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Review

Diverse heterochromatin states restricting cell identity and reprogramming

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Heterochromatin is defined as a chromosomal domain harboring repressive H3K9me2/3 or H3K27me3 histone modifications and relevant factors that physically compact the chromatin. Heterochromatin can restrict where transcription factors bind, providing a barrier to gene activation and changes in cell identity. While heterochromatin thus helps maintain cell differentiation, it presents a barrier to overcome during efforts to reprogram cells for biomedical purposes. Recent findings have revealed complexity in the composition and regulation of heterochromatin, and shown that transiently disrupting the machinery of heterochromatin can enhance reprogramming. Here, we discuss how heterochromatin is established and maintained during development, and how our growing understanding of the mechanisms regulating H3K9me3 heterochromatin can be leveraged to improve our ability to direct changes in cell identity.

Highlights

Various categories of heterochromatin exist and are regulated by different proteins, RNAs, and mechanisms to restrict the access of transcription factors in different ways and degrees.

Activation of a gene during reprogramming that was in heterochromatin requires the opening of the heterochromatin and the activating factors.

Disruptive mechanisms required for maintenance of heterochromatin make sites receptive to the binding and activation of transcription factors, but can activate off-target genes and repeat elements.

Heterochromatin: restricting access to the genome

Despite all cells containing the same genetic information, each cell type in multicellular organisms expresses a subset of genes corresponding to its distinct cellular function. The expression of cell type-specific genes relies upon transcription factors acting in the context of chromatin. During development, the progressive expression of sets of transcription factors drives changes in cell identity and lineage commitment. Reprogramming involves the activation of a new cell identity out of the normal developmental or regenerative context, typically by the ectopic expression of a cocktail of transcription factors that activate genes characteristic of an alternative lineage. The ability to reprogram cells was originally discovered through the observation that nuclear transfer can change a cell's identity [1]. Reprogramming through the direct expression of transcription factors was first demonstrated by the ability of *MyoD* to convert fibroblasts to myoblasts [2] and was later shown by reprogramming B cells into macrophages through the expression of *C/EBPα* and *C/EBPβ* [3]. Finally, fibroblasts were converted to pluripotent stem cells following expression of the *Oct4*, *Sox2*, *Klf4*, and *c-Myc* transcription factors [4]. Reprogramming from one somatic cell lineage to another somatic cell lineage, also referred to as transdifferentiation, has been used to generate many cell types including hepatocytes [5], cardiomyocytes [6], and neurons [7].

Transcription factors can be restricted from binding to heterochromatic regions of the genome that are compact and inaccessible, and hence transcriptionally silent. By contrast, euchromatin is more open, accessible, and generally transcriptionally active. Transcription factors vary in their abilities to bind to free DNA, euchromatin, and silent, unmarked chromatin regions, but are largely blocked from activating target genes in heterochromatin regions during reprogramming [8,9]. Thus, learning how to overcome heterochromatin repression to enable the binding of transcription factors would help improve our ability to reprogram cells for basic science and therapeutic applications [10–12].

Here, we reveal an emerging view that heterochromatin is complex in composition. After reviewing such complexity, we will focus on the H3K9me3 heterochromatin subtype in

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mammalian cells, including how it is established and rearranged during early development, how it resists activation during reprogramming, and how it can be disrupted to enhance reprogramming (Figure 1). It appears that H3K9me3 heterochromatin achieves gene silencing through diverse mechanisms, resulting in structures and biochemical parameters that may modulate the binding of specific classes or families of transcription factors. Unraveling such specificity is a major goal for the future.

Diverse types of heterochromatin

Functionally, heterochromatin silences alternative lineage genes during development [13–16], represses repeat elements, and promotes the genome stability by suppressing recombination among different repeats across the genome [17]. The repressive function of heterochromatin is driven by its structure, biochemical modifications, and chromatin-associated proteins and RNAs.

