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H3K9me3-Dependent Heterochromatin: Barrier to Cell Fate Changes

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Abstract

Establishing and maintaining cell identity depends upon the proper regulation of gene expression, as specified by transcription factors and reinforced by epigenetic mechanisms. Among the epigenetic mechanisms, heterochromatin formation is critical for the preservation of genome stability and the cell type-specific silencing of genes. The heterochromatin-associated histone mark H3K9me3, although traditionally associated with the noncoding portions of the genome, has emerged as a key player in repressing lineage-inappropriate genes and shielding them from activation by transcription factors. Here we describe the role of H3K9me3 heterochromatin in impeding the reprogramming of cell identity and the mechanisms by which H3K9me3 is reorganized during development and cell fate determination.

Keywords

heterochromatin; H3K9me3; reprogramming; cell identity; development

Modes of Developmental Gene Silencing

The diverse repertoire of cell types in multicellular organisms is achieved by the differential regulation of gene expression. While much effort has been expended to study how genes are activated, less is known about mechanisms by which cell type-inappropriate genes are repressed, even though this is a crucial aspect of cell fate control [1–3]. It has been long been appreciated that genetic material in the nucleus can be partitioned into two general categories: open 'euchromatin,' which has a relatively low density of DNA and high rates of gene transcription, and '**heterochromatin,**' which has a relatively high density of DNA and low rates of gene transcription (see Glossary). Heterochromatin was originally discerned cytologically by the intensity of dark staining with DNA dyes [4]. The physically condensed state of these regions, reflected by their increased resistance to nucleases [5] and their

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compact properties in biophysical assays [6,7], is mechanistically linked to gene silencing, since compaction renders the DNA template less accessible to binding by the transcriptional machinery. Heterochromatin also has the property of spreading along chromosomes, which is illustrated by the compaction and silencing of transgenes integrated proximal to endogenous heterochromatin regions [5,8].

A large fraction of mammalian genomes is taken up by repeat-rich sequences—including tandem-repeat satellites near centromeres and telomeres, retrotransposons, and endogenous retroviruses—which pose a risk to genome integrity through their potential for illicit recombination and self-duplication. Thus, in all cell types, there is utility in keeping such regions physically inaccessible and, consequently, transcriptionally silent, by packaging them in condensed heterochromatin. Such repeat-rich regions are classified as 'constitutive' heterochromatin, as their silencing is universal across developmental lineages [9]. By contrast, 'facultative' heterochromatin refers to regions whose compaction and silencing is dynamic in development, such as at cell type-specific genes and enhancers [10].

In organisms ranging from the fission yeast *Schizosaccharomyces pombe* to humans, repeat-rich constitutive heterochromatin is marked by di- and tri-methylation of histone 3 lysine 9 (H3K9me2 and **H3K9me3**) [11–13]. These covalent modifications are catalyzed by a family of SET-domain containing methyltransferases, of which there are five in mammals. SETDB1 and the related enzymes SUV39H1 and SUV39H2 contribute to both H3K9me2 and H3K9me3 [11,14], while GLP and G9a (also called EHMT1 and EHMT2, respectively) catalyze H3K9me1 and H3K9me2 [15–17]. H3K9me2/me3 are bound by the chromodomain of **Heterochromatin Protein 1** (HP1, 3 isoforms in mammals), which can self-oligomerize and recruit repressive histone modifiers, contributing to heterochromatin compaction and spread [18–20]. The methyltransferases that deposit H3K9me2 and H3K9me3 are required to establish high levels of DNA methylation at CpG dinucleotides and low levels of histone acetylation, two other hallmarks of heterochromatin [21,22]. By contrast, cell type-specific repression of many genes requires trimethylation of a different H3 residue, lysine 27 (H3K27me3), which is catalyzed by the Polycomb repressive complex 2 (PRC2) [23–26]. This mode of "facultative" silencing is particularly prominent at many lineage-specifying transcription factor genes, such as the homeobox (HOX) family (for detailed review of the role of PRC2 and H3K27me3 in development, see: [27,28]).

The presence of H3K27me3 over gene promoters is highly correlated with gene repression [27,29], yet it has been shown that H3K27me3-marked promoters remain accessible to binding by general transcription factors and a paused RNA polymerase [30,31]. This contrasts with chromatin marked by H3K9me3, which occludes the DNA from binding by transcription factors with diverse DNA-binding domains [32]. Thus, H3K9me3 and H3K27me3-dependent silencing appear to be mechanistically different based on the extent to which the chromatin is accessible to other factors (see Table 1).

