stem cells. This “epigenetic landscape” governs the fine-tuning and precision of gene expression programs that define the molecular basis of stem cell pluripotency, differentiation and reprogramming. This review will focus on recent progress in our understanding of the processes that govern this landscape in stem cells, such as histone modification, DNA methylation, alterations of chromatin structure due to chromatin remodeling and non-coding RNA activity. Further investigation into stem cell epigenetics promises to provide novel advances in the diagnosis and treatment of a wide array of human diseases.

Introduction

Stem cells are defined by two fundamental properties: self-renewal and pluripotency or multipotency. Until recently, the analysis of stem cells and their lineages has largely focused on transcriptional regulation. Current data, however, suggests that the genome undergoes major epigenetic alterations during embryonic stem cell (ES cell) differentiation in mammalian development.¹ Exciting progress in multiple studies of specific epigenetic features of human and mouse stem cells has provided insights into the unique properties of pluripotent and niche-restricted stem cells, and outlined the importance of the molecular mechanisms that control these epigenetic events. Complex regulations of the self-renewal and differentiation states of embryonic and adult stem cells and their “stemness” not only heavily rely on transcriptional factor networks, but also on the properties of the chromatin within the cells, the so-called “epigenetic landscape.” This heritable landscape is indispensable for establishing different degrees of chromatin compaction and conveying specialized gene expression patterns, which define the molecular basis of pluripotency, reprogramming and early human development. The importance of epigenetic regulation in maintaining gene expression and, therefore, cell fate determination is well established.

Epigenetic mechanisms operating within the cell include: post-translational modifications of histones (histone PTMs) and incorporation of histone variants, changes in DNA methylation,

ATP-dependent chromatin remodeling and the implementation of RNAi pathways and non-protein coding RNAs. There is a highly orchestrated and collaborative action between different epigenetic pathways to establish unique epigenetic states and to drive the final outcome of the transcriptional hierarchy mediated by transcriptional factors. This synergistic regulation allows for alterations of gene expression without changes to the DNA sequence. Perturbation of these epigenetic components may result in changes to local chromatin configuration and nuclear architecture within the stem cell, collapsing the self-renewal circuitry and triggering loss of stemness by promoting differentiation.²⁻⁵ Somatic cell nuclear transfer experiments have also unambiguously demonstrated that reprogramming to a pluripotent state requires large-scale epigenetic changes within the cell.⁶⁻⁸ By examining the abundance of modified histones and binding patterns of their modifying complexes, such as Polycomb group (PcG) and Trithorax group (TrxG) proteins, as well as replication timing and chromatin accessibility, new studies have revealed that stem cells manage their status through multiple layers of epigenetic events that impose flexible but precise control over the expression of important regulatory genes.⁸⁻¹¹ For instance, this complex regulation promotes the expression of pluripotency-associated factors, such as OCT3/4 (POU5F1) and NANOG, while transiently prohibiting activation of the genes that drive cellular differentiation along specific differentiation pathways.

In this review, we will discuss recent progress that points to an active role of epigenetic regulation in pluripotency and stemness, as well as in driving cell fate specification. We will also discuss recent discoveries that have shaped the emerging viewpoints in the field, focusing on the following questions: (1) How are epigenetic pathways involved in retaining stem cell potential? (2) How does a stem cell rapidly transition into a morphologically and molecularly distinct cell type, and is this event driven by epigenetic alterations? And (3) is this process reversible?

Stem Cells and Epigenetics

According to accepted terminology, stem cells are immature cells with the ability to self-renew and differentiate into multiple cell types. They can be classified, based on their relevance to developmental events, as embryonic or adult stem cells. Induced pluripotent stem (iPS) cells represent an additional class of stem cells, artificially derived from non-pluripotent cells (e.g., adult somatic

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or stem cells).¹² Stem cells of all types are characterized by stable, heritable states, allowing for multiple developmental pathways. During development, the potency of stem cells is reduced over time from totipotent (morula) to pluripotent (embryonic stem cells) to multipotent (fetal and adult stem cells) to omnipotent (precursor cells) due to progressive gene silencing. Genes active in earlier progenitors are gradually silenced at later stages during development, and subsets of cell type-specific genes are turned on. This progression is the result of selective expression of transcription factors and orchestrated action of the classic “corner stones” of epigenetics: chromatin remodeling and chromatin modifications, DNA methylation of CpG dinucleotides and activity of non-coding RNA.¹³⁻¹⁵ An important property of the epigenetic changes within the cell is that they are heritable. Once established, epigenetic modifications can be maintained and propagated through cellular division. Establishment of specific epigenetic signatures within the cell requires coordinated action of numerous enzymatic machineries responsible for deposition (writers) and removal (erasers) of epigenetic modifications, as well as protein complexes that recognize these modifications (readers).

Multiple levels of epigenetic regulation converge in the chromatin to establish transcriptionally permissive, less condensed euchromatin, and highly condensed and often repressed heterochromatin.¹⁶ Such complex nuclear architecture of stem cells is important for regulating transcriptional outcomes. Several recent studies suggest that nuclear structure experiences tremendous morphological alterations when ES cells progress along the differentiation axis. These alterations range from changes in nuclear lamina,¹⁷ size and shape of the nuclei, nucleolus, nuclear speckles (domains enriched in splicing factors) and Cajal bodies.¹⁸ As a result of epigenetic events, the level of chromatin compaction and its accessibility and positioning within specialized nuclear domains undergoes dynamic changes upon stem cell differentiation, as shown by changes in chromatin organization components, such as heterochromatin,¹⁹ promyelocytic leukemia bodies (PML NBs)¹⁸ and centromere positioning.²⁰ The first line of evidence for these remarkable dynamics came from visualization of chromatin in mouse ES cells using electron microscopy. The chromatin of pluripotent stem cells was noticeably devoid of heterochromatin, though prevalent in differentiated cells, suggesting that ES cells have an open or “loose” chromatin structure. Later, it was proven that chromatin of pluripotent ES cells is characterized by high rates of histone protein exchange, coupled with dispersed and very dynamic localization of heterochromatic markers, such as heterochromatic histone modifications like trimethylation on lysine 9 of histone H3 (H3K9me3) and chromatin bound protein HP1.¹⁹ Open chromatin correlates with a globally permissive transcriptional state, and has been proposed to contribute to the developmental plasticity, or pluripotency, of ES cells.²¹

As differentiation advances, cells undergo global chromatin reorganization,²² leading to accumulation of more rigid heterochromatin, driven by compaction of major satellite repeats and the pericentric regions of some chromosomes, resulting in concentrated heterochromatic foci detectable upon cytological

analysis.¹⁹ This suggests that the pluripotent nature of the ES cell genome becomes more transcriptionally restrained, due to chromatin condensation and maturation of heterochromatin upon differentiation. It has also been experimentally confirmed that the pluripotency-specific genes *OCT3/4* and *NANOG*, as well as lineage specific genes, undergo changes in their relative positioning between the transcriptionally-restrictive nuclear periphery and the transcriptionally-permissive nuclear interior,^{14,20} thus suggesting that three dimensional nuclear architecture is an important regulator of gene transcriptional activity in stem cells.

Understanding how this nuclear organization is established and how it influences gene expression might subsequently allow for a better understanding of pluripotency as a cellular state.

Stem Cell Chromatin Composition: Nucleosome Composition and Histone Variants

Before we take a dive into the ocean of epigenetic players and mechanisms that control pluripotency, self-renewal and differentiation, it is important to discuss the basics of chromatin composition. Early work on the nuclear packaging of chromosomal DNA has defined the basic unit of the DNA/protein complex known as chromatin. This fundamental unit, the nucleosome, is comprised of two copies of the histone proteins H3, H4, H2A and H2B creating a bead-like structure. Then, 146 bp of DNA is wrapped around the surface of this structure formed by these core histone proteins. The linker histone, H1, binds the nucleosome at the entry and exit sites of the DNA wrapped around the nucleosome core particle, thus locking the nucleosomal particle in place (Fig. 1A).

In the context of chromatin, nucleosomes can be: (1) covalently modified by chromatin modifying complexes, which provide histone tail and globular domain modifications or (2) repositioned by chromatin remodeling complexes, which cause an alteration of DNA-histone contacts. This occurs in a highly combinatorial and, sometimes, mutually exclusive fashion. Nucleosomal packaging and histone modifications dictate the different degrees of primary chromatin compaction, e.g., six nucleosomes per 11 nm in the euchromatic chromatin fiber vs. 12–15 nucleosomes per 11 nm in heterochromatin, which is achieved by additional chromatin structural proteins.²³

The core histones are subjected to numerous different PTMs including acetylation, methylation, phosphorylation, poly-ADP-ribosylation, ubiquitination and sumoylation (Fig. 1A).²⁴ Different chromatin states are defined by combinatorial patterns of these histone modifications that are often referred to as the “histone code.”²⁵ Each histone modification can induce or inhibit subsequent PTMs, and such cross talk can operate on the same nucleosome or can be established between nucleosomes.²⁶ To add to this complexity, the chromatin structure is also influenced by effector or “reader” proteins that recognize single or multiple histone PTMs (Fig. 1B). Moreover, this recognition can occur with PTMs on a single nucleosome, or several nucleosomes that can be either present on the same or different chromatin fibers (inter-chromosomal interactions). We will address some of these specific reader proteins at a later point.