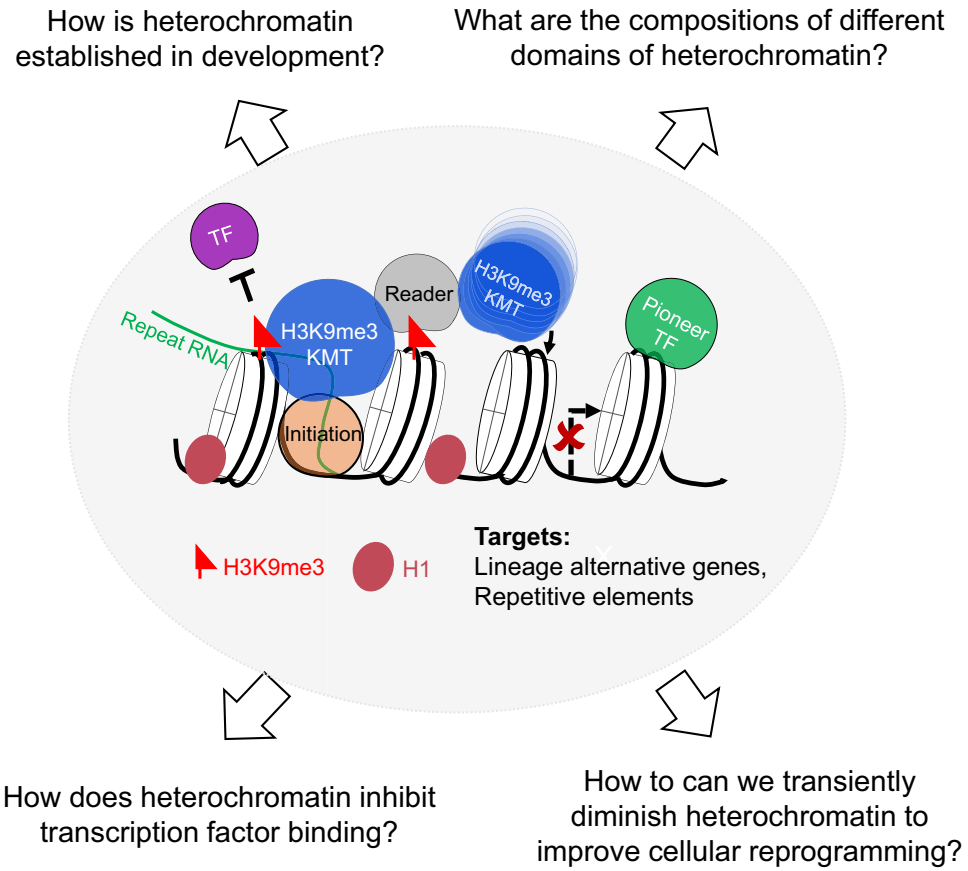

Figure 1. H3K9me3 heterochromatin as a barrier to cell fate change. Central to the functions of H3K9me3 heterochromatin is the 'reader–writer' module, in which the H3K9me3 mark deposited by H3K9me3 methyltransferases is recognized by reader proteins, including HP1α/β/γ, which further recruit methyltransferases to modify the neighboring nucleosomes. This leads to the spread of heterochromatin domains and the stable maintenance of H3K9me3 domains over the cell cycle. Further enrichment of the linker histone H1, HP1 proteins, and other heterochromatin-associated proteins leads to the compaction of heterochromatin and restricting the transcription factors from activating their targets. Building from this basic principle, we discussed how heterochromatin is established and maintained during development, the different compositions of heterochromatin domains, how it molds transcription factor binding, and finally how this knowledge can be used to enhance cellular reprogramming.

Our understanding of the structure of heterochromatin has undergone a dramatic shift, thanks to new insights provided by novel imaging, genomics, and biochemical advances. Compared with the uniform nucleosome compaction observed *in vitro,* recent experiments *in vivo* have revealed a more complex picture of heterochromatin's structures, with heterochromatin assuming multiple nucleosome configurations [18,19] and forming various higher-order structures [20]. Integrating how chromatin's structural configurations correspond to specific histone modifications, and protein and genomic compositions, as well as their impact on transcription factor binding will provide key insights into the regulation and function of heterochromatin during development and reprogramming.

Heterochromatin is often characterized by the associated biochemical modifications that decorate the DNA and histones. The first heterochromatic mark discovered was DNA methylation, which is generally associated with transcriptional repression when it occurs at CpG islands of gene promoters, but its function depends upon the genomic context [21]. Covalent modification of the histone tails, including di- and trimethylation of histone 3 lysine 9 (H3K9me2/3) [22], and trimethylation of histone 3 lysine 27 (H3K27me3), are the most extensively studied histone modifications associated with heterochromatin. H3K27me3, catalyzed by Polycomb Repressive Complex 2 (PRC2), has been associated with heterochromatin at developmental genes, including Hox clusters, which are dynamically regulated during development [23,24]. H3K9me2, which is catalyzed by the histone methyltransferase (HMT) G9a/GLP, and H3K9me3, which is catalyzed by the HMTs SETDB1 and SUV39H1/H2 [22], have long been known to repress repetitive elements. H3K9me2 and H3K9me3 are differentially distributed in the nucleus, with H3K9me2 mainly detected at the nuclear periphery and interacting with nuclear lamina through adaptor proteins [25,26], and H3K9me3 detected at both the nuclear periphery and other more centrally located heterochromatin compartments, including the perinucleolar and pericentric heterochromatin [27]. Upon loss of H3K9me3 in *Caenorhabditis elegans*, H3K9me2 can maintain the repression of some, but not all previously H3K9me3-repressed genes and repeats, indicating an overlapping but not redundant repressive function [15]. Growing evidence has shown that H3K9me2/3 is dynamically-regulated at genes and enhancers during development to enable lineage specification and restrict alternative lineages [13,22,28,29]. H3K9me3 will be the major focus of this review. Additional repressive marks including H4K20me3 [30,31], H3K64me3, H2AK119ub1 [24,32], and histone variants [33], together contribute to the complex organization and regulation of heterochromatin.

