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# Chromatin and Genome Organization in Reprogramming

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**Key Words.** Chromatin • Reprogramming • Epigenetics • Induced pluripotent stem cells • Pluripotency • Embryonic stem cells

ABSTRACT

**The ability to reprogram somatic cells to pluripotency is continuingly attracting increasing amounts of attention, providing both potential opportunities for regenerative medicine, as well as an intriguing model to study basic mechanisms of developmental reversal and epigenetic erasure. Currently, nuclear reprogramming is an inefficient process and a better understanding of its components and the underlying mechanisms will no doubt enable us to increase its robustness and to gain a deeper understanding of its regulation. Here we focus on the reprogramming process from the chromatin and genome organization perspective, describing the chromatin changes that occur both globally and locally. At the global level, chromatin condenses toward the characteristic 'open' state, while locally, chromatin reorganization supports the silencing of lineage-specific genes and the activation of pluripotency-related genes. Importantly, the proteins that regulate this process are being identified, revealing different layers of chromatin regulation, including histone modifications, histone variants, chromatin remodeling and genomic DNA methylation. The emerging theme is that chromatin and genome organization are not only altered during the transition from a somatic to a pluripotent state, but also play active, regulatory roles during the reprogramming process.** STEM CELLS *2012;30:1793–1799*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Embryonic stem cells (ESCs) possess the distinctive characteristics of self-renewal and pluripotency. Their capacity to differentiate into any cell type of the mammalian body makes them an excellent tool to model normal development and disease. While ESCs are derived from embryos, remarkably, the somatic cell state, which is stable in vivo, can be manipulated into a pluripotent state experimentally. This so called "nuclear reprogramming" can be achieved by three main techniques [1, 2]: somatic cell nuclear transfer (SCNT) [3], cell fusion [4, 5], and ectopic expression of the pluripotency-related transcription factors OCT4, KLF4, and SOX2 (with or without c-MYC; OKSM) [6]. The possibility to reprogram adult somatic cells to a "stem cell-like" state introduced a new wave of interest in this field as a source for patient-specific cells for regenerative medicine [7].

Pluripotent cells have a unique, "open" chromatin structure [8], characterized by relatively few condensed heterochromatin areas, a higher ratio of active (i.e., H3K9ac; H3K4me3) versus repressive (i.e., H3K9me3) chromatin marks, and a hyperdynamic state of the core chromatin proteins [9, 10]. In addition, developmental genes are "bivalent," marked by both the "active" histone H3 trimethylation on lysine 4 (H3K4me3; Fig. 1) and the "repressive" H3K27me3 [11, 12]. Upon differentiation, heterochromatin spreading takes place and chromatin plasticity is diminished [13].

When somatic cells are coaxed to reprogram into "induced pluripotent stem cells" (iPSCs), the resulting cells are extremely similar to ESCs both functionally and molecularly [14, 15]. However, the low efficiency of the reprogramming process and its reverse correlation with the differentiation stage imply an epigenetic barrier that confers resistance to reprogramming and which needs to be overcome by specific mechanisms [16]. In this review, we will focus on nuclear reprogramming from a chromatin and genome organization perspective, and we will try to integrate the chromatin changes during reprogramming with the proteins that were identified to play a role in this intriguing process.

## GLOBAL CHROMATIN REORGANIZATION

The reprogramming process entails major changes in chromatin structure, and the differentiated cells must reacquire the open chromatin state. Direct evidence for this change in chromatin structure from highly defined, compacted chromocenter domains in somatic cells to loosely packed 10 nm chromatin fiber domains in iPSCs was demonstrated using electron spectroscopic imaging in mouse cells [17]. In this study, the level of heterochromatin compaction was shown to inversely correlate with NANOG levels in ESCs, iPSCs, and blastocyst cells, illustrating the tight connection between the pluripotent state and chromatin structure [17]. In accordance, the distribution of heterochromatin protein 1$\alpha$ (HP1$\alpha$) and H3K9me3 in iPSCs and ESCs is more diffuse with less distinct foci and a higher nucleoplasmic content [18] (Fig. 2). The changes in