Although the H3K9me3 modification has been most often studied in the context of constitutive heterochromatin, genome-wide mapping studies have made clear its role in cell type-specific regulation of facultative heterochromatin [29,33–35]. In differentiated human cells, H3K9me3 forms large contiguous domains ranging in size from the kilobase to the

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megabase scale [29,32,33] (Figure 1). These domains or 'patches' expand in both number and size during differentiation from **pluripotency**, and they span numerous genes repressed in a cell type-specific manner [29]. In particular, there is enrichment for H3K9me3 over gene family clusters, such as those for zinc finger transcription factors, olfactory receptors, and neurotransmitter-related genes (in non-neuronal cell types) [29,33,34], raising the possibility that H3K9me3 protects repetitive gene clusters from illicit recombination similar to noncoding repeats, while also suppressing transcription. Such H3K9me3 domains are largely exclusive of the H3K27me3 domains that also expand during development [29,36,37], highlighting the different functions of these marks, although some developmental transcription factor genes are decorated by both modifications [29].

The related repressive modification, H3K9me2, similarly forms megabase-scale domains that include genes; the domains have been called Large Organized Chromatin K9-modifications (LOCKs) [38]. Interestingly, binding sites for the transcription factor CTCF were detected at the boundaries of these large domains, suggesting that presence of such H3K9me2-decorated patches might be intimately connected to higher-order chromatin structures maintained by CTCF [38]. Whether the boundaries of these H3K9me2 domains expand during differentiation from pluripotency has been a matter of dispute [38–40], with some groups favoring a model of mostly invariant domains during development but local gain of H3K9me2 over select genes [40]. Nevertheless, the dimethyl mark is important for the silencing of lineage-inappropriate genes during differentiation [38,40,41], and mass spectrometry-based quantification of histone marks reveals an increase in both H3K9me3 and H3K9me2 in mouse fibroblasts compared to pluripotent cells [42]. Taken together, the findings in this section indicate that H3K9me2/3 deposition is patterned according to cell identity and must be reset to specify new fates.

Heterochromatin: a barrier to cell reprogramming and cell fate plasticity

The hallmarks of cell identity are erased when differentiated cells are reprogrammed to **induced pluripotent stem (iPS) cells** (see Box 1) [43]. This conversion process requires that **reprogramming** transcription factors bind to their targets in DNA and reactivate pluripotency genes that were silenced in development, suggesting that accessing heterochromatic regions is a necessary step to fully reprogram cells. However, only a minor fraction of the starting cells (<0.1%) successfully complete this process [44,45], raising the question of what chromatin features contribute to such inefficiency.

Box 1

Methods of Cellular Reprogramming

Reprogramming refers to the erasure of a cell's identity to convert it to a different kind of cell, most commonly the conversion of a specialized fate to an earlier, undifferentiated state. Multiple techniques (see [130]) now exist to transform differentiated cells into cells that are pluripotent, which means that they can give rise to any cell type in the embryo.

Somatic cell nuclear transfer (SCNT)

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Seminal work in the 1950s established that transfer of a nucleus from a differentiated cell into an enucleated egg induces a restoration of developmental potential and the production of viable embryos [131]. SCNT has been used to successfully clone mammals, such as sheep [132] and mice [133]. Nonetheless, the frequency at which SCNT gives rise to viable organisms is low, with most resulting embryos exhibiting phenotypic and gene expression abnormalities [74,134]. Elegant studies have revealed specific molecular events required to complete reprogramming after SCNT [73,135], but given the complexity of the egg cytoplasm that is mediating the process, the underlying mechanism is likely to involve myriad factors acting in concert.

Generating induced pluripotent stem (iPS) cells

Yamanaka and colleagues made a critical breakthrough by defining a specific set of four transcription factors that, when ectopically overexpressed, are sufficient to convert a differentiated cell into an induced pluripotent stem (iPS) cell [43]. The factors originally identified—Oct4, Sox2, Klf4, and cMyc—are central regulators of the pluripotency gene network in ES cells [48], and additional combinations of factors capable of generating iPS cells have since been reported [50,136]. In all cases, the resulting iPS cells meet stringent criteria for pluripotency, such as ability to rescue tetraploid blastocysts and contribute to the germline, and on the transcriptional level most iPS lines are highly similar to ES cells derived from the pluripotent inner cell mass [50,136,137]. However, iPS reprogramming is a highly inefficient process, as it proceeds to completion only in a small fraction of cells (generally <0.1%) and at long latency (weeks to months) [44,45].

Direct cell fate conversion

The strategy of ectopically expressing defined cocktails of lineage-specific TFs has been used to convert or transdifferentiate differentiated cells to other developmental lineages, without going through a pluripotent intermediate [138–140]. Despite the promise of these techniques, the reprogrammed cells generally exhibit substantial gene expression differences from their native counterparts, limiting their in vivo functionality and therapeutic utility [141,142].

H3K9me3 heterochromatin impedes iPS reprogramming

Insights into chromatin impediments to reprogramming emerged from determining where the canonical iPS reprogramming factors (Oct4, Sox2, Klf4, and cMyc; henceforth OSKM) first bind the genome shortly after they are expressed in human fibroblasts [32]. All four factors target open chromatin sites, but only OSK, while not M, also target sites containing nucleosomes and lacking evident histone marks, making them pioneer factors [32,46,47]. However, there are megabase-scale chromatin regions in which none of the four factors can target DNA in fibroblasts, even though these same domains have binding sites for the factors in pluripotent cells [32]. Thus, the domains were called **Differentially Bound Regions** (DBRs). The DBRs overlap with domains enriched for H3K9me3 in fibroblasts but not in **embryonic stem** (**ES) cells** (see Figure 1), suggesting that H3K9me3 heterochromatin may mediate the impediment to OSKM binding. Indeed, knockdown of the SUV39H1/H2 methyltransferases increases Oct4 and Sox2 binding in these regions [32].