H3K9me3 heterochromatin can be further decorated by associated proteins and RNAs, to enforce repression. The linker histone H1 associates with the 'linker' DNA region between nucleosomes throughout most of the chromatin (i.e., both euchromatic and heterochromatin), but a higher density of H1 in the heterochromatin domains contributes to the compaction of chromatin [34,35]. The so-called histone modification-reader proteins include the heterochromatin-binding proteins HP1α, HP1β, and HP1γ, which bind methylated lysines through their chromodomain and recruit SUV39H1/H2 and SETDB1 to spread H3K9me3 marks to the neighboring nucleosomes, compacting the chromatin, and reinforcing repression through the cell cycle [36]. Chromatin-associated noncoding RNAs also play important roles in establishing and maintaining heterochromatin, such as the Xist RNA in the inactivation of the X chromosome [37], satellite RNAs in the recruitment of SUV39H1 and SUV39H2 [38,39], pseudogene lncRNAs in the recruitment of SUV39H1 [40], and endogenous siRNAs which recruit HMTs through nuclear Argonaute [41]. The specific protein compositions of different heterochromatin compartments [8,37,42] may explain how heterochromatin can be uniquely deposited and rearranged during development and reprogramming.

Heterochromatin remodeling enables zygotic genome activation and totipotency

Mammalian embryos undergo extensive epigenetic reprogramming during pre-implantation development, erasing epigenetic information from the past generation and establishing new epigenetic programs to enable developmental progression [43]. Therefore, early development offers an important model for investigating the molecular mechanisms of heterochromatin initiation, establishment, and maintenance, and its impact on cell potential (Figure 2).

In the zygote, the paternal genome in the sperm is largely packaged with protamines, while the remaining canonical histones are largely devoid of H3K9me3 [28,44]. The zygotic maternal genome possesses canonical H3K9me3-, H3K40me3-, and H3K64me3-marked heterochromatin at the centromeric, pericentromeric, and telomeric regions [44,45]. *De novo* H3K9me3 on the paternal genomes by SUV39H2 starts as early as the late zygote stage [46], although the association of the SUV39H2 RNA-binding domain with the pericentromeric RNA transcribed from the paternal genome limits its methyltransferase activities [46,47]. SUV39H1 lacks RNA-binding domains [39], and the overexpression of SUV39H1 induces precocious H3K9me3 heterochromatin in zygotes, causing a developmental arrest at the two-cell stage and reducing the efficiency of nuclear transfer by the oocyte [46,48]. Similarly, a depletion of KDM4a in oocyte, the major H3K9me3 demethylase expressed in mouse and human oocytes, leads to invasion of the H3K9me3 domains into euchromatin and disrupts zygotic gene activation [49]. We can conclude that precisely coordinated heterochromatin resetting is crucial for establishing a permissive chromatin environment for zygotic genome activation and establishing totipotency (Figure 2).

Re-establishment of heterochromatin in the early embryo drives the transition from totipotency to pluripotency

Heterochromatin maintains genome integrity by preventing recombination between repeat sequences and silencing transcription from repetitive elements to prevent the formation of RNA:DNA hybrids (reviewed in [17]). However, the newly established heterochromatin domains before the eight-cell stage lack HP1α [30,50] and most of the linker histone H1 variants [51], which are normally molecular hallmarks of compact heterochromatin domains [34], consistent with the notion that heterochromatin domains prior to the eight-cell stage harbor a noncanonical, nonrepressive structure [46]. Consequently, the resetting of H3K9me3, along with the erasure of other heterochromatin marks, namely H3K64me3 and H4K20me3, and DNA methylation (reviewed in [52]) from the two-cell to blastocyst stage lead to transient activation of satellite repeats and many retrotransposons during pre-implantation development [53].

Interestingly, a pulse of major satellite RNA transcribed from the paternal genome during the zygote stage recruits SUV39H2 to the pericentromeric regions [39], and the transcription from both strands may lead to dsRNA formation, reminiscent of the RNAi mechanisms in *Schizosaccharomyces pombe* and *C. elegans* (reviewed in [22]). Retrotransposons constitute a large proportion of the mammalian genome, and mounting evidence suggests that RNAs transcribed from diverse classes of retrotransposons can direct different heterochromatin machineries to silence the repetitive DNA and target genes [54–57] (Figure 2B). The retrotransposons can be broadly divided into non-long terminal repeat (non-LTR) elements, including long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs), and LTR elements, including endogenous retroviruses (ERVs) ERV1, ERV2, ERV3, and MaLR (reviewed in [58]). LINEs constitute 10–30% of eutherian genomes [59] and its transcripts, which are abundant in two-cell embryos, can recruit nucleolin and KAP1 (TRIM28) to repress *Dux*, the master regulator of two-cell totipotency genes, and therefore drive the exit from totipotency [55].