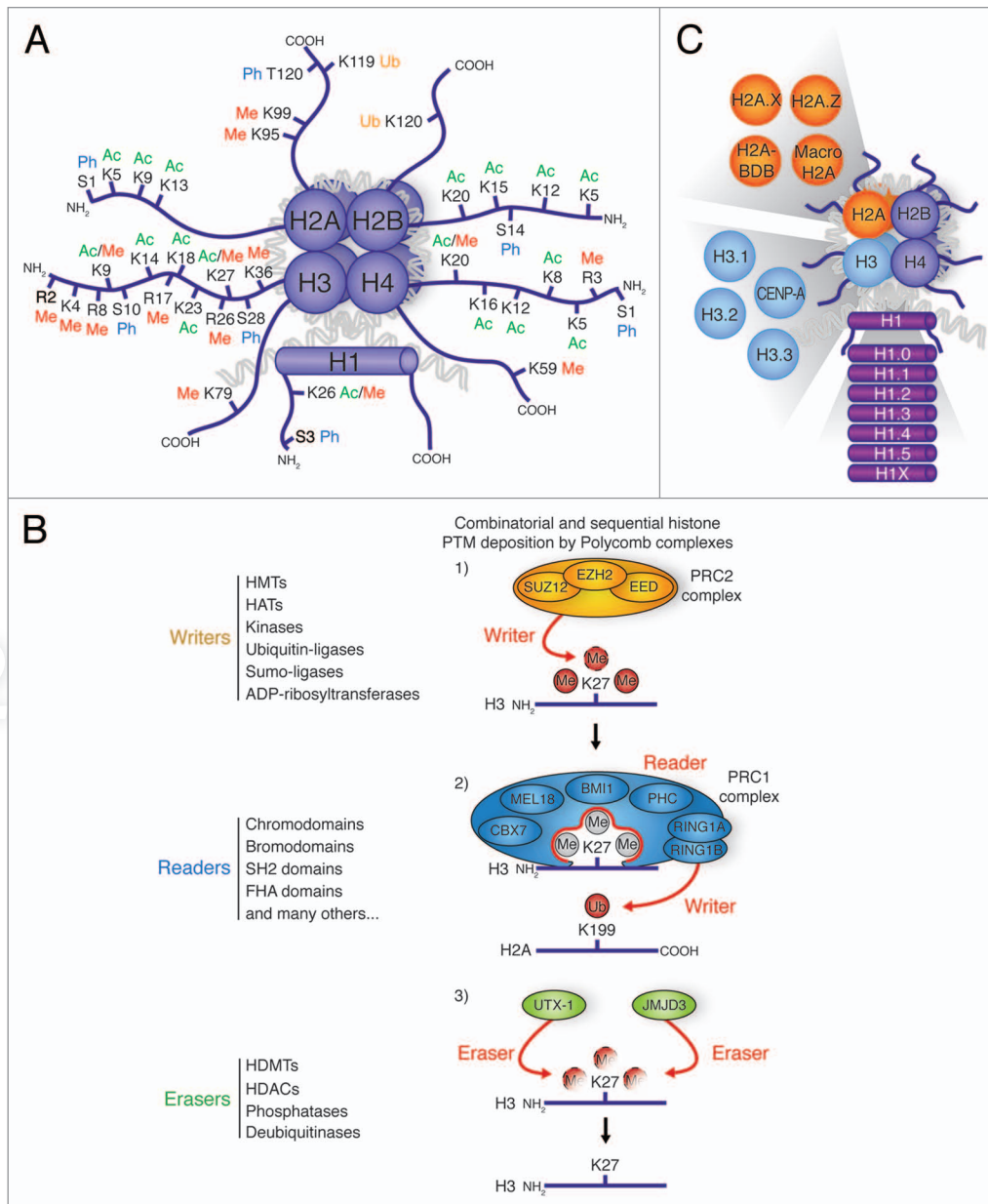


Figure 1. Histone post-translational modifications and variants. (A) Schematic drawing of a nucleosome with the four canonical histones (H2A, H2B, H3 and H4) and the linker histone H1. The covalent PTMs [methylation (Me), acetylation (Ac), ubiquitination (Ub), and phosphorylation (Ph)] are highlighted on the N- and C-terminal tails of each histone. (B) Graphical representation of histone PTM writers, which add covalent PTMs to histone tails, readers, which recognize and bind histone PTMs, and erasers, which remove histone PTMs. Protein families associated with these steps are listed on the left. In this example, the PRC2 complex adds (writes) tri-methylation on lysine 27 of histone H3. This is then recognized by the reader complex PRC1. The RING1A and RING1B subunits of PRC1 (writers) subsequently act to ubiquitinate lysine 199 of histone H2A. UTX-1 and JMJD3 can act to remove (erase) histone H3 K27 tri-methylation. (C) The known histone variants in ESCs are represented next to the canonical histones they replace.

The majority of histone PTMs have been shown to be reversible (Fig. 1B). The balance between enzymatic machineries responsible for establishment, maintenance and removal of histone PTMs significantly contributes to chromatin dynamics in stem cells and is indispensable for driving cell-type specific biological outcomes.

In addition to the four canonical histone proteins (H2A, H2B, H3 and H4), many variant forms of histones exist in different organisms. H1.0, H1.1, H1.2, H1.3, H1.4, H1.5 and H1X are

the variants of H1; H2A.X, H2A.Z, H2A-BDB and Macro H2A are replacement variants for H2A; and H3.1, H3.2, H3.3 and CENP-A are variants of the core histone H3 (Fig. 1C, for review see ref. 27). The variants are usually present as single-copy genes that are not restricted in their expression to the S-phase, but are expressed throughout the cell cycle. Unlike the major subtypes, the variant histone genes contain introns and their transcripts are often polyadenylated. These features are thought to be important in the post-transcriptional regulation of these proteins.²⁸

Some variants exchange with the pre-existing histones during development and differentiation, and are therefore referred to as replacement histones. Currently, the majority of studies aimed at elucidating the functions of histone variants are based on correlation between the localization of variants and the transcriptional activity of certain loci, or on analyses of phenotypes associated with the loss of variants.

The large number of histone variants leads to the question of how many different nucleosomal structures exist, and whether structural alterations can account for differences in function and localization of these nucleosomes. Use of fluorescent recovery after photobleaching (FRAP) technology in recent investigations of chromatin in mouse ES cells and their differentiated progeny has allowed for the assessment of nucleosomal structure.²⁹ The published study has demonstrated that ES cells preserve their differentiation potential by maintaining a loosely bound fraction of histones and other chromatin-associated proteins, which through free exchange with bound histones and chromatin generate a state of active, “breathing” chromatin. Consistent with high levels of transcription in ES cells, the same group reported that the only structural chromatin protein lacking hyperdynamic behavior is histone variant H3.3. The histone variant H3.3 preferentially associates with transcriptionally active regions.³⁰ This finding is also consistent with the observed accelerated differentiation of cells lacking histone chaperone HIRA (TUP1). *Hira*^{-/-} cells are marked by a drastic reduction of H3.3 incorporated into open chromatin, and are predisposed to the formation of heterochromatin, thus promoting cellular differentiation. Ultimately, histone variant H3.3 deposition into the chromatin of actively transcribed genes can contribute to the cellular memory phenomena. Experiments performed by Ng and Gurdon in *Xenopus laevis* provide the first documented evidence of the persistence of epigenetic memory of a transcriptionally active state and propose the role of histone variant H3.3 in this process,³¹ thus further highlighting the important role of histone variants in the regulation of stem cell epigenetics.

DNA Methylation Status as Major Epigenetic Player in Stem Cells

Although there is a strong correlation between transcriptionally favorable states of chromatin and the pluripotency of stem cells, the chain of molecular events, as well as the full spectrum of molecular players providing for chromatin plasticity, remains largely unknown. Recent discoveries indicate that chromatin structural