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The DBRs encode diverse genes and repeat elements, including transcription factor genes essential for pluripotency, such as NANOG, SOX2, DPPA2, DPPA4, GDF3, and ZFP42 [32,48]. Strikingly, all of these genes were independently shown to be delayed in activation until the late phases of reprogramming [49,50], with endogenous SOX2 and NANOG highly restricted to cells that successfully reprogram [50]. The discovery that all four OSKM factors fail to bind within large patches of H3K9me3 heterochromatin [32] that include key pluripotency genes provides mechanistic insight into the observation that these genes are more refractory to activation than others [49,50]. The DBRs also encompass 21 out of 22 of the domains found to have aberrant non-CpG methylation in human iPS cells, compared to ES cells [32,51]. This indicates that some H3K9me3 domains, in addition to impeding the rate or efficiency of reprogramming, have a persistent effect in the final reprogrammed state of iPS cells, rendering the conversion to the ES state incomplete.

These findings suggested that H3K9me3 removal might be an effective strategy to enhance the efficiency of reprogramming. Indeed, knockdown of the SUV39H1/H2 methyltransferases, thereby reducing H3K9me3, causes a dramatic increase in the number and rate of appearance of human iPS colonies [32]. Independently, in a screen of 22 chromatin modifiers, short hairpin RNA (shRNA) against SUV39H1 was found to cause the strongest increase in human iPS colony formation [52]. Similar results have been obtained for the other H3K9 methyltransferases, in that reprogramming efficiency is improved in murine neural progenitor cells after G9a inhibition [53] and in murine fibroblasts after depletion of G9a, GLP, or SETDB1 (with additive effects in combination) [42]. It is thus unclear which methyltransferase is most responsible for stabilizing the differentiated state. The yield of fully reprogrammed murine iPS colonies is also enhanced by perturbation of other heterochromatin components, such as knockdown of individual HP1 isoforms (e.g., HP1γ/*Cbx3*) [42], inhibition of histone deacetylases [54–56], or inhibition of DNA methylation [57]. Loss of DNA methylation enhances removal of H3K9me3 in the presence of a transcriptional stimulus [58], and thus the effects of DNA methyltransferase inhibition on reprogramming efficiency may act through similar mechanisms as SUV39H1 knockdown, although this has not been definitively investigated.

Other components of repressive chromatin that oppose iPS reprogramming appear to act at sites outside of DBRs. Demethylation of H3K27me3 by Utx is required for reprogramming [59], while the repressive histone variant macroH2A inhibits it [60,61], but both observations are linked to a common class of pluripotency genes that activate in early reprogramming [59,61], in contrast to most DBR genes [32,50]. In further contrast to H3K9me3, the H3K27me3 methyltransferase EZH2 is required for iPS reprogramming, consistent with its role in maintaining pluripotency [50,52,62]. Thus, iPS reprogramming depends upon continued deposition of H3K27me3 at certain loci, simultaneous with H3K27me3 removal by Utx at other loci. Finally, reduction of another mediator of gene silencing, MBD3 (a component of the NuRD histone remodeling and deacetylase complex), can allow a high fraction of cells to reprogram to the iPS state and to do so in a more synchronous manner [63,64]. However, this co-repressor thwarts reprogramming factor activity at sites they already bind [63], and its role in regulating H3K9me3 domains or preventing factor binding to heterochromatic genes has not been explored.

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Paucity of heterochromatin defines the pluripotent state

A reduction in inaccessible H3K9me3-marked heterochromatin not only speeds conversion to pluripotency by enhancing transcription factor binding, but it also appears to be a fundamental hallmark of the pluripotent state. Using electron spectroscopy imaging (ESI), Bazett-Jones and colleagues identified remarkable differences in chromatin compaction between embryonic epiblast cells and subsequent lineage-restricted stages of development (primitive endoderm and trophectoderm), with the former characterized by a highly dispersed network of 10-nm fibers and the latter showing blocks of highly compacted chromatin [65]. Studies of mouse ES cells in culture revealed similar findings by ESI [66] and a reduction in the number and intensity of nuclear foci that stain positively for H3K9me3 [67]. Furthermore, the chromatin of pluripotent cells shows a higher rate of exchange of chromosomal proteins such as linker histone and HP1, indicative of a more dynamic and accessible chromatin state [67]. Consistent with such accessibility, ES cells have elevated levels of global transcriptional activity, including expression of **repetitive sequences** and mobile elements, which are repressed in differentiated cells [68]. Importantly, depletion of proteins involved in maintaining chromatin accessibility [68] or introduction of elements that promote heterochromatin formation [69] results in impaired ES cell self-renewal and altered differentiation capacity. Thus, the developmental plasticity of early embryonic cells, much like the ability of differentiated cells to complete reprogramming, is tightly linked to the accessibility of chromatin.