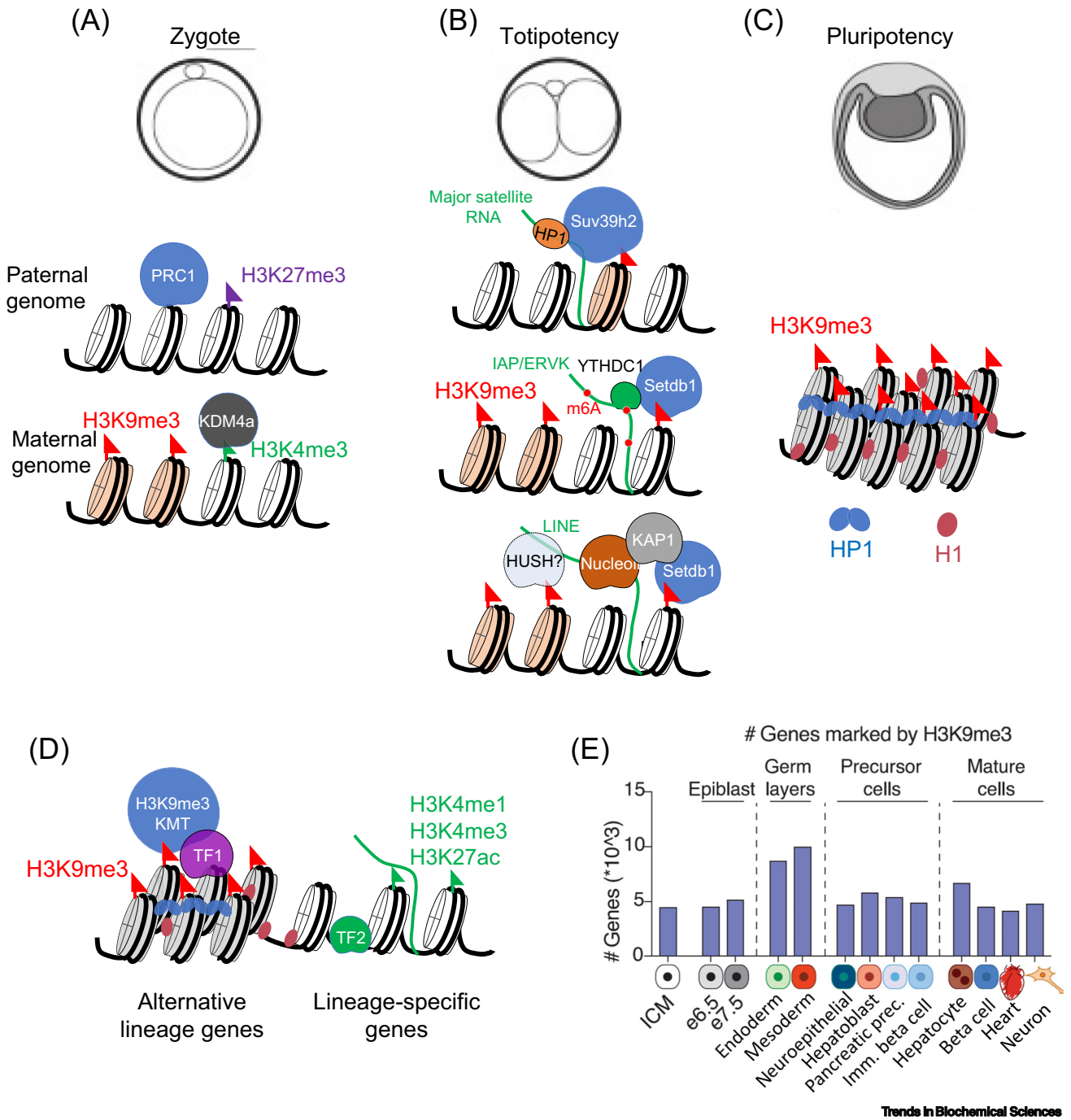

Figure 2. Heterochromatin is dynamic during development. (A) Heterochromatin remodeling accompanies developmental progression during early mouse development. In the zygote, the maternal genome has H3K9me3 heterochromatin marks at the centromeric and pericentromeric regions, whereas the paternal genome does not. (B) The ensuing heterochromatin remodeling creates an open chromatin environment, a hallmark of totipotent states, and leads to activation of repeat regions, which recruit heterochromatin machinery to establish heterochromatin and promote the transition from totipotency to pluripotency. (C) Heterochromatin domains in pluripotent stem cells are decorated with H3K9me3 marks, compacted by the linker histone H1, and recruit heterochromatin-associated proteins, including HP1. (D) During lineage specification in mouse development, transcription factors, including KRAB-ZNF proteins, direct heterochromatin machinery to repress alternative lineage-specific genes to maintain the cell fate. (E) Genes are increasingly marked by H3K9me3 for repression during germ layer development, but this mark is removed from key functional genes upon lineage specification [13].

In addition, the HUSH complex recognizes L1 (LINE) RNAs and recruits SETDB1 to silence L1 retrotransposons, although direct evidence for the function of the HUSH complex during early development is still lacking. LTR elements represent around 25% of the retrotransposons in the mammalian genome, and their transcripts are detected from the zygote to morula stages, some of which show remarkable stage-specific expression (reviewed in [59]). ERV2 families, including intracisternal A-particle (IAP) and ERVK, are among the most abundant ERV elements in the mouse genome [58,60]. RNA m6A modifications by Mttl3/4 on IAPs RNAs can mark the RNAs for degradation [57] and recruit YTHDC1, which further recruits SETDB1 to initiate the formation of heterochromatin at the IAP elements [54]. It is currently unknown if the RNA-directed mechanism also plays a role in silencing other LTR families.

It is possible that RNA transcribed at the heterochromatin domains may also directly recruit HP1 proteins through interactions with the HP1 hinge domains [61]. Taken together, the extant studies indicate that RNA is at the core of heterochromatin initiation and maintenance, and provides targeting specificity for heterochromatin machineries to silence diverse repeat families. Recently, of 172 proteins found to be associated with H3K9me3 heterochromatin in human fibroblast cells, many are strongly enriched for the RGG RNA-binding motif [8], hinting that the RNA binding is a common mechanism for the formation and maintenance of heterochromatin.