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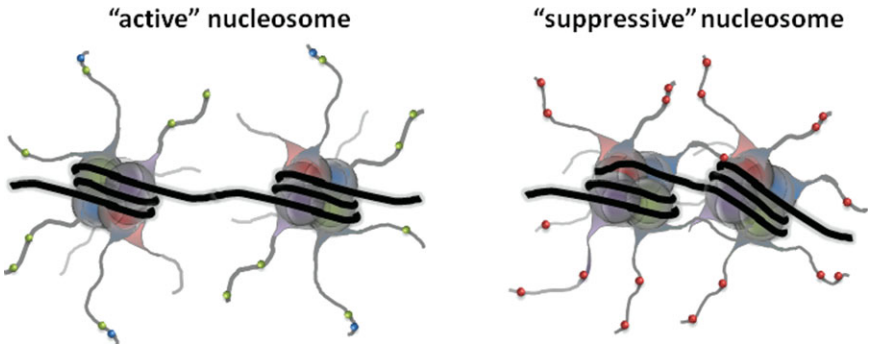


**Figure 1.** Nucleosome structure. The nucleosome is the fundamental unit of chromatin. It consists of a histone octamer, assembled from two copies each of the core histones H2A, H2B, H3, and H4, wrapped by DNA (black line). From each core histone, a long N terminus amino acid "tail" is extended outward from the nucleosome structure. The tail (especially of histone H3) is exposed to various post-translational modifications including (but not limited to) acetylation, phosphorylation, methylation, and ubiquitination, all of which play a role in chromatin structure regulation. Some histone tail modifications, such as acetylation (green dots) and H3K4 methylation (blue dots) are correlated with active gene transcription and an open chromatin conformation (left), while others, including H3K9 and H3K27 methylations (red dots) are associated with gene suppression and condensed chromatin structure (right).

heterochromatin organization seem to be an early event during reprogramming since they can be observed in a small fraction of cells already at day 7 of the reprogramming process—as observed using time course experiments in mouse cells transduced with a single lentiviral cassette expressing the OKSM—before the cells express NANOG. In contrast, a sharp increase in euchromatin marks, including H3ac, H3K9ac, H3K27ac, H4ac, H4K5ac, H3K4me3, and H3K36me2, always appeared concomitantly with NANOG, indicating that heterochromatin precedes euchromatin changes during reprogramming [18] (Fig. 2). Also in human cells, a global decrease in the repressive domains marked by H3K9me3 and H3K27me3 in ESCs and iPSCs was demonstrated by ChIP-seq analysis, when compared with human fibroblasts [19]. ChIP-seq analyses also demonstrated that at the very early stages of the reprogramming process (96 hours), an increase in the active mark H3K4me2 is already detected at enhancers and promoters of pluripotency genes in mouse cells. This increase precedes the changes in the H3K27me3 mark as well as the changes in gene expression [20]. By the end of the reprogramming process, the reprogrammed cells acquire the same global pattern of the "bivalent" modifications [19, 21]. Along the same lines, a global increase in acetylated histones H3 and H4 as well as H3K4me3 was also observed in mouse cells following reprogramming by cell fusion [22]. Interestingly, the transition to a pluripotent chromatin state was also accompanied by an increase in the nucleoplasmic fraction of several core histone marks and HP1$\alpha$ [18], potentially explaining the loosely bound fraction of chromatin proteins observed in pluripotent ESCs [23].

## Histone Modifying Complexes

The gradual change in the patterns of histone modifications that accompany the reprogramming process implies regulation by histone modifying complexes. Indeed, a growing number of investigations implicate histone modifiers as key regulators of reprogramming. One of the earliest such studies demonstrated that the interplay between the H3K9 lysine methyltransferase KMT1C (a.k.a. G9A) and the H3K9 lysine demethylase KDM3A (a.k.a. JHDM2A) affects the reactivation of OCT4 during reprogramming by cell fusion: knockdown of KMT1C or overexpression of KDM3A accelerates fusion-mediated reprogramming [24]. Along these lines, chemical inhibition of KMT1C enables reprogramming of mouse embryonic fibroblasts (MEFs) to iPSCs using OCT4 and KLF4 alone [25]. In a knockdown screen searching for chromatin-related proteins that regulate reprogramming, the euchromatin-related H3K9 methyltransferases KMT1D (a.k.a. EHMT1) and KMT1E (a.k.a. SETDB1) were both shown to enhance reprogramming efficiency [26]. This implies that the interplay between euchromatin and heterochromatin plays a role in shaping the pluripotent genome toward an open chromatin conformation but at the same time, factors that promote the silencing of lineage-specific genes are also essential for a successful reprogramming process. Supporting this notion is the recent finding that suppression of KMT4 (a.k.a. DOT1L), the methyltransferase of the active H3K79 mark, enhances reprogramming. KMT4 catalyzes H3K79 methylation on lineage-specific genes and its inhibition (by either shRNA, conditional knockout or small molecules) significantly reduces H3K79me2 levels on MEF-specific genes (e.g., Snail1, Tgf$\beta$1, Hoxa9, and Hoxc5) and increases reprogramming efficiency [26].