The necessity of heterochromatin erasure for the pluripotent state is further illustrated by studies of 'partially reprogrammed' cells that appear during iPS conversion. These cells lack induction of the pluripotency gene network and have limited developmental potential, but express the reprogramming factors and have downregulated their somatic program [57,70]. Nuclear imaging with ESI revealed that partially reprogrammed cells, but not iPS cells, have highly compartmentalized heterochromatin structures containing dense chromatin fibers, similar to differentiated cells [71]. This is consistent with the persistence of DNA methylation and H3K9me3 over specific pluripotency loci, including *Oct4* and *Nanog*, in these cells [57,71,72]. Meanwhile, erasure of H3K9me3, via depletion of SETDB1 or SUV39H1 or overexpression of the Kdm4b demethylase, is sufficient to allow partially reprogrammed cells to progress to full iPS cells [72]. These findings suggest that H3K9me3 is not only a barrier to pluripotency factor binding in the earliest stages of reprogramming [32], but also opposes late maturation steps necessary for pluripotency.

Heterochromatin opposes reprogramming by somatic cell nuclear transfer

In contrast to the reliance of iPS reprogramming on defined factors, **somatic cell nuclear transfer** (SCNT, see Box 2) utilizes the diverse factors of the egg cytoplasm, acting *en masse*, to restore pluripotency, and the resulting process proceeds more rapidly [73]. Yet, recent evidence suggests that H3K9me3 heterochromatin presents a barrier to even this form of reprogramming. Zhang and colleagues performed detailed transcriptomic analysis of 2-cell mouse embryos derived by normal fertilization and SCNT, and they identified '**reprogramming resistant regions**' (RRRs) containing transcripts that were silenced only in the SCNT condition [74]. The authors found that the RRRs had features of heterochromatin including selective marking by H3K9me3 and enrichment for LINE and

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LTR-type repeat elements in the genome. Injection of mRNA for the H3K9 demethylase *Kdm4d* into the embryo or knockdown of *Suv39h1/h2* in donor nuclei improved the expression of genes within the RRRs. Importantly, either approach for reducing H3K9me3 caused dramatic improvements in the developmental potential of the SCNT-derived embryos, with as much as 80% of the embryos reaching the blastocyst stage, compared to less than 20% in controls [74]. Similarly, dramatic increases in the number of cleavage-stage SCNT-derived embryos was observed for donor nuclei lacking G9a [22]. Other methods to reduce heterochromatin integrity—including inhibition of histone deacetylases [75,76], reduction in DNA methylation [77], or depletion of macroH2A [78]—all improved embryo derivation by SCNT.

Taken together, the current evidence suggests that heterochromatin, and in particular H3K9me3-marked domains, presents a barrier to reprogramming to pluripotency. The H3K9me3 heterochromatic barrier applies regardless of the cell conversion methodology (SCNT versus defined factors) and impairs both the efficiency of reprogramming and the quality of the cells produced (Figure 2).

H3K9me3 as a regulator of cell fate in vivo

The critical function of H3K9me3 in impeding cell reprogramming [32,72,74] and in silencing lineage-specific genes [29,35] suggests that heterochromatin helps maintain cellular identity. Thus, patterns of H3K9me3 must be reorganized during cell fate transitions in development, both in the early embryo (see [79,80] for review) and in terminal lineage maturation.

Maintaining and exiting pluripotency: H3K9me3 and transcription factor crosstalk

In pluripotent stem cells, transcription factor networks ensure that H3K9me2/me3 is deposited over genes for cell differentiation and removed from essential pluripotency regulators. In murine ES cells, SETDB1 has been shown to occupy and repress genes encoding developmental regulators [81] and to act as a co-repressor of Oct4, thereby suppressing trophoblast genes [82–84]. Similarly, Loh et al. elegantly demonstrated that in murine ES cells Oct3/4 positively regulates the expression of the demethylases Kdm3a and Kdm4c to remove H3K9me2 and H3K9me3, respectively, from *Tcl1* and *Nanog*, guaranteeing the maintenance of cell renewal in ES cells [85].

Upon implantation of embryos *in vivo* or differentiation of ES cells *in vitro*, there is a progressive and irreversible silencing of *Oct3/4* and other pluripotency-associated genes, including *Nanog*, *Stella*, and *Rx-1*. Deposition of H3K9me2 at these sites, and in turn DNA methylation, is dependent on the methyltransferases GLP and G9a [22,41,86]. G9a prevents *Oct3/4* reactivation when differentiated ES cells are returned to pluripotency culture conditions [86] (Figure 2, dashed line). Meanwhile, mutations in GLP that disrupt its H3K9me1-recognition domain result in decreased H3K9me2, a delay in silencing of pluripotent genes during ES differentiation, and abnormal embryonic development *in vivo* [41]. The reverse H3K9me2 dynamics are seen at the master germ-line regulator genes *Ddx4* and *DazI*, which show high levels of H3K9me2 in ES cells and lose the modification in *in vitro*-generated mature primordial germ cell-like cells [87]. A reduction in H3K9me2 occurs

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at lamina-associated domains (LADs), which normally associate with the nuclear periphery, and is coupled to a relative depletion in H3K27me3, a mark enriched at LADs' borders [88]. The overall picture highlights crosstalk between H3K9me2 and H3K27me3 and a direct role for H3K9me2/me3 in the developmental control of gene expression (Figure 2).