In addition to the RNA-directed heterochromatin initiation mechanisms mentioned previously, many transcription factors directly interact with SUV39H1/H2, SETDB1, and HP1 to recruit the heterochromatin machinery to repress diverse retrotransposon families and lineage-specific genes (Figure 2D) [62–64]. Kruppel-associated box zinc finger proteins (KRAB-ZFP) represent a repertoire of constantly evolving transcription factors that recruit SETDB1 through the bridging factor KAP1 to silence invading retrotransposons (reviewed in [65]). Some zinc finger proteins, including ZFP809 [66], KLF4, KLF17 [67], and ZFP93 [68], are highly expressed in early embryos and bind to specific families of retrotransposons, indicating that ZFPs can recruit the machineries of H3K9me3 to establish H3K9me3 heterochromatin at specific retrotransposons during early development. Interestingly, the maturation of heterochromatin domains requires additional heterochromatin-associated proteins, including CAF-1, linker histone H1, and the SUMOylation pathway (Figure 2C) [46,55,69]. A depletion of SETDB1 and the aforementioned heterochromatin-associated proteins in early embryos causes a developmental arrest at the two-cell stage and derepresses the totipotent genes *Dux* and *Zscan4* in pluripotent ES cells, causing a reversion to a two-cell-like totipotent state. Therefore, the re-establishment of the H3K9me3 heterochromatin directed by RNA and transcription factors plays important roles in repressing the two-cell totipotency program and driving the transition to pluripotency at the blastocyst stage.

Dynamic changes in heterochromatin enable lineage specification during development

In addition to repressing retrotransposons, H3K9me3-marked heterochromatin plays important roles in delineating lineage specification during and after gastrulation (Figure 2D,E) [13]. Mapping the changes in H3K9me3 at protein-coding genes from the germ layer stage to endoderm progenitors, and then to differentiated hepatic and pancreatic cells, reveals that, in addition to the expected acquisition of H3K9me3 by genes that become silent during terminal differentiation, surprisingly, many genes are marked by H3K9me3 heterochromatin at the germ layer stage and gradually lose the mark during lineage progression (Figure 2E). Further genetic studies with *Suv39h1/Suv39h2* and *Setdb1* triple knockout (KO) or Setdb1 knockdown show that H3K9me3 heterochromatin functions to restrict late developmental genes and repress alternative lineages [13,28] (comprehensively reviewed in [22]). Thus, the dynamics of H3K9me3 at protein-coding genes are critical for embryologic differentiation to progress properly. Interestingly, in

addition to the roles of ZFPs in the initiation of heterochromatin, as mentioned previously, some of the KRAB-ZNF proteins also show lineage-specific expression patterns and functions, such as ZNF417/ZNF587 in human neurons [70], ZNF558 in the human neural progenitors [71], ZNF589 during human hematopoietic system [72], and ZNF808 in human pancreatic development [73]. Thus, KRAB-ZNF proteins and the transposable elements that they target can be co-opted by the host genome to expand the lineage and species-specific regulatory network [74,75].

In summary, the dynamics of H3K9me3 heterochromatin are critical for early development. Interestingly, the drastic heterochromatin remodeling in early development does not necessarily lead to genome instability; similarly, no genome-wide genome instabilities in the liver were observed after a global loss of H3K9me3 caused by compound SUV39H1/2 and SETDB1 deletions [13], suggesting that the roles of H3K9me3 heterochromatin in safeguarding the genome's stability depend on the cellular context. Understanding how different heterochromatin-associated proteins direct diverse heterochromatin patterns during development has inspired novel screens to perturb heterochromatin machineries to help reprogram cells [10].

Heterochromatin blocks transcription factor-binding and gene activation

To elicit cellular reprogramming, transcription factors are induced to bind and activate the genes of a new cell identity. Many reprogramming protocols have been developed to enable the conversion to diverse cell identities, including pluripotent stem cells [4], macrophages [3], hepatocytes [5], cardiomyocytes [6], and neurons [7]. However, in most cases, the reprogramming elicited by the ectopic expression of transcription factors is limited and does not reflect the fully differentiated cell state desired [76]. Indeed, reprogramming transcription factors are often impeded from binding the terminal differentiation genes of alternative fates because of repressive chromatin at important differentiation genes, particularly H3K9me3 heterochromatin [8,9,15,77]. Transcription factors have different capacities to bind and open closed chromatin.

Pioneer transcription factors have DNA-binding domains that can bind a partial motif displayed on the surface of a nucleosome [78], leading to the opening of chromatin and enabling additional factors to bind [79]. Hence, pioneer factors can scan closed chromatin regions, in contrast to transcription factors that primarily target open chromatin regions [80,81]. Analysis of heterochromatin compartments and diverse transcription factors by single-molecule tracking demonstrated that the pioneer transcription factors' nonspecific DNA and nucleosome-binding ability enable their access to the most restricted heterochromatin [82]. Loss of OCT4 nucleosome-binding ability, without compromising the free DNA-binding affinity, was sufficient to exclude OCT4 from binding closed chromatin and abolish its reprogramming capacities [83]. Therefore, binding of pioneer factors initiates structural changes between DNA and histones [84,85] and facilitates the binding of other transcription factors and remodelers [86].