Two important groups of histone methyltransferases (KMTs) that act in this interplay are the Trithorax group (TrxG) and Polycomb group (PcG) protein complexes, which are key regulators of chromatin structure required both for proper differentiation of ESCs and for the maintenance of pluripotency. TrxG complexes execute H3K4 trimethylation, a modification which marks active gene promoters. The PcG complex PRC2, composed of EED, SUZ12, and KMT6 (a.k.a. EZH2), establishes the repressive H3K27 trimethylation. H3K27me3 is recognized by the PRC1 complex which, in turn, brings about the repressive mark H2AK119ub [27].

As described earlier, H3K4 methylation is a prominent mark during early reprogramming, thus it is reasonable to suspect that the enzymes that catalyze it will also be important for establishing pluripotency. Indeed, the TrxG protein WDR5, which is part of the mixed-lineage leukemia (MLL) KMT complex [27], was recently shown to be highly expressed in ESCs/iPSCs and through its binding to the promoters of pluripotency genes, to regulate pluripotency and self-renewal. Moreover, WDR5 is activated during reprogramming, and knocking it down during reprogramming causes a significant reduction in the reprogramming efficiency [28].

PcG proteins EED and SUZ12 were also shown to be important for fusion-mediated reprogramming of human B cells. Knockout for SUZ12 or EED in mouse ESCs significantly

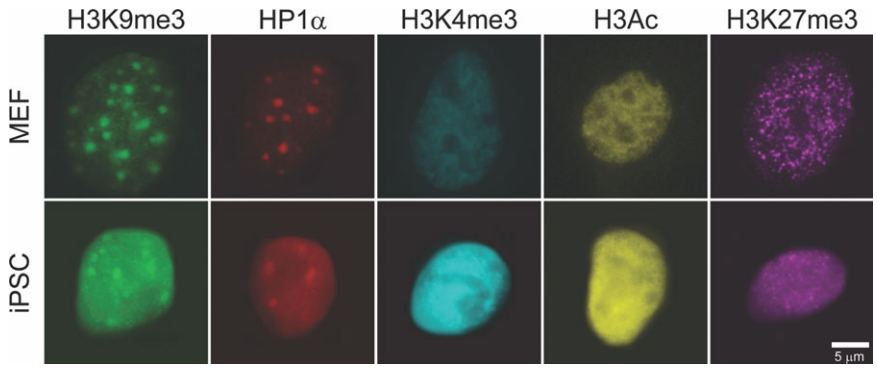


**Figure 2.** Chromatin in fibroblasts versus iPSCs. Chromatin features before (MEF, top) and after (iPSC, bottom) induced reprogramming. Shown are immunofluorescence images of different chromatin markers. In all cases, a fluorescent secondary antibody was used. Pseudocolor is used in these images. From left to right: the cells are labeled with anti-H3K9me3 (green) and anti-HP1α (red), two heterochromatin-related marks, which appear more diffuse with less heterochromatin foci in iPSCs (bottom) compared to MEFs (top); anti-H3K4me3 (blue) and anti-H3ac (yellow), two euchromatin-related histone modifications, which are globally elevated in iPSCs (bottom) compared with MEFs (top), and H3K27me3 (purple), a repressive mark with comparable levels between both cell types. For complete description of growth and staining conditions see [18]. Abbreviations: HP1α, heterochromatin protein 1α; iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblast.

decreased their capacity to reprogram B cells. Since the expression of key pluripotency factors was unaltered in the knockout ESCs, the reprogramming defect was not due to insufficient pluripotency factors but likely due to depletion of the ESC PRC2 complex components [29]. Recently, additional ESC-specific PRC2 subunits were identified, including EZH1, JARID2, MTF2, and esPRC2p48 (ESC-specific *PRC2* subunit *p48*). The latter three were found to mediate H3K27 methylation of MEF-specific genes when added together with OCT4, SOX2, and KLF4, increasing the reprogramming efficiency by threefold [30].

The emerging theme from these studies is the notion that both TrxG and PcG proteins are required for the process of resetting the epigenome of differentiated cells to that of pluripotent cells, but while PcG complexes are required for an early silencing of lineage-specific genes, the TrxG complexes are necessary for the activation of pluripotency genes. The mode by which reprogramming factors bind and alter the chromatin state of polycomb repressed genes was recently proposed to occur through the initial binding to a nucleosome-depleted region in the enhancers of PcG targets and the subsequent recruitment of the cohesin complex, which facilitates enhancer-promoter interaction by DNA looping [31].