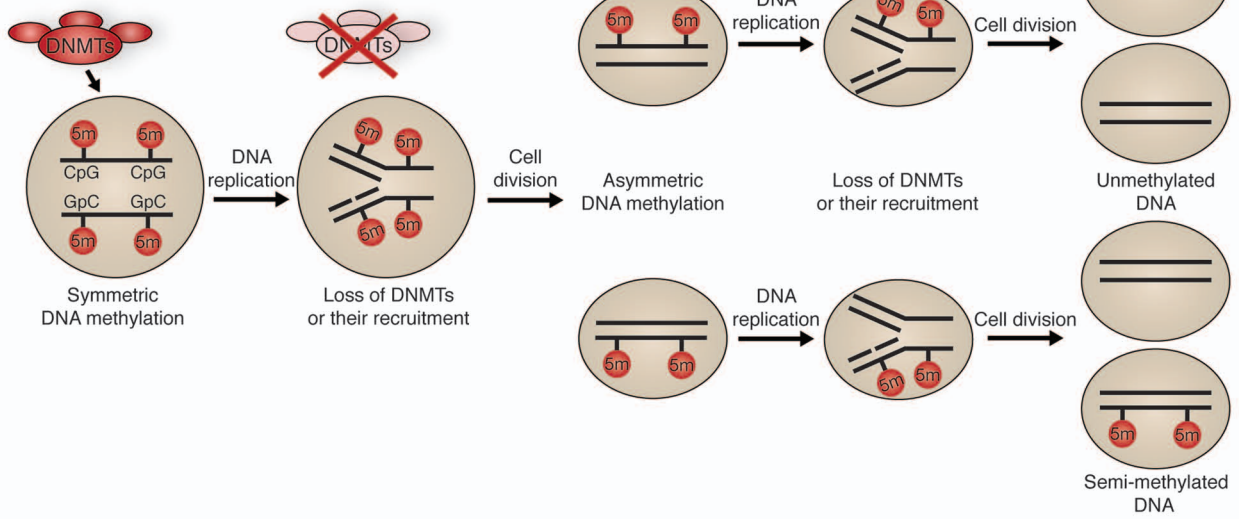
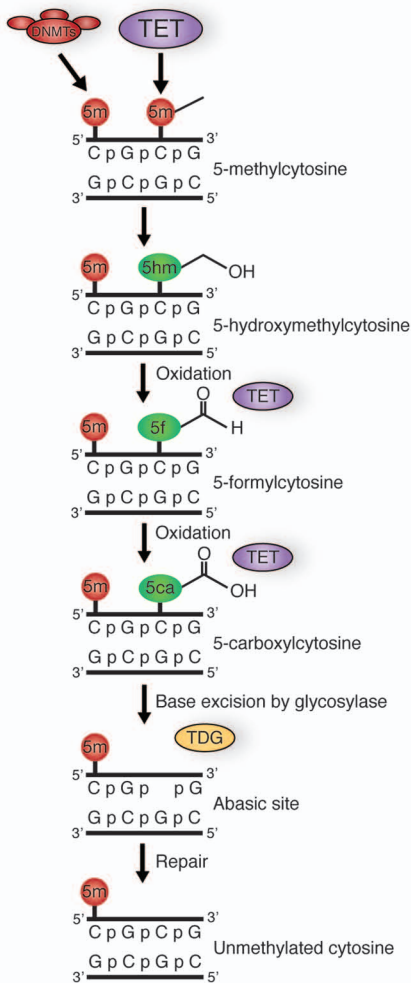
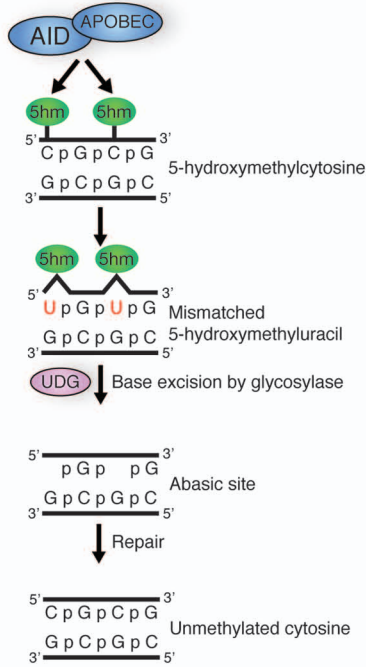
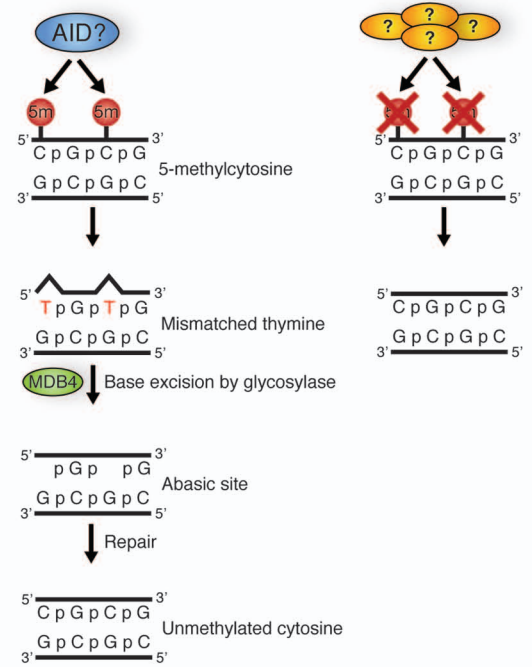
proteins and chromatin modifying activities, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (HDMs), as well as DNA methyltransferases (DNMTs), are essential regulators of open chromatin state and pluripotency.

DNA methylation. DNA methylation is a classic example of epigenetic inheritance of gene expression during development, and therefore it is not surprising that recent evidence indicates its key role in stem cell function. DNA methylation patterns are directed and preserved by the action of the DNA methyltransferase (DNMT) family, whereas the effects of DNA methylation are mediated by recruitment of the “reader” methyl-CpG-binding domain (MBD) family,³² containing proteins such as MBD2, MBD3, MBD4, MeCP2 and KAISO or, alternatively, by blocking the binding of transcriptional factors to their cognate response elements. In most eukaryotes, methylation of the 5' site of cytosines (5-mC) represses transcription through association with MBD proteins, which in turn are part of chromatin remodeling complexes.³²⁻³⁴ Since removal of the methyl group from 5-methylated cytosine is a thermodynamically unfavorable event, the existence of a bona fide DNA demethylase has been a subject of debate.³⁵ Until recently, DNA methylation was regarded as stable and irreversible. However, new evidence indicates that DNA demethylation can occur passively, when DNA methylation enzymes and/or their complexes are denied access to the newly replicated DNA (Fig. 2A), or actively through the selective recruitment of various enzymes, in both animals and plants.^{36,37} For example, in plants, the DNA glycosylase enzymes ROS1 and DEMETER are two well-characterized DNA demethylases.^{38,39} In animals, DNA demethylation is performed by cytidine deaminases in concert with DNA glycosylases (Fig. 2B).^{40,41}

In animal germ cells, genes that control cell differentiation are methylated and transcriptionally inactive.⁴² However, there are at least two developmental periods, both in germ cells and in pre-implantation embryos, during which methylation patterns are reprogrammed genome-wide, generating cells with a broad developmental potential. After fertilization, the parental genome undergoes a rapid loss of DNA methylation in the first several rounds of cellular division, suggesting active DNA demethylation events.^{40,41} Contrary to this, the maternal genome is gradually demethylated and this demethylation appears to be mediated through passive mechanisms.⁴³

As a consequence of this genome-wide demethylation during embryonic development, genes that are essential for stem cell renewal are activated, suggesting that existing DNA methylation

Figure 2 (See opposite page). Pathways of DNA methylation and demethylation. (A) Passive DNA demethylation. DNA in a cell is 5'methylated at CpG islands in a symmetrical fashion by DNA methyl transferases (DNMTs). If there is a loss of DNMT function, followed by DNA replication and cell division, cells containing asymmetric DNA methylation will arise. If there is continued loss of DNMT function and further DNA replication and cell division, this will give rise to cells with unmethylated DNA. This action is passive, as it relies on DNA replication and cellular division. (B) Active DNA demethylation. Several pathways can lead to active demethylation of DNA without the need for DNA replication, and in the presence of DNMTs. Hydroxymethylation (1) of 5-methylcytosines (5-mC) is performed by TET proteins. 5-hydroxymethylcytosine (5-hmC) may act as a substrate for further modifications, or may itself be sufficient to prevent factors that interact with methyl-cytosine from having an effect. 5-hmC can be further oxidized by TET proteins to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).⁵⁴ 5caC is a substrate for TDG glycosylase, creating an abasic site through base excision, which is then repaired with an unmethylated cytosine.^{54,55} Both 5-mC and 5-hmC can act as substrates for deaminases (2). The deaminases AID/APOBEC can convert 5-hmC to 5-hydroxymethyl-uracil (5hmU).⁵⁷ This is then repaired by mismatch-repair pathways, beginning with base excision by the glycosylase UDG. AID may also be able to convert 5-mC to thymine, which is repaired beginning with the glycosylase MBD4. The presence of a bona fide DNA demethylase (3) is a controversial topic, but several groups have proposed candidates.³⁵

A**Passive DNA demethylation****B****Active DNA demethylation****(1) Hydroxymethylation****(2) Deamination****(3) Demethylation?**

must be erased, especially at the promoters of genes that are essential for pluripotency, such as *NANOG* and *OCT3/4*.⁴⁴ Studies have indicated that mouse *Nanog* promoter methylation is erased by active and passive demethylation after fertilization, before expression commences in the morula. In mouse ES cells, the normally active *Nanog* promoter is silenced when targeted by de novo methylation.⁴⁵ Interestingly, DNA methylation at genes that are essential for stem cell renewal are primarily associated with coding sequences, not gene promoters. Furthermore, nearly one-quarter of all methylation identified in embryonic stem cells was in a non-CG context, suggesting that embryonic stem cells may use different methylation mechanisms to affect gene regulation.^{46,47}

DNA hydroxymethylation. Several lines of evidence demonstrate that Ten-11 translocation family proteins, TET1–3, have the capacity to convert 5-mC to 5-hydroxymethyl-cytosine (5-hmC) (Fig. 2B).^{48,49} It has been reported that ES cells deficient in the three enzymes that are involved in de novo DNA methylation and its maintenance (TKO cells) are also deficient in 5-hmC, thus suggesting that 5-hmC arises from the processing of pre-existing 5-mC within the gene body during transcription.^{50,51} Hydroxymethylation of cytosines may lead to passive demethylation during cell division, as 5-hmC is a poor substrate for DNMT1 recognition (Fig. 2A).⁵² However, an emerging consensus in the DNA methylation field is that hydroxymethylation leads to active replacement of methylated cytosines via DNA repair pathways, in the absence of cell division (Fig. 2B).⁵³ These pathways rely, in part, on the further enzymatic modification of 5-hmC to 5-formyl-cytosine (5-fC) and, subsequently, 5-carboxyl-cytosine (5-caC). 5-caC can then be removed by the base-excision repair pathway, leading to its replacement with unmodified cytosine (Fig. 2B).^{54,55} Recent studies have identified enrichment of 5-fC and 5-caC in the genomic DNA of mES cells and mouse organs, suggesting that this pathway is active in stem cells.⁵⁶ Another pathway of active DNA demethylation involves the base excision repair of 5-hmC by the activation-induced deaminase (AID)/apolipoprotein B mRNA-editing enzyme complex (APOBEC) family of cytidine deaminases (Fig. 2B).^{54,57}

These observations raise the possibility that 5-hmC may act as a distinct epigenetic state contributing to dynamic changes in DNA methylation and transcriptional regulation during embryonic development. This possibility is supported by the finding that *Tet1* is highly expressed in mouse ES cells, which is concurrent with elevated 5-hmC levels relative to differentiated cells.^{50,58} Ito et al. first suggested that cytosine hydroxymethylation might be involved in the maintenance of pluripotency in stem cells through their observation that *Tet1* knock-down in mES cells correlates with downregulation of *Nanog* and methylation of the *Nanog* promoter, thus supporting a role for TET1 in regulating DNA methylation status.⁵⁶ The levels of TET1 and TET2 are dramatically downregulated upon differentiation of ES cells and embryoid body (EB) formation. Declining levels of TETs during differentiation are associated with a decrease in hydroxymethylation at the promoters of pluripotency genes. This event is coupled with increased 5-mC methylation and gene silencing. It was also reported that 5-hmC is mostly associated with euchromatin and, whereas 5-mC is under-represented at gene promoters and

CpG islands, 5-hmC is enriched in gene bodies and is associated with increased transcriptional levels.^{50,51,59} Most, if not all, 5-hmC in the genome depends on pre-existing 5-mC, and the balance between these two modifications varies depending on the genomic region. In initial studies, knockdown of *Tet1* and *Tet2* causes downregulation of a group of genes that includes pluripotency-related genes (including *Esrrb*, *Prdm14*, *Dppa3*, *Klf2*, *Tcl1* and *Zfp42*).⁵⁰ Concomitant with this event, it has been shown that increased methylation of pluripotency gene promoters shifts ES cell to extra-embryonic lineage differentiation.