Despite their abilities to bind nucleosomes, the pioneer transcriptional factors SOX2 and OCT4 are largely excluded from the H3K9me3 marked heterochromatin during reprogramming [9,87]. For instance, in human pluripotent cells, OCT4, SOX2, and KLF4 are bound to pluripotency genes such as *Nanog* and *Prdm14*, but these genes are buried in H3K9me3-marked heterochromatin domains in human fibroblasts. The activation of such pluripotent genes in H3K9me3 heterochromatin occurs at the final stage of induced pluripotent stem cell (iPSC) reprogramming and is a rate-limiting step. Similarly, during fibroblast to hepatic cell reprogramming by the pioneer factor FoxA3 with the transcription factors HNF1α and HNF4α, the hepatic genes repressed by H3K9me3-marked heterochromatin are more resistant to activation than the genes marked by H3K27me3 or the silenced chromatin marked by neither H3K9me3 nor H3K27me3 [8]. During

differentiation from pro-opiomelanocortin to melanotropes, the binding of the pioneer factor PAX7 was also blocked from regions with high levels of H3K9me3 [88]. H3K27me3 heterochromatin can also block MyoD in undifferentiated muscle cells [89] and multiple lineage-specific transcription factors during early mouse and human embryonic stem cell differentiation [90]. Although heterochromatin has been shown to exclude transcription factor-binding in many cell contexts, the ability to bind or being excluded from specific chromatin contexts varies among specific pioneer factors [82,91].

Heterochromatin can be derepressed to enhance the activation of genes during cellular reprogramming

Complete loss of H3K9 methylation in early liver development, through disruption and deletion of all H3K9 lysine methyltransferases, leads to global chromatin decompaction, including the loss of electron-dense heterochromatin and the derepression of protein-coding genes and repeat elements [92]. Transiently depleting diverse nonenzymatic proteins that are important for maintaining H3K9me3 enhances the activation of genes in heterochromatin and improves reprogramming [8–10,12,15,93] (Figure 3A). However, the inhibition of H3K27me3 through knockdown of the PRC2 components EED, EZH2, or SUZ12 decreased the reprogramming of iPSC, potentially because of a failure to silence fibroblast-specific transcripts which gain H3K27me3 during successful iPSC reprogramming [11] (Figure 3A). Disruption of MBD3 or GATAD2A in the NuRD complex, which normally facilitates repression through histone deacetylation and remodeling, enhanced iPSC reprogramming [94]. GATAD2A siRNA knockdown was also shown to improve the activation of the genes located in H3K27me3 heterochromatin during fibroblast to hepatocyte reprogramming [10], potentially through decreased H3K27 deacetylation [94]. Gene derepression alone is typically not sufficient for activation during reprogramming, which requires both derepression as well as the presence of an activating transcription factor [10,15].

The rationale here is that a transient diminution of heterochromatin proteins can allow the reprogramming factors to activate new genetic networks, and the subsequent restoration of heterochromatin proteins, after transient diminution, can allow a new genetic network to re-establish the heterochromatin appropriate for the new cell type.

However, such manipulations can be a dangerous game. Heterochromatin opening during reprogramming can lead to activation of off-target lineages and repeat elements [10]. To lessen this problem, recent findings have revealed that groups of heterochromatin proteins co-repress distinct sets of genes located in heterochromatin and each gene set possesses a particular chromatin signature [10]. While H3K9me3 HMTs and complexes such as HUSH target H3K9me3 to broad classes of genes and repeat elements for repression, recent findings have identified that heterochromatin proteins are necessary for subsets of the targets of H3K9me3 heterochromatin (Figure 3B). Thus, to more precisely open the heterochromatin domains and reduce the undesired consequences, it is necessary to learn more about the mechanisms by which the heterochromatin machinery is targeted in a locus- and gene-specific manner.

Recently it was demonstrated that depletion of Enhancer of Rudimentary Homolog (ERH) in human cells, the *S. pombe* homolog, which is a known regulator of H3K9 methylation [95,96], leads to a global loss of H3K9me3 in human cells, the activation of heterochromatic protein-coding genes during induced hepatocyte reprogramming, and the activation of satellite repeats [10] (Figure 3A). In *S. pombe,* Erh1 interacts with the YTH domain-containing protein Mmi1 and is recruited in an RNA-dependent manner to meiotic genes to maintain H3K9me3 heterochromatin and silencing [95,96]. Despite a conserved protein sequence with Erh1 [95] and role in H3K9me3 regulation [10], the mechanism of ERH recruitment in humans is unknown, as the