The enzymes that counteract the action of the KMTs are the family of lysine demethylases (KDMs), which were also implicated in the reprogramming process. The H3K4/H3K9 demethylase KDM1 (a.k.a. LSD1), for example, which acts as a repressor of bivalent genes in hESCs [32] and facilitates differentiation through its action on enhancers of pluripotency genes in mouse ESCs [33], was implicated in lithium-enhanced reprogramming [34]. Lithium promoted reprogramming by over threefold, partly by inhibiting KDM1. Knockdown of KDM1 partially mimicked the lithium effect demonstrating its direct involvement [34]. The action of KDM1 in ESCs is further facilitated by the KDM1 complex subunit RCOR2. Knockdown of RCOR2 attenuates cell proliferation, increases apoptosis, and causes downregulation of pluripotency-related factors, especially SOX2. Importantly, reprogramming efficiency is significantly impaired when RCOR2 levels are decreased and significantly improved when RCOR2 is overexpressed [35], providing a link between KDM1 activity and reprogramming efficiency. Another KDM that increases reprogramming efficiency is the H3K36me2/3 demethylase KDM2B (a.k.a. JHDM1B). It does so by suppressing expression from the *Ink4*/*Arf* locus and activating the mir302/367 cluster [36]. These data support an active and crucial role for KDMs during reprogramming and suggest that the balance between KMTs and KDMs is important to reset the differentiated genome.

Histone acetylation is a mark of active transcription localized at euchromatin regions mostly encompassing promoters, enhancers, and gene bodies. It is catalyzed by lysine acetyltransferases (KATs, previously known as HATs) and counteracted by histone deacetylases (HDACs), and the interplay between them determines the acetylation level at any given genomic region [37]. Histone acetylation is predominant in undifferentiated ESCs [9], and increasing acetylation levels using HDAC inhibitors (HDACi) increases the efficiency of nuclear reprogramming in nuclear transfer experiments [38], cell fusion assays [39], and induced reprogramming [40–42]. In SCNT experiments in mice, HDACi significantly increases the efficiency and quality of cloned embryos by facilitating correct embryonic stage-specific gene activation [38]. In fusion experiments, HDACi increased the capacity of ESCs with a relatively low acetylation level, to reprogram MEFs. Curiously, although H3K9 acetylation was markedly increased genome-wide following a 16 hour HDACi treatment, very little overall change was observed in gene expression [39]. Similarly, in induced reprogramming experiments of human fibroblasts, HDACi increased the efficiency of iPSC generation dramatically and substituted for c-MYC and KLF4 in the reprogramming cocktail [40, 41, 43]. The effect on reprogramming with retrovirus-transduced OKSM factors was found to be more profound when the inhibitor was added at later stages during reprogramming (days 6–10), leading to elevated acetylation levels and DNA demethylation specifically on pluripotent gene promoters [41].

These data suggest that increased acetylation supports the hyperacetylated state of the pluripotent genome thereby enhancing reprogramming, although emerging studies indicate that the ability to regulate the acetylation state of specific genes and/or genomic regions, as well as the exact timing of manipulation, should no doubt increase our ability to reprogram cells more efficiently.

## Histone Variants

The canonical replication-dependent core histones are clustered together in several different chromosomes in both mouse

and human. They are the only protein-coding transcripts that are not polyadenylated and are primarily produced during S-phase. By contrast, the replication-independent histone variants are solitary and undergo conventional transcription and RNA processing. Histone variants can replace canonical histones, regulating transcription, chromatin structure, DNA damage and repair, epigenetic silencing, and ESC differentiation [44]. It is therefore likely that they play a role in the regulation of local and global chromatin structure during reprogramming. Indeed, 4 hours after SCNT in mouse oocytes, all H3 variants as well as H2A and H2A.Z are displaced, and the oocyte-stored histones, mainly H3.3 and H2A.X (respectively), are incorporated instead, in a replication-independent manner [45]. This rapid histone replacement enables an efficient erasure of the epigenetic state of the somatic nucleus.