Requirement of H3K9me2/me3 deposition for normal embryonic development

The importance of H3K9me2/me3 establishment in completing developmental transitions is illustrated by genetic loss-of-function studies in mouse embryos. G9a- and GLP-null embryos show early lethality, characterized by dramatic morphological abnormalities associated with alteration in gene expression and chromatin organization [16,17]. Homozygous inactivation of SETDB1 also leads to embryonic lethality around the time of implantation, an even earlier stage compared to G9a and GLP mutants, as well as defects in inner cell mass growth [89]. Although single knockouts of either SUV39H1 or SUV39H2 in mice show no developmental defects, double-null mice are born at sub-Mendelian ratios and show prenatal lethality linked to genome instability [90]. Furthermore, knockout of HP1β results in dramatic genomic instability and leads to perinatal lethality, likely caused by defects in the development of neuromuscular junctions and cerebral cortex [91].

The distinct lethal phenotypes seen for the different classes of H3K9me-related methyltransferases and associated factors reflect their diverse contributions during development. G9a, GLP, and SETDB1 regulate early lineage commitment [81–84,86,22,17], while SUV39H1/H2 are involved in genome stability [90] and maintenance of fully differentiated cell identity [32,52,74].

H3K9me3 contributes to lineage restriction in mature cell types

The role of H3K9me3-decorated heterochromatin in controlling terminal differentiation and ensuring the stability of cell identity emerges in two recent studies. Amigorea and coworkers, studying the molecular mechanisms underlying naive T cell differentiation into distinct T helper (Th) cells subtypes, revealed an interplay between SUV39H1 and HP1α to maintain a high ratio of H3K9me3 over H3K9ac at key Th1 genes, the latter of which must be silenced in Th2 cells [92]. Applying both genetic and pharmacologic loss-of-function approaches, the authors showed that in SUV39H1- and HP1α-deficient conditions, Th2 cell lineage stability is compromised and cellular plasticity towards the Th1 fate is increased. This phenotype is also seen in disease-related conditions: a Th2-mediated allergic lung inflammation is reduced upon depletion of H3K9me3 [92]. In a genome-wide approach, Casaccia and collaborators analyzed differentiation processes in the brain and showed that silencing of H3K9-related, but not H3K27-related, methyltransferases impairs oligodendrocyte differentiation, altering their response to electric stimulation [93]. Taken together, these studies indicate that H3K9me3 and H3K27me3 have different roles in developmental gene silencing and cell identity maintenance, depending on the cell lineage.

Molecular control of H3K9me3 deposition

Since H3K9 methyltransferases are broadly expressed [15,94] and are not known to make specific base contacts with DNA [95], additional factors are required to explain the site-

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selectivity of H3K9me3 deposition and the developmental dynamics of H3K9me3 domains. In this section, we consider protein and RNA-based mechanisms by which H3K9me3-based heterochromatic domains are established.

Transcription factor-based recruitment of heterochromatin

A growing number of sequence-specific transcription factors have been found to recruit the heterochromatin machinery to particular gene promoters. The retinoblastoma (Rb) protein interacts with both SUV39H1 and HP1, and it is required for cell cycle-regulated H3K9me3 at the cyclin E promoter [96]. Also important for heterochromatin establishment is a large, tetrapod-specific family of zinc finger (ZNF) transcription factors containing Krüppel-associated box (KRAB) domains. **KRAB-ZNFs**, which mostly have lineage-specific expression, repress transcription of target genes by binding the co-repressor KAP1 (also known as TRIM28 and TIF1β), which in turn interacts with HP1, SETDB1, and histone deacetylases [14,97,98]. Experimental tethering of a KRAB domain to a genomic site results in spreading of H3K9me3 and silencing of gene promoters as far as 15 kb away [99]. Yet mutant forms of KAP1 that cannot bind KRAB-ZNFs nonetheless retain many of their genomic binding sites [100], suggesting that we still have much to learn about KAP1 recruitment.

Murine satellite repeats contain reiterated binding sequences for transcription factors, such as Gfi1b, Sall1, Zeb1, and select Pax family members [101–103]. Specifically, Pax3 and Pax9 contribute to H3K9me3 deposition and transcriptional repression at major satellites and are required for the integrity of pericentric heterochromatin [103], a startling finding given that these factors are expressed only in select cell types. The alternative factors that sustain constitutive heterochromatin in Pax3/9-negative cell types, and the role of these factors in recruiting H3K9me3 to domains containing genes, are not presently understood.