However, these observations have recently been challenged. Several groups have reported that although TET1 and TET2 levels are high in ES cells, these proteins may regulate lineage-specific genes, rather than pluripotency factors such as NANOG.^{50,53,60} Newly reported data suggest that TET1 is dispensable for ES cell maintenance, and that its loss is compatible with embryonic development and postnatal survival.⁶¹ This conclusion is drawn from the observation that *Tet1*^{-/-} mES cells: (1) express markers of pluripotency, such as OCT3/4, SOX2 and NANOG; (2) remain in an undifferentiated state and; (3) can support normal development of the embryo proper in a tetraploid complementation assay.⁶¹ Furthermore, TET1 homozygous mutant mice are both viable and fertile. Taken together, this suggests that TET1 is not essential for postnatal survival. This is most likely due to a level of functional redundancy between TET family members. Although *Tet2* expression in mES cells is 5-fold lower than *Tet1*, and that there is no observed increase in *Tet2* expression in *Tet1*^{-/-} mES cells, it is likely that expression of *Tet2* can compensate for *Tet1* loss.⁶¹ This is consistent with the observation that *Tet1* knockout mES cells exhibit only a 35% reduction in 5-hmC levels; however, a 60% knockdown of *Tet2* on this background further reduces 5-hmC levels.⁶¹ It will be necessary to develop double and triple knockouts of the *Tet* genes in order to fully understand the roles of TET proteins and 5-hmC in vivo.

The studies outlined in this section have made great progress into understanding the function of TET proteins and have answered a number of fundamental questions regarding the mechanisms of DNA demethylation. However, it is as yet unclear what the precise functions of these proteins are in ES cells. The body of evidence discussed herein suggests that the roles played by TET proteins in ES cells may not be trivial, and further studies will be required to understand the precise role of hydroxymethylation in these cells.

Histone Post-Translational Modifications and Chromatin Modifying Activities in the Regulation of the Stem Cell Chromatin Landscape

Chromatin states: euchromatin, heterochromatin and bivalent chromatin. Recent genome-wide studies have indicated that chromatin in ES cells has a specific histone PTM profile, characterized by an abundance of histone modifications associated with “open” and transcriptionally permissive euchromatin, such as H3K4me3, H3K9ac and H4ac (Fig. 3A). “Silent” transcriptionally repressive heterochromatin is associated with PTMs such as H3K9me3 and H3K27me3 (Fig. 3B).

In stem cell chromatin, less accessible lineage-specific genes are maintained in a silent but transcriptionally available (poised) state, which is characterized by the simultaneous presence of both the repressive H3K27me3 and the activating H3K4me3, creating so-called bivalent domains (Fig. 3C). Such bivalent domains are thought to mark a subset of key developmentally regulated genes in ES cells that are kept at a low transcriptional rate.^{9,11} The enrichment of the bivalent marks at conserved domains overlapping with sites for the recruitment of the core pluripotency factors OCT3/4, SOX2 and NANOG originally suggested a functional relationship between such bivalent domains and pluripotency. The initially proposed model of bivalent domains implied that their formation and/or maintenance might be regulated through these “master regulators.” However, it has recently been reported that bivalency is not a unique feature of pluripotent cells. Bivalent chromatin is not limited to ES cells; multipotent adult hematopoietic stem (HS) and progenitor cells, as well as neural progenitors and terminal neurons also exhibit bivalent domains at specific sites targeted by Polycomb group proteins.⁶²⁻⁶⁴ Interestingly, during cellular differentiation, the active mark within bivalent chromatin, H3K4me3, is lost and de novo DNA methylation locks genes controlled by this type of chromatin in a silent state.⁶³ It cannot be excluded that several other bivalent or multivalent chromatin modification states exist in cells. It is important to keep in mind that only a small fraction of the known histone PTMs have been examined in current studies, and a more comprehensive analysis of histone PTMs, together with their relationship to gene expression, may help to decipher the enigma of bivalent chromatin. It is also important to mention that the combinatorial patterns of bivalency might simply reflect allelic differences in chromatin modifications, explaining the apparent coexistence of both histone marks at the same loci, as shown in Figure 3C.

Chromatin bivalency poses an interesting hypothesis tailored to explain the molecular mechanisms involved in the regulation of accessibility and transcriptional competence of differentiation-specific genes in ES and adult stem and progenitor cells; however, several examples bypass the need for bivalency in pluripotent cells, such as epigenetic regulation of some loci during B-cell development.⁶⁵ In this study, the intergenic and cis-acting element in the mouse *Lambda5-VpreB1* locus does not exhibit bivalency. On the contrary, it is marked by histone H3 acetylation and histone H3 lysine 4 methylation at a discrete site in ES cells. The epigenetic modifications spread from this site toward the *VpreB1* and *Lambda5* genes at later stages in B-cell development, and a large, active chromatin domain is established in pre-B cells when the genes are fully expressed. These results suggest that localized and unambiguous rather than bivalent epigenetic marking is important for establishing the region of transcriptional competence for the *Lambda5* and *VpreB1* genes as early as the pluripotent ES cell stage.

Future investigations will be needed to demonstrate the significance of chromatin bivalency in the stem cells pluripotency, lineage commitment and control of the developmental regulators.

Histone acetyltransferases and deacetylases. The acetylation of histones H3 and H4 are catalyzed by histone acetyltransferases

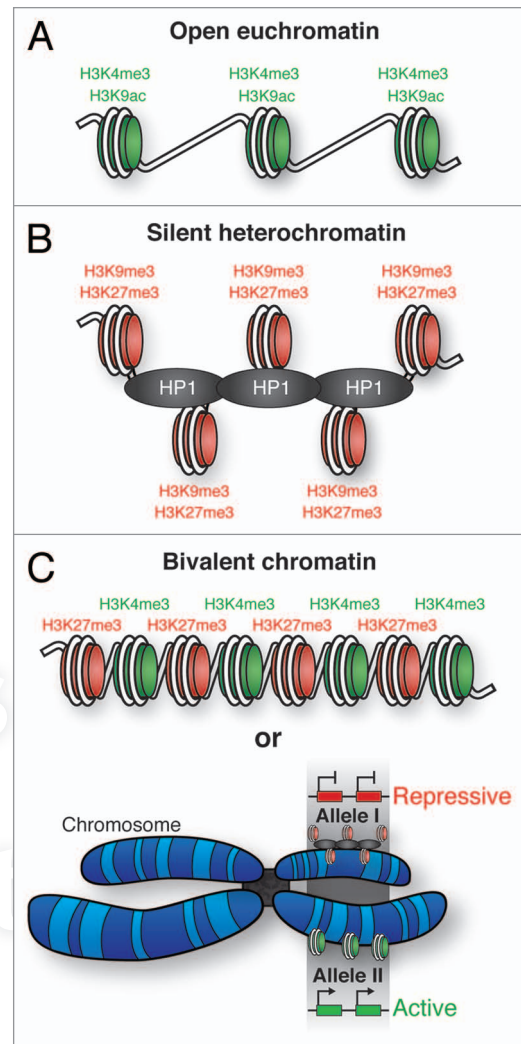


Figure 3. Chromatin states. (A) Open, active euchromatin is characterized by histone H3 lysine 4 methylation (H3K4Me3) and lysine 9 acetylation (H3K9Ac). (B) Silent, repressive heterochromatin is characterized by methylation of histone H3 lysine 9 (H3K9Me3) and lysine 27 (H3K27Me3). Heterochromatic DNA is more densely packed than euchromatin, facilitated by heterochromatin protein 1 (HP1). (C) Regions of the genome have been identified with both activating (H3K4me3) and repressive (H3K27me3) histone marks; termed bivalent chromatin. It is possible that these PTMs occur on neighboring nucleosomes, however, it is also possible that one allele of the gene is associated with heterochromatin, and the other euchromatin. This would give the appearance of bivalent chromatin.

(HATs/KAT) and the removal of acetyl-group is achieved by action of histone deacetylases (HDACs).⁶⁶ In the past 10 years, multiple studies have indicated an interplay between HATs and HDACs, and research has advanced significantly as these enzymatic activities have become more amenable to molecular and biochemical analysis. Since both HATs and HDACs are integral components of transcriptional co-activator and co-repressor complexes respectively, it is no surprise that, despite their opposing activities, both acetylation and deacetylation are required for proper ES cell differentiation and adult stem cell function.⁶⁷ For instance, inhibition of HDACs prevents the differentiation of ES

cells,⁶⁸ thus suggesting histone deacetylation events are part of cell-type specification. Similarly, the histone acetyltransferase KAT3B (p300) is required for differentiation of ES cells, but is dispensable to maintain their self-renewal properties.⁶⁹ Since HDACs and HATs are also involved in a wide array of cellular events, including transcriptional and post-transcriptional regulation, and even post-translational modifications, their regulatory role might extend not only to modification of histones, but also to dynamic acetylation/deacetylation of key regulatory ES cell differentiation modulators like members of the SOX family, TGF- β family, WNT and NOTCH.