The H2A variant macroH2A was shown to play a role in the silencing of the inactive X chromosome (Xi) in SCNT experiments in Xenopus. Injected fibroblasts failed to activate the Xi, whereas in Epiblast stem cells (EpiSCs) the Xi became activated. The authors showed that only the Xi in MEFs, and not in EpiSCs, is packaged with macroH2A. Knockdown of macroH2A not only enabled partial Xi activation but also activated the pluripotency genes *Sox2* and *Oct4* [46]. In another study, the same group showed that incorporation of the Xenopus oocyte linker histone variant B4 increases the dynamic association of histone H1 with chromatin during reprogramming [47], a situation that resembles the hyperdynamic nature of histones in undifferentiated ESCs [10, 23].

These examples provide compelling evidence that histone variants play important roles during reprogramming by SCNT. It will now be interesting to examine the potential roles of different histone variants during the induction of pluripotency by the four pluripotency factors since this process is slower and gradual and unlike SCNT requires cell division.

## Chromatin Remodelers

Chromatin remodelers are ATP-dependent protein complexes, which catalyze nucleosome insertion, displacement, and eviction as well as histone replacement [48]. Among the prominent chromatin remodeling families is the switch/sucrose non-fermenting (SWI/SNF) family, composed of the brahma-associated factor (BAF) complex, which assumes different compositions in different cell types. In ESCs, a specific complex is present, termed esBAF, which includes BRG1 as the catalytic subunit. BRG1 and other members of the esBAF complex (e.g., BAF155) are important for the maintenance of pluripotency and the differentiation capacity of ESCs [49]. The same proteins were also identified in a screen of nuclear fractions that enhances reprogramming. Addition of BRG1 and BAF155 to the reprogramming cocktail increased reprogramming efficiency by twofold to fivefold in MEFs [50]. Congruently, liver progenitors displayed over 100-fold elevated reprogramming efficiency over differentiated liver cells due to the endogenous expression of KLF4, c-MYC, BRG1, and BAF155 [51]. At the molecular level, BRG1 and BAF155 act synergistically to promote euchromatin formation and DNA demethylation at promoters of pluripotency-related genes, thus facilitating the reprogramming process [50]. Two additional proteomic screens identified members of the SWI/SNF complex as facilitators of reprogramming. In one study, KLF4 was found to enhance reprogramming through direct interaction with the SWI/SNF catalytic subunit brahma (BRM), thus promoting active chromatin remodeling [52]. In a more recent proteomic screen, the SWI/SNF subunit BAF155 was identified, again, in a protein complex that was suggested to promote reprogramming [53]. Finally, the ATP-dependent chromatin remodeler CHD1 was shown to promote reprogramming to iPSCs, likely by regulating open chromatin of the pluripotent state [54].

Although still sparse, these emerging studies indicate the importance of chromatin remodeling proteins in obtaining the pluripotent state and hint that they do so by promoting the decondensed chromatin nature of the pluripotent genome. We anticipate that additional chromatin remodelers and their mechanisms of actions will be revealed in the years to come.

## DNA Methylation-Demethylation

DNA cytosine methylation is a major epigenetic mark regulating gene expression and chromatin structure [55]. In mammalian cells, DNA methylation is catalyzed by a “maintenance” DNA methyltransferase, DNMT1, which transfers the existing methylation patterns from mother to daughter cells, and “de novo" DNA methyltransferases, DNMT3A/B, which methylate unmethylated cytosines. During reprogramming, a gradual change in DNA methylation patterns takes place from the somatic to the pluripotent state, shifting from hypermethylation to hypomethylation in pluripotency genes, and vice versa in lineage-specific genes [56, 57]. The resetting of DNA methylation to the pluripotent pattern seems to be more efficient by SCNT than by induced reprogramming, and an incomplete reprogramming of the methylome affects the differentiation capacity of the cells [58–62]. Rearrangement of the methylation pattern in iPSCs continues during passaging until the resetting of the methylation is reached [60, 63]. How this vast change in DNA methylation is achieved and the underlying mechanisms are still not clear, but we do know that an interplay between passive and active DNA demethylation takes place. Inhibition or knockdown of DNMT1, which leads to demethylation by dilution, accelerates the reprogramming process [56, 61], and somewhat unexpectedly, DNMT3A and DNMT3B were found to be dispensable for reprogramming, although the generated iPSCs had a restricted developmental potential until DNMT3A/B were reintroduced back into the cells [64].