Contribution of RNA to heterochromatin formation

In addition to the role of transcription factors, **noncoding RNA** (ncRNA) can function as a binding platform to establish heterochromatin at specific genomic positions. In the fission yeast *S. pombe*, heterochromatin formation at pericentromeres [104] and other sites [105] depends upon the components of the RNA interference pathway (RNAi) and, paradoxically, requires transcription of the locus to be silenced [106,107]. Double-stranded RNAs transcribed from these regions are processed into small interfering RNAs (siRNAs) by Dicer, which in turn guide the silencing machinery, including an H3K9 methyltransferase, to the site of nascent heterochromatin transcription by RNA-RNA base-pairing [108–110]. (For review of the role of RNAi in heterochromatin, see [111,112]). In *S. pombe*, there are additional mechanisms by which ncRNA can establish heterochromatin, independent of RNAi, involving a growing number of RNA processing factors and components of the RNA exosome [113,114]. Meanwhile, in mammals, despite initial reports that Dicer was required for silencing of pericentric heterochromatin [115,116], heterochromatin-derived dsRNA has not been consistently detected across cell systems [9].

While the mechanisms by which ncRNA may establish heterochromatin in mammals remain poorly understood, emerging evidence suggests its contribution is significant. Transcription

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of mammalian heterochromatin has been observed despite the presence of H3K9me3 over the same regions [13,117,118]. The localization of HP1α at pericentromeric heterochromatin is dependent on its interaction with RNA, specifically its binding via its hinge domains to sense-oriented repeat transcripts [119–121]. Strikingly, injection of pericentromere-derived dsRNA in the early mouse embryo is sufficient to rescue the phenotype of a mutant with defects in constitutive heterochromatin [122]. During normal development, an early burst of major satellite transcription precedes and is required for H3K9me3 deposition [123,124]. Also, dynamics in major satellite transcription, which in turn modulate pericentromeric binding of HP1, are important for the cell fate transition of epithelial-to-mesenchymal transition [125]. Along the chromosome arms, the transcription of LINE-1 repeats early in development is required for the subsequent silencing of the LINE-1 elements themselves [126] as well as for the spreading of facultative heterochromatin to nearby genes on the inactive X-chromosome [127]. Finally, another ncRNA derived from rDNA arrays in human ES cells is sufficient to induce widespread H3K9me3 deposition outside the nucleolus and its expression is required for exit from pluripotency [69]. These findings suggest an intimate relationship between RNA and H3K9me3 establishment, though the nature of the interactions and the RNA-binding proteins involved are in need of further elucidation.

Concluding Remarks

Recent work suggests that large domains of H3K9me2/3 form in a cell type-specific manner [29,32], but the protein machinery responsible for such precise developmental dynamics remain largely mysterious (see Outstanding Questions). First, the mechanisms and relative contributions of RNA-dependent versus transcription factor-dependent H3K9me3 recruitment must be defined for these regions, and it is not presently understood how either process can nucleate H3K9me3 deposition over a domain as large as multiple megabases [29,35]. In addition, simple inspection of the large H3K9me3 patches on a genomic level shows that they can terminate precisely over a local domain, suggesting a kind of boundary. Elucidating these mechanisms for the initiation and delimitation of H3K9me2/3 domains will enable more targeted strategies to perturb H3K9me3-dependent heterochromatin at specific sites, possibly to enhance reprogramming in a manner tailored to the starting and desired cell types.

Outstanding Questions Box

- What are the proteins and ncRNAs that control the cell type-specific locations and boundaries of large H3K9me3 domains across the mammalian genome?
- To what extent do Suv39h and Setdb1 have specific, non-redundant roles in repressing cell identity genes, and how does this relate to the distinct embryonic phenotypes upon deletion of these H3K9me3-related methyltransferases?
- How do H3K9me2- and H3K9me3-marked chromatin differ in their exclusion of transcription factors and resistance to gene activation?
- What are the relative contributions of H3K27me3 and H3K9me3 to developmental gene regulation in different lineages, and to what extent to they cooperate in cell fate establishment?

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- Does H3K9me3-dependent heterochromatin impede directed conversion between differentiated cell types, similar to its role in limiting reprogramming to pluripotency?

As RNAi-based knockdown of all five H3K9 methyltransferases has been found to promote reprogramming to pluripotency [32,52,70,72], it will be important to carefully dissect the unique and redundant roles of each enzyme in the establishment of specific H3K9me2/3 domains in diverse cellular contexts, and the relative contributions of the dimethyl and trimethyl forms. This would be facilitated by the creation of conditional knockouts of these genes, alone and in combination. Mapping of H3K9me2/3 domains in specific lineages and developmental stages, coupled with conditional deletion of methyltransferases, will reveal the enzymes responsible for tissue-specific domains and their contribution to developmental gene regulation.