Histone methyltransferases. Historically, cellular inheritance was explained by the methylation of promoter DNA; however, a new wave of published data argues that DNA methylation is not the only mechanism utilized by the cell to impart epigenetic memory. How is the epigenetic memory of silent chromatin handled?

In multiple organisms, genes encoding developmental regulators are tightly controlled. Such control is mediated not only through transcriptional factors, which participate in auto- and cross-regulation self-renewal circuitry (for review, see ref. 70), but also by a handful of chromatin regulators catalyzing histone methylation and demethylation. Multiprotein repressive Polycomb group (PcG) and activating trithorax (TrxG) chromatin modifying complexes have long been known for their significance in the regulation of the lineage-specific genes during *Drosophila* development. Current research suggests that both of these complexes regulate the nuclear organization of their target genes, and mechanistically cross-talk with noncoding RNAs and the RNAi machinery.⁷¹ Next, we will discuss the role of these complexes and their integral chromatin modifying activities in the stem cell biology.

Polycomb group (PcG) protein complexes. A series of recent studies have revealed that, in order to maintain pluripotency, mouse and human ES cells deploy mechanisms for dynamic repression of genes regulating developmental pathways in such a way that this repression can be epigenetically maintained through cell division.⁷² The epigenetic modifier PcG complex proteins can perform this function.^{10,73-76} The PcG complex is an evolutionary conserved family of chromatin regulators known best for their role in establishing and maintaining the silent state of homeotic gene expression during embryonic development.⁷⁷ Mammalian PcG proteins assemble at least three biochemically distinct complexes PRC1, PRC2 and PhoRC. Polycomb repressive complex 2 (PRC2) acts to stabilize repressive chromatin structure through the function of chromatin modifiers, such as enhancer of zeste (EZH2), embryonic ectoderm development protein (EED), and suppressor of zeste 12 (SUZ12), all of which are histone methyltransferases responsible for depositing H3K27me2 and H3K27me3 marks onto chromatin (Fig. 1B).^{78,79} By performing ChIP-on-CHIP analysis for SUZ12 and EED proteins in ES cells, Lee and coworkers have demonstrated that genome-wide binding of these modifiers overlaps with the chromosomal region of H3K27me3 deposition within the highly evolutionary-conserved genomic segments in the vicinity of transcriptionally silent genes.⁷⁴ The 1,800 genes identified as targets include the majority

of *OCT3/4*, *NANOG* and *SOX2* regulated genes in human ES cells,⁸⁰ including the regulators of differentiation *GATA4* and *CDX2*. These results, together with the observation that EZH2 is required for maintaining the proper H3K27me3 marks in pluripotent epiblast cells,^{81,82} suggest that PRC2 could be potentially viewed as a component of epigenetic memory strategies required for ES cell maintenance.

Nucleosomes containing H3K27me3 provide a binding platform for the recruitment of the PRC1 complex containing PHC, CBX, BMI1 and RING1A, RING1B (RNF2) and MEL-18 (PCGF2) via the affinity of chromodomain containing proteins to these PTMs.¹⁰ The activity of the PRC1 complex was implicated in the establishing of high-order chromatin structure.⁸³ How does PRC1 complex compact chromatin? Evidence indicates that the E3-ligase activity of the RING1A and RING1B proteins present in the PRC1 complex can mono-ubiquitinate H2AK199.⁸⁴ This activity appears to be stimulated by the BMI1 and MEL-18 (PCGF2) subunits of PRC1 (Fig. 1B).

This logical interdependency between PRC2 and PRC1 functions does not, however, fully explain the phenotypes of ES cell models that are deficient for one of the PRC2 components (e.g., EED). Mutant ES cells demonstrate gross loss of H3K27 methylation, but still retain their ability to self-renew and maintain normal morphology.^{10,85} This occurs despite the fact that several neuron-specific genes, and *GATA4* and *GATA6* factors, are transcriptionally upregulated on the background of *Eed* deficiency. The mutant ES cells simply manifest high level of spontaneous differentiation¹⁰ and are still capable of producing all the three germ layers upon injection in blastocysts.⁸⁵⁻⁸⁷ Similar to this, *SUZ12*^{-/-} ES cells do not demonstrate a full requirement for PRC2 in maintenance of ES cell pluripotency.⁷⁴

It is probable that both H3K27 methylating enzymes and DNA methylation regulate the timing of differentiation and maintenance of cell-type identity, in a coordinated and semi-redundant fashion.⁸⁸⁻⁹⁰ As a result, cellular inheritance could be achieved by means of methylation on the DNA in the absence of a functional PRC2 complex. It is known that PcG proteins can directly control DNA methylation,⁹¹ thus providing another important role as connectors between key epigenetic events. Future analysis of the functional links between DNA methylation status and the context-dependent action of PcG complexes in ES cell models will extend our understanding of molecular memory of a silent state and its link to pluripotency.

Trithorax group (TrxG) protein complexes. As we have discussed above, maintenance of ES cell self-renewal relies upon two equally important events: repression of lineage-specific genes mediated by PcG complexes and the transcriptional activity of self-renewal genes, such as *OCT3/4*, *SOX2* and *KLF*, which is mediated by the trithorax group of histone modifiers. In contrast to PcG complexes, TrxG complexes mediate deposition of histone PTMs that mark active transcription, such as H3K4me3.⁷⁷ Until now, little was known about TrxG-associated members in the context of ES cell self-renewal and pluripotency maintenance, or somatic cell reprogramming. The SET/MLL (mixed-lineage leukemia) histone methyltransferase family are mammalian homologs of *Drosophila* TrxG, which function as

conserved, multisubunit ensembles that catalyze methylation of H3K4. SET/MLL histone methyltransferases alone are catalytically inept and require the core subunits ASH2L, RBBP5 and WDR5 for HMT activity. RBBP5 and ASH2L form a heterodimer to provide for HMT activity of the MLL1 complex,⁹² and ASH2L has been reportedly required for mouse embryogenesis.⁹³ On the other hand, WDR5, which recognizes and interacts with H3K4me2, has been shown to be indispensable for SET/MLL complex assembly and for the transition of H3K4me2 to the trimethylated state (H3K4me3).⁹⁴ This unique ability of WDR5 to bind unmethylated or dimethylated H3K4 is indicative of its participation in both reading and writing of H3K4 methylation. Although the exact logistics and chain of molecular events of this process need to be fully unraveled, new evidence indicates that WDR5 expression levels positively correlate with the undifferentiated ES cell state, thus suggesting a specific WDR5 function in ES and iPS cell maintenance.⁸ WDR5 knockdown in ES cells induces changes in stem cell morphology and increased ectodermal and trophodermal gene expression, suggesting that WDR5 depletion induces the collapse of the transcriptional network of ES cells.⁸ Genome-wide analysis indicates that WDR5 is critical for maintenance of global and localized H3K4me3, as well as for transcriptional activation of specific targets in ES cells. Interestingly, WDR5 directly interacts with the master pluripotency factor OCT3/4, and this interaction is stabilized upon formation of multimeric complexes, even in the absence of ASH2L or RBBP5,⁸ thus suggesting that the function of the WDR5-OCT3/4 partnership might extend beyond H3K4 methylation. Nevertheless, WDR5 and OCT3/4 share overlapping gene regulatory functions, where both of these factors co-localize with RBBP5 and H3K4me3 in genome-wide mapping experiments.⁸ This work represents the first unbiased, high-resolution mapping of core TrxG members, and demonstrates their interconnectivity with the core transcriptional network, which is required for maintenance of ES cell self-renewal. Of interest, WDR5 has also recently been shown to function as a subunit of other nuclear complexes, such as the histone acetyltransferase ATAC2⁹⁵ and human chromodomain helicase DNA binding protein 8 (CHD8), important for ATP-dependent chromatin remodeling.⁹⁶ It remains unclear, however, whether these two complexes contribute to the observed WDR5 phenotype in ES cells.

Histone demethylases. In a similar manner to stemness, the differentiation of pluripotent stem cells into tissue-specific lineages has proven to be controlled by epigenetic components. The exit from the self-renewing state is accompanied by changes in the covalent modifications of histones, for example, an increase in the silencing-associated histone H3K9me2 and H3K9me3 marks on the chromatin and removal of H3K27me3. DNA sequence-specific factors can act as a landing pad for the recruitment of specialized enzymatic machineries that either deposit⁷⁵ or remove the PTMs on chromatin (for details of a multitude of histone PTMs and the substrate-specificity of enzymes responsible for their deposition see refs 24,97 and Figure 1). While it has been known for a number of years that histone acetylation and

phosphorylation are reversible, only relatively recently has it been shown that methyl-groups can be enzymatically removed from lysine residues, and demethylation enzymes have been identified.^{24,98-101} The debate as to whether H3K27me3 can be actively removed has been settled by a series of papers identifying two related jumonji-family proteins, JMJD3 and UTX, which specifically demethylate H3K27me3 (Fig. 1B).¹⁰²⁻¹⁰⁶ Both of these demethylases are members of MLL protein complexes, which antagonize PcG-mediated gene silencing. Interestingly, JMJD3 is a direct gene target of silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), which, through its interaction with retinoic acid receptors (RARs), represses JMJD3 expression to maintain a neural stem cell state.¹⁰⁵ Retinoic acid (RA) treatment of neural progenitors results in upregulation of JMJD3 and decreases in H3K27me3 levels on the promoter of the *DLX5* gene, a marker of differentiated neurons.