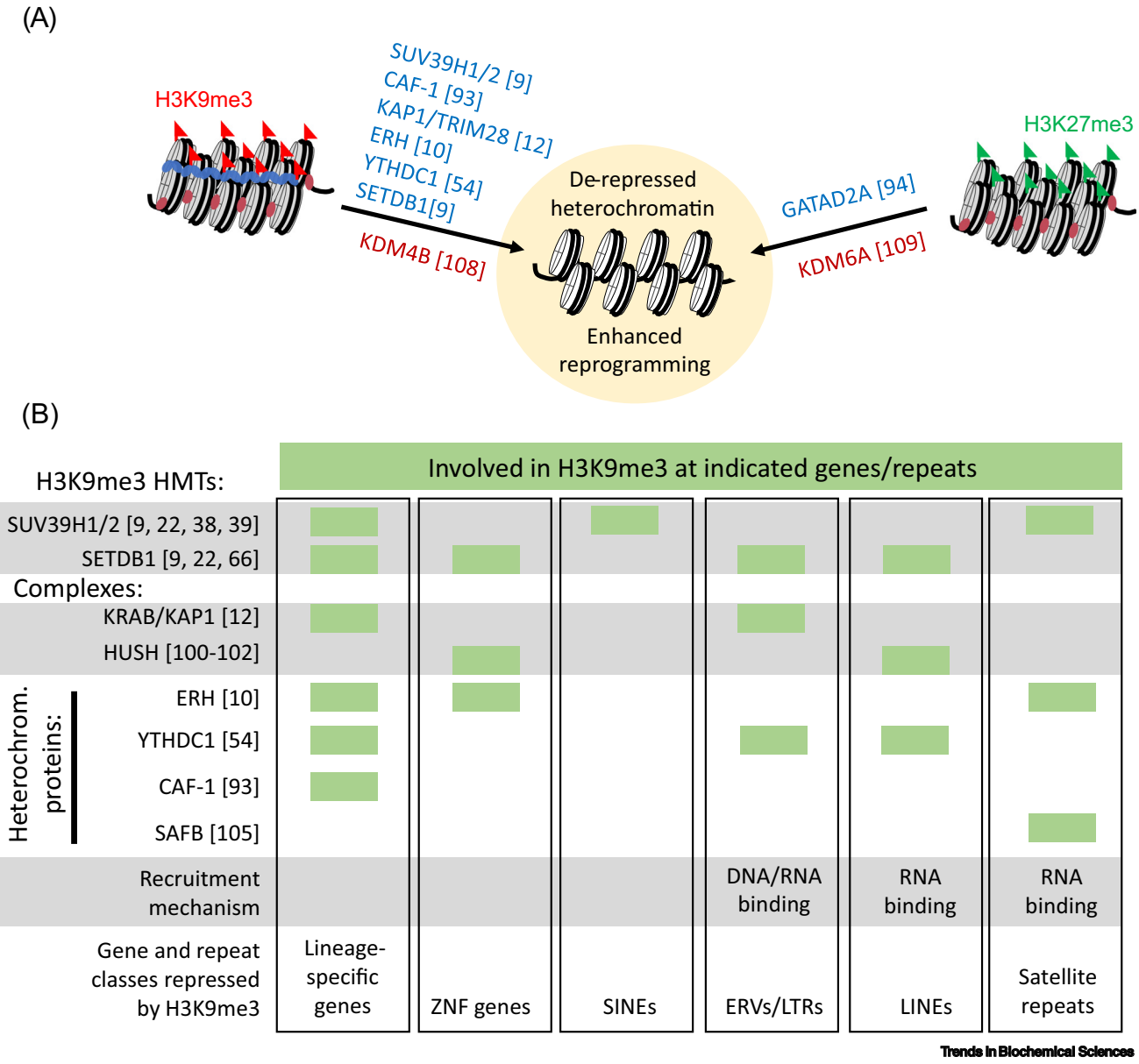

Figure 3. Groups of heterochromatin proteins regulate distinct classes of heterochromatin and can be disrupted to facilitate gene activation. (A) Published results showing knockdowns or knockout (blue) and overexpression (red) experiments that led to the derepression of heterochromatin and enhanced reprogramming. (B) Regulation of H3K9me3 at genes and repeat classes by H3K9me3 HMTs, protein complexes, and selected heterochromatin proteins. Green boxes indicate that the indicated protein or complex has been experimentally demonstrated to regulate H3K9me3 at the designated gene or repeat class. Abbreviations: ERV, endogenous retrovirus; LINE, long interspersed nuclear element; LTR, long terminal repeat; SINE, short interspersed nuclear element; ZNF, zinc finger protein. See [9,10,12,22,38,39,54,65,89,90,101–103,107–109].

direct ortholog of Mmi1 is absent in mammals [97]. Surprisingly, human ERH was found to repress genes in the heterochromatic and euchromatic H3K9me3 domains, indicating that it may function in targeting many or most of H3K9me3 deposition mechanisms [10].

Although heterochromatin is partially defined by its transcriptionally silent nature, recent findings have demonstrated the role of RNAs in the establishment and maintenance of heterochromatin,

beyond the canonical role of the XIST RNA in X inactivation [37]. RNA-directed establishment of heterochromatin is of particular interest because of the potential for uncovering target specificity, which could allow specific RNAs to be disrupted to unlock specific heterochromatin domains. An example of such sequence specificity can be observed in RNAi-directed post-transcriptional gene silencing, by which nuclear Argonaute proteins establish repression in *S. pombe, Drosophila melanogaster, Arabidopsis thaliana,* and *C. elegans* [22,41]; nuclear Argonaute proteins in mammals, however, may be involved in both activation and repression [98].