Finally, studies of lineage-specific H3K9me2/3 domains should investigate if they similarly impede directed conversions or transdifferentiation between two differentiated fates (see Box 1). Whether perturbation of heterochromatin components can universally improve the fidelity of these direct conversions, or whether the role of H3K9 methylation in reprogramming is pluripotency- or tissue-specific, will be an exciting avenue for further investigation.

Glossary

Differentially Bound Regions (DBRs): large regions of the genome that are not targeted by iPS reprogramming transcription factors (Oct4, Sox2, Klf4, and c-Myc) in terminally differentiated fibroblasts, but allow binding by the factors in human ES cells, thus impeding efficient reprogramming in fibroblasts. These domains correspond to regions marked by H3K9me3 [32]

Embryonic stem (ES) cells: undifferentiated cells derived from the inner cell mass of the early embryo, which can be cultured in vitro and give rise to any cell type in the embryo

H3K9me3: trimethylation of histone 3 lysine 9, a chemical modification of the histone proteins around which DNA is wrapped. H3K9me3-marked chromatin is associated with inhibition of gene transcription

Heterochromatin: regions of the chromosomes that are especially compacted and transcriptionally repressed. Heterochromatin can be “constitutive” (meaning present in all cell types and phases of the cell cycle) or “facultative” (meaning that repression is cell type-specific or cell cycle phase-specific)

Heterochromatin protein 1 (HP1): proteins required for heterochromatin formation that bind methylated H3K9 via their chromodomain. HP1 proteins act as

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	a scaffold, interacting with H3K9me-related methyltransferases and other proteins via the chromoshadow domain
Induced pluripotent stem (iPS) cell	A cell that has been reverted from a differentiate state to an embryonic stem cell-like state, by overexpression of specific transcription factors
Krüppel associated box zinc finger proteins (KRAB-ZFPs)	C2H2 zinc-finger transcription factors containing an N-terminus KRAB domain, leading to transcriptional repression of genes and recruitment of H3K9me3 upon binding to co-repressor proteins
Noncoding RNAs (ncRNAs)	RNA molecules that are not translated into proteins but can be involved in a variety of cellular processes including regulation of gene activity
Pluripotency	the property of being able to give rise to all tissue types in the embryo
Repetitive sequences	DNA sequences with high copy numbers organized in adjacent near-identical units (tandem repeats, satellite repeats at telomeres and centromeres) or dispersed throughout the genome (DNA transposons, retrotransposons, and endogenous retroviruses)
Reprogramming	erasure of epigenetics states converting a differentiated cell into a different kind of cell, such as a pluripotent stem cell
Reprogramming Resistant Regions (RRRs)	regions of the genome containing genes active in normal 2-cell mouse embryos but repressed in embryos derived by somatic cell nuclear transfer, indicating that the reprogramming process was incomplete [74]
Somatic Cell Nuclear Transfer (SCNT)	Laboratory technique in which the nucleus of a differentiated cell is transferred to the cytoplasm of an enucleated egg. Maternal components reprogram the donor nucleus to pluripotency, allowing the generation of cloned organisms

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Trends Box

- H3K9me3, a histone modification associated with heterochromatin, contributes to gene regulation by forming large repressive domains on the chromosomes that can be dynamic in mammalian development.
- H3K9me3 domains in chromatin prevent binding by diverse transcription factors and constitute a major barrier to reprogram cell identity either by transcription factor overexpression or by somatic cell nuclear transfer.
- H3K9me3 deposition provides a restriction on developmental potency in the early embryo and promotes the stability of specific differentiated cell fates.
- Transcription factors and noncoding RNAs have been found to recruit H3K9me3 to particular genomic locations, but a thorough accounting of the mechanisms of tissue-specific variation in H3K9me3 domains is lacking.

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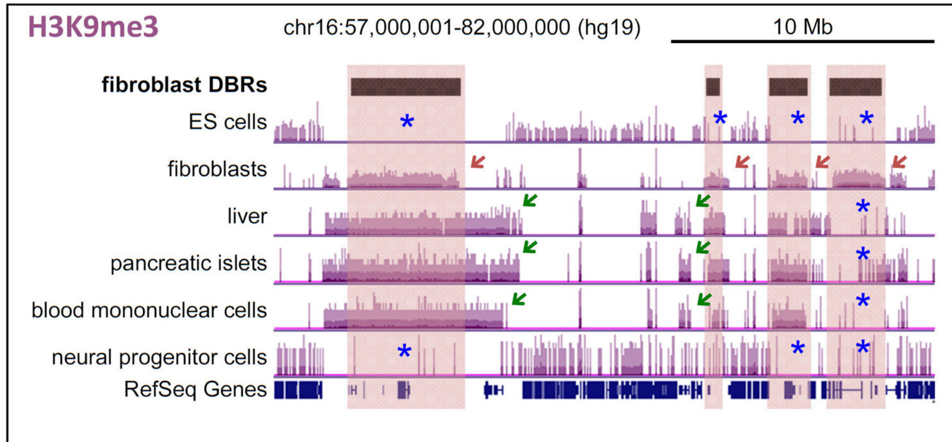