It has also been shown that the H3K9me2 and H3K9me3 demethylase genes, *JMJD1A* and *JMJD2C*, are positively regulated by the ES cell transcription factor, OCT3/4. Interestingly, *JMJD1A* or *JMJD2C* depletion leads to ES cell differentiation and is accompanied by a reduction in expression of pluripotency genes, favoring induction of lineage marker genes. *JMJD1A* demethylates H3K9me2 at the promoter regions of *TCL1*, *TCFCP2L1* and *ZFP57*, leading to upregulation of the expression of these pluripotency-associated genes. *JMJD2C* also acts as a positive regulator for *NANOG*.⁴

In general, histone demethylases are tightly integrated in the transcriptional factor regulatory networks in ES cells.^{107,108} To give one example, the high-ranking gene *JARID2* is the target for seven core regulators of ES cells. *JARID2*, also known as Jumonji (JM), is highly expressed in ES cells; however, it is downregulated in the whole embryonic body at the onset of differentiation. Later during cellular differentiation events, the compartments where *JM* is expressed expand gradually, with expression detectable in almost all adult tissues, although the intensities vary among cell types.^{108,109}

These observations suggest that histone demethylases link core transcription factor networks to the regulation of chromatin status during cellular differentiation. Once again, the actions of histone methylases and demethylases seem to be interconnected with DNA methylation. For instance, TET1 binds a significant proportion of Polycomb and Trithorax group target genes. Remarkably, 5-hmC is significantly enriched predominantly at two groups of promoters. First, at inactive promoters, many of which contain bivalent chromatin domains with both activating H3K4me3, and repressive H3K27me3.^{51,110} In contrast, in the second group of promoters, TET1 is associated with active histone marks, including H3K4me3, H3K4me1 and H3K36me, a mark associated with transcriptional elongation.^{51,59,111} These data indicate that 5-hmC can be associated with both actively transcribed and repressed target genes. The relationship of DNA methyltransferases and hydroxymethylases with histone demethylases awaits further investigation, but one can speculate that their combinatorial action provides for the balancing act of histone PTMs in stem cells.

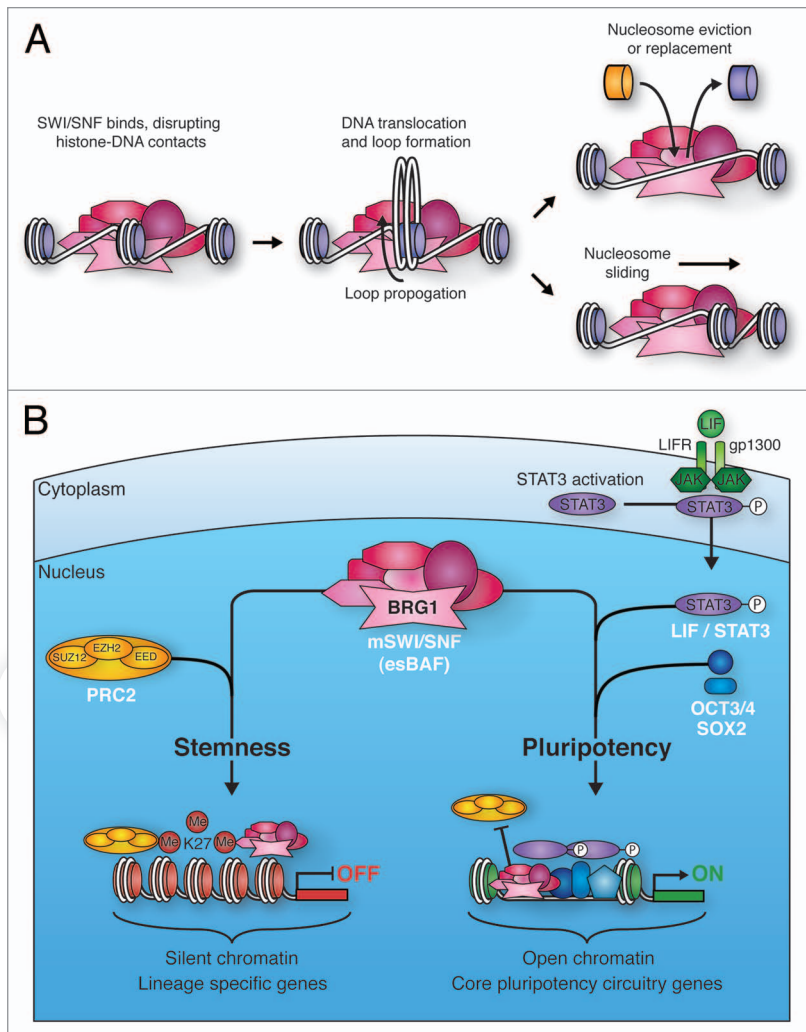


Figure 4. Mechanisms of SWI/SNF-mediated chromatin remodeling. (A) SWI/SNF binding to chromatin disrupts histone-DNA contacts. This allows for the creation of a loop of DNA that propagates around the nucleosome, ultimately resulting in repositioning of the DNA with respect to the nucleosome (sliding). This sliding can lead to eviction of adjacent nucleosomes. DNA looping also facilitates histone replacement with histone variants. (B) BRG1 containing mSWI/SNF in ES cells (esBAF) plays vital roles in the pathways promoting ES cell stemness and pluripotency. esBAF enforces repressive H3K27me3 marks deposited by the PRC2 complex at many lineage specific genes that would otherwise promote differentiation. In contrast, esBAF antagonizes PRC2 action at LIF targets, such as core pluripotency circuitry genes. By creating more accessible chromatin at these locations, esBAF prepares the chromatin environment for the cooperative action of phospho-STAT3 and master pluripotency regulators, such as OCT3/4 and SOX2.

ATP-Dependent Chromatin Remodeling Complexes in the Regulation of the Stem Cell Chromatin Landscape

When it comes to the discussion of the chromatin landscape and its regulation, it is important to emphasize the role played by chromatin remodeling complexes, which change the chromatin architecture by modulating interaction between nucleosomal particles and DNA. These chromatin remodeling complexes are enzymes that transiently disrupt the association between DNA

and histones in an ATP-dependent manner. This allows for nucleosome sliding, translocation and eviction, as well as changes in nucleosome composition through the exchange of canonical histones with histone variants. This in turn may induce conformational changes in nucleosomes and control different degrees of the condensation state of chromatin (Fig. 4A).¹¹²

Chromatin remodeling complexes are numerous and highly abundant, with each complex displaying distinct patterns of activity. These complexes can be divided into families based on their subunit composition and biochemical activity.¹⁵ The precise subunit composition of these complexes can be fine-tuned to confer functional specificity within the cell. For the purpose of this review, we shall discuss two of the most well studied ATP-dependent chromatin remodelers: SWI/SNF and CHD1. For a thorough review all ATP-dependent chromatin remodeling complexes, see a recent review by Hargreaves and Crabtree.¹¹³

SWI/SNF chromatin remodeling activity. Perhaps one of the most well studied examples of ATP-dependent chromatin remodelers is the SWI/SNF protein complex (mSWI/SNF in mammals, also known as BAF). The 9–12 subunits of mSWI/SNF are gene families and are combinatorially assembled, with one of two mutually exclusive catalytic ATPase subunits, brahma homolog (BRM, also known as SMARCA2) or BRM/SWI2-related gene 1 (BRG1, also known as SMARCA4). Variations in mSWI/SNF subunit composition contribute to targeting, assembly and regulation of lineage-specific functions during ES cell differentiation.^{114,115}

Unlike the yeast SWI/SNF complex, the mammalian SWI/SNF complex is not monomorphic. mSWI/SNF subunit exchange assists the transition of stem cells from pluripotency to a multipotent state, and further to terminal differentiation.¹¹⁶⁻¹¹⁹ For example, ES cells express the subunits BRG1, BAF155 and BAF60A; however, upon differentiation, switch in subunit expression occurs, and these are replaced by BRM, BAF170 and BAF60C, respectively.^{118,120} Moreover, differential usage of the mSWI/SNF subunits BAF53A and BAF53B has also been shown upon transitioning of neural

precursors to terminally differentiated neurons.¹¹⁷ Inactivation of mouse SWI/SNF subunits such as BRG1, BAF47, BAF57, BAF60, BAF155, BAF180 and BAF250A leads to embryonic lethality and BRG1, BAF47 and BAF155 to the failure of formation of pluripotent cells (individual phenotypes outlined in more detail in Table 1).^{15,119,121-126} In human ES cells, the BRG-containing mSWI/SNF complex is required for the ability of ES cells to maintain self-renewal and remain pluripotent (Fig. 4B).^{118,127}

Genome-wide analysis of mSWI/SNF binding in mouse ES cells conducted by Ho et al. suggests that the complexes bind

Table 1. Roles of mSWI/SNF complex components in mammalian development

Name	Alias	Lethality	Observed phenotype	Reference(s)
BAF250a	ARID1A	Embryonic lethal at E6.5	Inhibition of self-renewal in ES cells. Absence/impaired differentiation of mesoderm. Promotes primitive ectoderm and endoderm.	114
BAF47	INI1, SNF5	Embryonic lethal at E3.5 to E5.5: peri-implantation lethal	Peri-implantation defects. Nervous system and soft tissue sarcomas in heterozygotes.	119, 121
BAF60c	SMARCD3	-	Heart defects; defects in establishment of left-right asymmetry.	117, 164
BAF155	SRG3	Trophoblast stage	Defects in formation of inner cell mass. Heterozygotes display brain organization problems (exencephaly) due to failure of neural tube closure.	118
BAF53a	-	-	Required for neuronal stem-cell proliferation.	115
BAF53b	-	-	Required for activity-dependent dendritic outgrowth.	165
BAF57	Smarcae1	-	Dominant-negative mutations prevent T-cell development.	166
BAF180	PBRM1	-	Impaired epithelial-to-mesenchymal-transition (EMT) and arrested maturation of epicardium at E11.5. Leads to defects in coronary vessel formation.	167
BRG1	-	Arrest at two-cell state (ZGA)	Required for Zygotic Genome Activation. H3K4me2 reduced. Required for differentiation of neurons, lymphocytes, and adipose and heart tissues. Required for beta-globin expression activation during embryonic erythropoiesis.	115, 117, 120, 168, 169, 170
BRM	-	None	Greater body mass.	171

roughly 3% of the genome, with a 2.1 kb footprint.¹²⁷ The majority of mSWI/SNF binding is not at transcriptional start sites, but rather at distal enhancer and silencer sites.¹²⁷ BRG1 binding overlaps more closely with H3K4me1 than H3K4me3, suggesting it occupies enhancers and regulatory elements rather than sites of active transcription.^{113,127} This is in contrast to yeast SWI/SNF that activates its targets by binding promoters.¹⁵ Further studies by Ho et al. have shown that the mSWI/SNF complex binds to the enhancers and promoters of genes encoding important pluripotency regulators, and cooperates with the master regulators of pluripotency, such as OCT3/4 and SOX2 for control of stemness circuitry (Fig. 4B).¹¹⁸ Such interaction suggests a functional overlap between mSWI/SNF and the core pluripotency pathways.¹¹⁸ In parallel, mSWI/SNF complexes also act as transcriptional repressors on a number of differentiation specific genes in ES cells (Fig. 4B).¹¹⁸ It has been shown that addition of the mSWI/SNF complex to the master pluripotency cocktail used to reprogram somatic cells into induced pluripotent stem cells (iPS cells) increases reprogramming efficiency dramatically.¹²⁸