Regardless, the fact that reprogramming by SCNT or cell fusion occurs without cell division [57, 65] indicates that active DNA demethylation must take place. Although a bona fide DNA demethylase has not been identified, several proteins have been implicated in DNA demethylation activity [66]. Among these, activation-induced deaminase (AID), which deaminates 5mC to thymidine (leading to a T:G mismatch, which is repaired by the base excision DNA repair pathway), was found to be essential for demethylation and expression of pluripotency genes during reprogramming through cell fusion [65]. Other potential “demethylases” are the ten-eleven translocation (TET) proteins. Although the function of these proteins, which convert 5-methyl-cytosine to 5-hydroxymethyl-cytosine (5hmC), has not been determined yet in nuclear reprogramming experiments, their participation in passive demethylation of mouse preimplantation embryos, in reprogramming of the paternal pronucleus and in SCNT [67–69], implies an active role during reprogramming.

These studies are no doubt just the beginning of our understanding of the control of DNA methylation/demethylation during reprogramming, but they nonetheless indicate an important role for the machinery controlling DNA methylation in the reprogramming process.

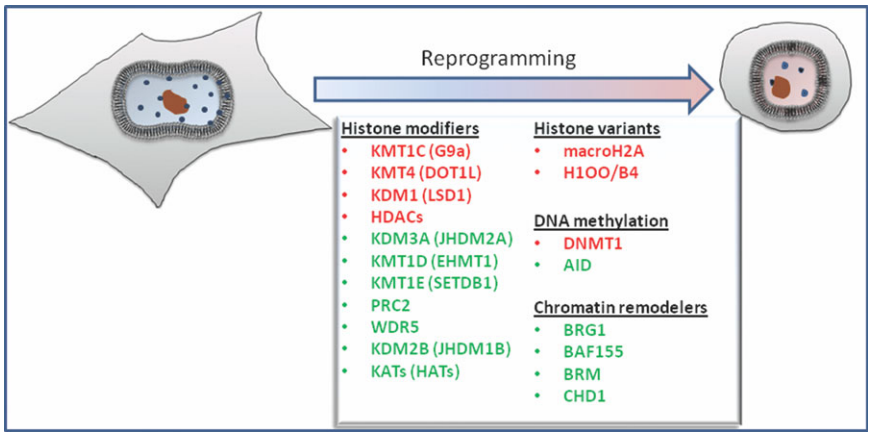


**Figure 3.** Chromatin-related modulators of reprogramming. The reprogramming of a somatic cell (left) to a pluripotent cell (right) requires a global transition to a more open chromatin structure. It also requires local chromatin changes that facilitate the silencing of lineage-specific genes and reactivation of pluripotency genes. Listed below are chromatin-related proteins that were shown to affect the reprogramming process. Promoters of reprogramming are shown in green and suppressors in red. Abbreviations: AID, activation-induced deaminase; BRM, brahma; HDACs, histone deacetylases; KAT, lysine acetyltransferase.

## Conclusions and Prospective

Chromatin plays a fundamental role in pluripotency and stem cell biology [70]. It is therefore not surprising that many studies focused on chromatin-related mechanisms in the conversion of somatic cells to pluripotency (Fig. 3). Since chromatin in pluripotent cells assumes a more decondensed conformation, it appears only natural that factors that act in this direction would assist the reprogramming process and vice versa. In addition, proteins and RNAs that act on specific genes or genomic regions, facilitating the expression of pluripotency genes or inhibiting differentiation genes, should also enhance reprogramming. Understanding chromatin-related changes that occur during reprogramming should lead to superior methods for induced pluripotency and direct conversion of one cell type to another [71]. One of the emerging fields in nuclear biology is the role of noncoding RNAs in regulating chromatin structure and function [72]. We anticipate that the years to come will shed light on the interactions between chromatin and RNA in both pluripotency and reprogramming and will lead to improved manipulations of both ESCs and iPSCs. One such long noncoding RNA, lincRNA-RoR, has already been shown to enhance reprogramming when overexpressed in human fibroblasts [73]. Overexpression of an additional noncoding RNA, the mir302/367 miRNA cluster, was reported to efficiently induce pluripotency, generating Oct4-green fluorescent protein positive colonies already at day 10 of the reprogramming process, partly by suppressing KDM1 expression [74, 75]. It thus seems likely that noncoding RNAs will emerge as central players in pluripotency and reprogramming, acting as chromatin regulators and/or scaffolds for chromatin modifying enzymes [76].

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## Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

## Note Added in Proof

During preparation of this article, the H3K27 demethylase Utx was reported to play a role in reprogramming of both germ cells and somatic cells (Mansour AA et al., Nature, 2012). Utx joins the growing list of chromatin modifying enzymes that regulate the reprogramming process, emphasizing, once again, the important role that histone modifications and chromatin-related proteins play in reprogramming.

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