Figure 1. Megabase-scale domains of H3K9me3 vary by cell type and match regions resistant to reprogramming factor binding

Shown is a 25-Mb segment of human chromosome 16, visualized in the UCSC Genome Browser. The purple tracks show H3K9me3 signals by chromatin immunoprecipitation and sequencing (ChIP-seq), normalized by input-subtraction, for the selected cell/tissue-types. All ChIP-seq data come from the Roadmap Epigenomics Mapping Consortium (GSE16368). Note the close correspondence between the H3K9me3-enriched domains in foreskin fibroblasts (red arrows) and the fibroblast Differentially Bound Regions (DBRs, black bars), which are regions that fail to be targeted by iPS reprogramming factors in fibroblasts but are bound in ES cells [32]. Each of these regions lack H3K9me3 enrichment in ES cells, as well as in select other tissues (blue asterisks). Green arrows indicate representative H3K9me3 domains in other tissues that are absent in fibroblasts.

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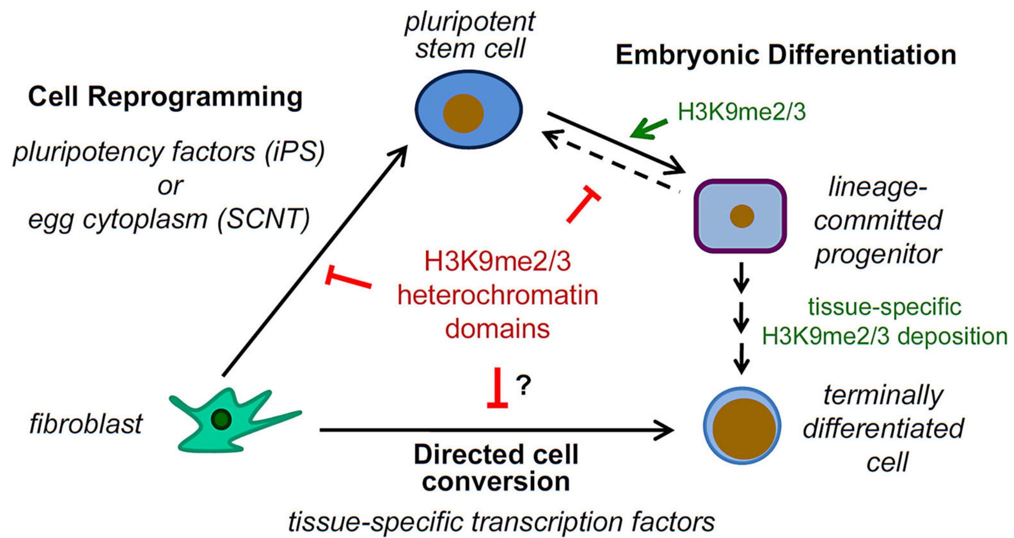

Figure 2. H3K9me2/3 heterochromatin domains impede diverse forms of cellular reprogramming

The diagram shows major cell fate transitions (black arrows) that occur during differentiation and reprogramming and the role of H3K9me2/3 in these transitions. The leftmost black arrow indicates conversion of differentiated cells to pluripotency, which can be carried out by nuclear transfer to an enucleated egg or by overexpression of pluripotency transcription factors. In both cases, pluripotency genes inside H3K9me3 domains are more resistant to activation, and the success rate is reprogramming is improved when H3K9me3 levels are reduced [32,52,72,74]. Thus, H3K9me3 domains impede reprogramming to pluripotency (red inhibitory arrows). When ES-derived differentiated cells are returned into ES culture conditions, thereby encouraging de-differentiation (dashed black arrow), the loss of a H3K9me2 methyltransferase increases the appearance of undifferentiated colonies and the expression of pluripotency genes [86]. In contrast to reprogramming, the differentiation of pluripotent cells in culture (upper black arrows) is promoted by increases in H3K9me2/3 [69,85]. Although H3K9me2/3 domains form in a tissue-specific fashion over the course of development (rightmost black arrows), the role of these domains in the directed conversion of cells across developmental lineages (bottom black arrow) remains to be investigated.

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Table 1

Differences between H3K9me3 and H3K27me3 Heterochromatin Domains

domain properties	**H3K9me3 domains**	**H3K27me3 domains**
genomic distribution	constitutive heterochromatin and tissue-specific sites [29,32,93]	tissue-specific sites [23–26,29,35]
chromatin accessibility	prevent binding by diverse TFs[a] [32]	allow binding by general TFs and paused RNA polymerase [30,31]
allowance of "poised" state, competent for activation	not seen	enhancers/promoters with dual K27me3 & K4methyl [128,129]
timing of gene reactivation during iPS reprogramming	latest stages of reprogramming [32,50,72]	early-to-mid stages of reprogramming [59]
major methyltransferases and role in reprogramming	SETDB1, SUV39H1/H2: impede iPS conversion [32,52,72]	PRC2 complex (EZH2 or EZH1): required for generating iPS [50,52,62]

[a]TFs = transcription factors