The mechanism by which mSWI/SNF acts to repress or activate transcription is not clear in mammals; however, it is proposed to function in a similar manner to yeast SWI/SNF activity, by mobilizing nucleosome sliding along DNA and by catalyzing the insertion and eviction of histone octamers.¹²⁹ This activity is probably indispensable for denying or allowing access of transcriptional factors to their cognate DNA binding sites.¹²⁹ Importantly, SWI/SNF complexes are also capable of recruiting histone deacetylases, which remove activating acetyl marks from histone tails, further promoting the repressive state.¹³⁰

CHD1 adaptor protein. Another example of a protein with chromatin remodeling activity required for mediating cellular stemness is chromodomain helicase DNA binding protein 1 (CHD1), which functions as substrate recognition component of the transcription regulatory histone acetylation (HAT) complex SAGA. Evidence indicates that CHD1 can be used as a molecular adaptor, bringing SNF2, the FACT complex and the PAF complex to H3K4me2/3.¹³¹ It has been suggested that such an adaptor may be required to maintain open chromatin in ES cells, thus providing for pluripotency. In fact, it is a target gene for OCT3/4, SOX2, NANOG, SMAD1, ZFX and E2F1.¹³² *Chd1* RNAi mouse ES cells have decreased self-renewal but maintain expression of markers of the undifferentiated state, such as alkaline phosphatase, SSEA1 and OCT3/4, as shown by immunofluorescence experiments.⁵ Since CHD1 is classically associated with active transcription, one would expect drastic downregulation of stemness genes at the transcriptional level upon knock-down. Surprisingly, however, experimental evidence indicates that only a few genes, other than *Oct3/4*, are downregulated. This could be due to residual levels of CHD1 in RNAi mutants, which could be sufficient for maintenance of the ES cell transcriptome. However, *Chd1* RNAi ES cells have skewed formation of primitive endoderm upon differentiation to embryoid bodies, which consequently leads to a loss of cardiac mesoderm differentiation and abnormally high levels of neural differentiation.⁵ Subsequent genome-wide location analysis for CHD1 indicated that CHD1 binding strongly correlates with RNAP II and H3K4me3. Surprisingly, bivalent domains were largely devoid of Chd1, and major H3K9 methyltransferases and demethylases are expressed in *Chd1* RNAi ES cells at similar levels to control ES

cells. However, rapid exchange of histone H1 is compromised in *Chd1* RNAi ES cells, indicating that chromatin is less breathable. This data suggests that formation of open chromatin, required for pluripotency, is dependent on CHD1 levels. Additional evidence to support this notion comes from the reduced capacity of *Chd1* knockdown fibroblasts to undergo induced pluripotency. Since CHD1 is viewed as an adaptor protein for targeting many other specialized complexes, the full spectrum of CHD1 action in ES cells has yet to be discovered.

Non-Coding RNAs in Stem Cell Regulation

A new, integrated regulatory network is currently emerging based on the dynamic interplay of chromatin modifying, chromatin remodeling and DNA methylation components with non-protein coding RNAs (ncRNAs). These mechanisms synergize to choreograph cellular stemness and to generate cellular diversity. New evidence indicates the existence of an extensive regulatory network involving RNA signaling.¹³³ This is based on the notion that although only 1.2% of the human genome encodes protein, a large fraction of it is transcribed. Indeed, as much as 98% of the transcriptional output in humans and other mammals has been proposed to consist of non-protein coding RNAs.¹³⁴ The number of known functional ncRNA genes has risen dramatically in recent years, and many of these appear to be expressed and function in a developmentally specific manner. Such ncRNAs include microRNAs (miRNAs), long non-coding RNAs (lncRNAs), PIWI RNAs (piRNAs), short heterochromatic RNAs (shRNAs), endogenous short interfering RNAs (endo-siRNAs) and transcripts originating from retrotransposon repeats and pseudogenes.^{135,136} There is also considerable evidence that ncRNAs regulate chromosome dynamics, chromatin modification and epigenetic memory, including imprinting, DNA methylation and transcriptional gene silencing (reviewed in ref. 137). We will now discuss several examples of ncRNAs and how their functions pertain to ES cell maintenance and differentiation.

microRNAs. miRNAs are a class of small, ~21 nt, non-coding RNAs that play an important role in post-transcriptional gene expression through the regulation of mRNA stability and, consequently, protein abundance. miRNAs recognize and bind their targets through short 2–8 nt seed sequences.¹³⁸ As each miRNA can recognize many hundreds of targets, and multiple miRNAs may target individual mRNAs, these gene regulatory networks can become rather complex.¹³⁸ For a review of miRNA biogenesis, see reference 139 and **Figure 5A**.

miRNAs are appealing candidates to control ES cell pluripotency and drive cellular differentiation. There are two main reasons for this argument. First, key regulators of pluripotency such as OCT3/4, SOX2 and NANOG have been found to occupy the promoters of a host of miRNAs, and act to either activate or repress their expression depending on the miRNA species.¹⁴⁰ Furthermore, histone marks associated with active transcription (H3K4me3 and H3K36me3) and silenced transcription (H3K27me3) are associated with active and silent miRNAs, respectively, almost mimicking the epigenetic patterns of protein coding genes.¹⁴⁰ Second, knockouts of the miRNA processing

enzymes *Dicer* and *Dgcr8* abolish the ability of ES cells to silence their self-renewal program and cause severe defects in their ability to differentiate.^{141,142}

Several miRNAs have been linked to cellular stemness, including those from miR-290 and miR-302 clusters. Expression of mature ES cell-specific miR-290 family members can rescue the *Dgcr8* null proliferation defects in ES cells by controlling the expression of negative regulators of ES cell cycle, such as CDKN1A, RBL2 and LATS2 (**Fig. 5B**).¹⁴³ miR-290 family miRNAs also maintain de novo DNA methylation through the control of RBL2, which transcriptionally represses DNA methyltransferases.¹⁴⁴ Furthermore, miR-290 family members antagonize the activity of differentiation-related miRNAs such as the *let-7* family.¹⁴⁵

Let-7 family members are a highly conserved group of miRNAs that repress C-MYC, SALL4 and LIN28 protein production, leading to a loss of ES cell self-renewing capacity and subsequent ES cell differentiation (**Fig. 5B**).¹⁴⁶ LIN28 plays an important role in ES cell maintenance by inhibiting pre-*let-7* miRNA cleavage and destabilization of pre-*let-7* miRNA.¹⁴⁷ Inhibition of *let-7* substantially enhances somatic cell reprogramming into iPS cells.¹⁴⁶ *miR-145* is another miRNA associated with ES cell differentiation, acting through inhibition of the master regulators of pluripotency OCT3/4, SOX2 and KLF4.¹⁴⁸ In self-renewing ES cells, however, OCT3/4 transcriptionally represses *miR-145* expression to maintain a pluripotent state.¹⁴⁹ Along this line, it has been shown that retinoic acid-inducible *miR-134* promotes differentiation of mouse ES cells to the ectodermal lineage, with probable targeting of *Sox2*, *LHRH1* and *Nanog* transcripts.^{150,151}

Finally, a number of lineage-specific miRNAs are responsible for maintaining ES cell differentiation (**Fig. 5B**). For example, *miR-1* and *miR-133* are muscle-specific miRNAs that are activated upon ES cell differentiation to cardiomyocytes.¹⁵² These miRNAs promote mesoderm differentiation by repressing non-muscle gene expression through downregulation of the Notch pathway ligand, DLL-1.¹⁵² Another miRNA, *miR-9/9**, has recently been linked to the molecular pathways of neural differentiation, and provides a good example in which several mechanisms of epigenetic regulation feedback on one another to mediate a specific function. In particular, as a part of neural differentiation circuitry, downregulation of the gene *Rest/Nr2f3*, responsible for the repression of neuronal-specific genes, lifts repression on *miR9/9** and *miR-124* promoters, resulting in elevated expression of these miRNAs. This in turn leads to a switch in mSWI/SNF subunit composition, due to miRNA mediated BAF53A repression, exit from cell cycle, and concomitant BAF53B activation.¹⁵³ As discussed above, this leads to transitioning of neural precursors to terminally differentiated neurons.

There is ever-growing evidence of the increased and complex network of miRNA function in ES cells. Although not all of the players have been identified to date, evidence points to three distinct mechanisms of their actions: (1) participation in maintenance of stem cell self-renewal and pluripotency through the inhibition of negative factors controlling these events, (2) initiation of stem cell differentiation through the inhibition of master pluripotency factors and (3) maintenance of lineage definition

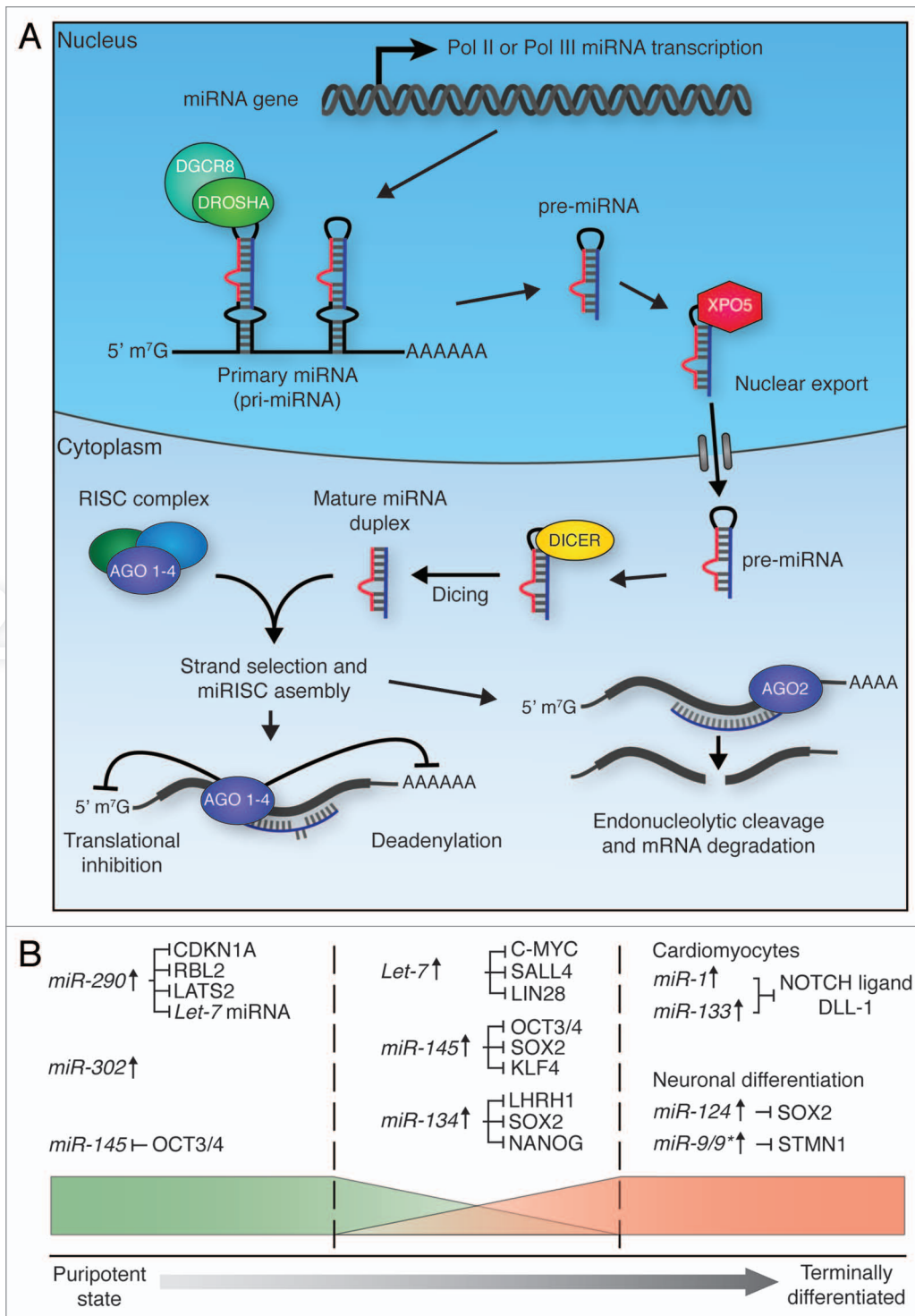


Figure 5. miRNA biogenesis and function in ES cell differentiation. (A) MicroRNA biogenesis. Primary transcripts (pri-miRNAs) are generated through RNA pol II or pol III transcription, which then undergo RNase III cleavage, mediated by the DROSHA/DGCR8 complex, to generate ~70 nt pre-miRNAs. XPO5 exports these pre-miRNAs to the cytoplasm, where they are further cleaved by DICER to generate mature double-stranded RNA duplexes. One strand of these duplexes is then bound by one of four Argonaute proteins (AGO 1–4) to form active RISC complexes, which can modulate gene expression through translational inhibition, or mRNA deadenylation. If the miRNA is perfectly matched to the target sequence, endonucleolytic cleavage of the mRNA transcript can occur through the “slicer” activity of AGO2.^{139,145} (B) miRNAs play roles in the maintenance of pluripotency (left section), the onset of differentiation (middle section), and the maintenance of terminal differentiation (right section). In order to maintain pluripotency, miRNAs act to promote maintenance of cell cycle progression (CDKN1A, RBL2 and LATS2 inhibition) and de novo DNA methylation (RBL2 inhibition), and suppress factors that promote differentiation (*Let-7* inhibition). In order to promote differentiation, miRNAs act to block self-renewal and core pluripotency factor production. miRNAs maintain terminal differentiation by suppressing gene expression associated with other lineages (*miR-1* and *miR-133*), block self-renewal (*miR-124*) and maintain the state of the specific lineage (*miR-9/9**).

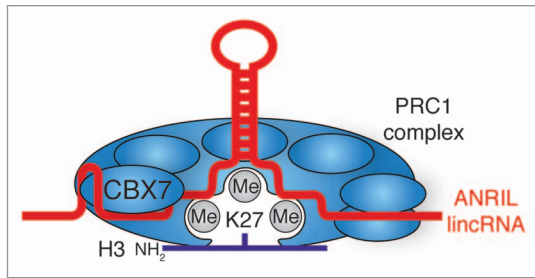


Figure 6. lincRNA stabilization of Polycomb Group complexes. ANRIL lincRNA bind and stabilizes the interaction of PRC1 with H3K27me3 through the CBX7 subunit.

through restricting the expression of genes from other lineages. There may be other, as yet undiscovered functions.

Polycomb-associated non-coding RNAs. A number of recent publications indicate that the Polycomb group complex PRC2 (discussed above) utilizes non-coding RNA co-factors as sequence specific guides to direct Polycomb group complexes to their cognate binding sites within the genome. In mouse ES cells, RNA immunoprecipitation (RIP), combined with RNA-sequencing (RIP-seq), has uncovered novel Polycomb group-interacting RNA genome-wide.¹⁵⁴ This study has identified at least 9,000 distinct ncRNA transcripts that bind PRC2, revealing a highly complex and abundant population of long non-coding RNAs, which may direct PRC2 to its target loci throughout the genome. Previous reports also indicate that large intergenic non-coding RNA (lincRNA) associated with members of the Polycomb group were cataloged from a number of human stem and somatic cells.¹⁵⁵ Although less than 2% of the PRC2 non-coding transcriptome identified by Zhao et al. intersects with lincRNAs, the jury is still out as to whether or not PRC2 associated non-coding RNA interactions have cell-type specificity, allowing PRC2 to employ a variety of ncRNAs to differentially suppress genomic loci in a cell-type specific manner. Similarly, the ncRNA *ANRIL* (also known as *CDKN2B-AS1*) reportedly interacts with the chromodomain of CBX7 (a component of the PRC1 complex), and modulates its binding to H3K27me3 in vitro (Fig. 6).¹⁵⁶ Relevance of *ANRIL* ncRNA in ES cell biology has yet to be investigated, but detailed investigations of the functional significance of individual ncRNAs that interact with PRC2, such as *Hotair*, *Xist* RNA, *Tsix* and *RepA*, demonstrate that long ncRNAs may contribute to the cellular epigenome through modulation of DNA methylation,^{157,158} changes in chromatin modifications^{154,155,159,160} or interception with RNAi and miRNA pathways.¹⁶¹⁻¹⁶⁴ These

observations suggest that long ncRNAs might represent a “flexible scaffold,” mediating interactions between DNA and protein complexes.^{165,166}

OCT3/4 and NANOG-associated ncRNAs. Of note, several lines of evidence indicate that master pluripotency regulators such as OCT3/4 and NANOG might be involved in the regulation of transcriptional activity of ES cell-specific non-coding RNAs.⁷⁰ The functional relevance of two of these RNAs, *Rncr2/Ak028326/Gomafu/Miat* (OCT3/4-activated) and *Ak141205* (NANOG-repressed), was recently investigated in the context of mES stemness and differentiation.¹⁶⁷ In particular, knockdown of these ncRNAs altered *Oct3/4* and/or *Nanog* transcript levels, and modulated mESC differentiation toward specific lineages in the presence of LIF. Overexpression of either long ncRNA led to enhancement of mesodermal, endodermal, and ectodermal differentiation in the presence of LIF. This data suggests that non-coding RNA can be integral part of transcriptional factor circuitry in ES cells.

Conclusions

The cumulative research data only briefly discussed in this review suggests an ever-expanding realm of epigenetic players intimately involved in a multilayered but interconnected network of epigenetic regulation within stem cells. This staggering complexity holds the key not only to the puzzle of mammalian development, but also tissue and organ regeneration and ultimately the emerging paradigms of human aging and age-related diseases. Epigenetic memory operates on combinatorial read-outs of histone modifications, DNA methylation, alterations of chromatin structure due to chromatin remodeling and non-coding RNAs. These represent another crucial mechanism, besides just a network of transcriptional factors, that governs the fine-tuning and precision of gene expression programs. The elucidation of epigenetic mechanisms promises to have important implications for novel advances in stem cell research and nuclear reprogramming, and may offer novel targets for combating human diseases, potentially leading to new diagnostic and therapeutic avenues.

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