Euchromatic H3K9me3 regions are transcriptionally dampened but not fully silenced by the HUSH complex, which recruits SETDB1 to repress evolutionarily young L1 retrotransposons, naively integrated lentiviruses, and tissue-specific genes including ZNF gene clusters [99]. The HUSH complex is recruited by intronless RNAs, a feature of retroelements, to repress transgenes and mobile elements [100]. In turn, the repression by HUSH also produces shorter nonpolyadenylated transcripts, which are favorable for the degradation by nuclear exosome targeting (NEXT) [101]. Suppression of L1 elements by the HUSH complex is required for the self-renewal of ground-state pluripotent stem cells [102], but depletion of the component Periphilin 1 in the HUSH complex enhanced activation of genes in heterochromatin during reprogramming to hepatocytes [10]. How HUSH is targeted to genes with introns such as the ZNF clusters remains unclear.

In parallel, heterochromatic H3K9me3 domains are highly enriched in HP1 proteins, which can bind RNA through HP1's hinge domain [61]. Recent *in vitro* modeling suggested that the affinities of IAP and satellite RNAs for HP1 proteins are fivefold higher than for Mediator complexes, thus partially explaining the different recruitment mechanisms to repeats versus gene promoters and enhancers [103]. Depletion of the HP1 proteins during reprogramming destabilizes the H3K9me3 heterochromatin domains that repress pluripotency genes, and therefore enhances reprogramming efficiency [104]. SAFB, a nuclear matrix-associated protein, binds major satellite RNA to promote phase separation at the boundaries of H3K9me3-marked heterochromatin domains [105]. Interestingly, SAFB has been demonstrated to interact with ERH [106] and may cooperate in miRNA processing [107]. In mouse ESCs, deletion of YTHDC1, which targets the RNA modification m^6A to direct SETDB1 to establish H3K9me3 at retrotransposons and totipotent genes, initiated reprogramming to a two-cell-like totipotent state [54] (Figure 3A).

Heterochromatin opening can be facilitated by the active removal of repressive marks and the addition of activating marks to histones. The ectopic expression of lysine demethylases, KDM6A and KDM4B, targeting the H3K27me3 and H3K9me3 domains, respectively, improved reprogramming [108,109] (Figure 3A). Similarly, increased histone acetylation, which is triggered through pathways downstream from MAP2K6 phosphorylation, can lead to improvements in Sox2 and Klf4 binding and reprogramming to pluripotency [110].

These studies revealed that the derepression of heterochromatin can be triggered by disrupting the maintenance functions or active heterochromatin removal, making the target chromatin more accessible and improving reprogramming by transcription factors.

Selectively derepressing heterochromatin domains

The activation of unintended transcripts, including repeat elements and alternative lineage genes [10], as well as the increased genome instability associated with widespread heterochromatin derepression [17,92,111], remains a major barrier to diminishing heterochromatin for cell therapy applications. The goal is to selectively derepress specific heterochromatic gene sets or domains while maintaining the repression of repeat regions and undesired genes. Further work to understand how the HUSH complex [99–101], ERH [10,95], or YTHDC1 [54] are recruited or maintained

in chromatin will be key. It is important to note that, as best as we understand, disrupting the maintenance of H3K9me3 heterochromatin still requires either dilution through cell division [112] or the action of demethylases, for the H3K9me3 mark to go away [113]. Understanding which H3K9me3 HMTs are targeted and how this targeting can be disrupted is complicated by their ability to function redundantly and compensate for partial losses of the other of the three H3K9me3 HMTs [13,22,92]. Another approach involves the identification of highly specific repressors, such as sequence-specific ZNFs [70–73] or the design of synthetic derepressors, which has been achieved recently by fusing epigenetic regulators to transcription activator-like effectors [111] and dCas9 [114].

The transcriptional outcome of derepressing H3K9me3 domains may be influenced by other marks that are either coincident with H3K9me3 or are established in a compensatory manner. For example, in mouse ESCs, dual H3K36me3/H3K9me3 domains, but not H3K9me3-only domains, increased in the interactions with active genes upon *Setdb1* KO [115]. H3K9me2 [15] and compensation by H3K27me3 [10,13,92] have been shown to maintain repression in a subset of sites after the loss of H3K9me3. A better understanding of the complex landscape of heterochromatin will be key to enabling precise and selective derepression.

Outstanding questions

How are heterochromatin proteins dynamic at particular genomic locations in a cell-type-specific manner (e.g., during development)?

What are the roles of RNA-binding proteins and RNAs in regulating heterochromatin at lineage-specific genes and how can they be targeted to enhance reprogramming?

How can heterochromatin at genes be derepressed while maintaining the repression of repeats and transposable elements?

How can the manipulation of heterochromatin be used to improve cellular reprogramming?

Concluding remarks

Despite the extensive rearrangement of heterochromatin during development, genome stability and the repression of repeats are maintained, indicating that different types of heterochromatin can be selectively modulated. Different heterochromatin complexes, directed by RNA and transcription factors, appear at different chromatin domains to accommodate various developmental needs. By discovering the mechanisms by which heterochromatin is selectively targeted during development, we hope to selectively derepress key genes in heterochromatin for reprogramming to diverse cell types, without activating repetitive regions and off-target genes, as seen with a global loss of heterochromatin [10,92]. Recent advances in human iPSC reprogramming suggest that the route(s) to pluripotency transiently goes through a totipotent state [116], which was recently captured *in vitro* [117], offering an opportunity to reconstitute early human development *in vitro* and investigate the heterochromatin remodeling underlying cell fate transitions in greater detail. Future studies are required to dissect the upstream signaling pathways and examine the functional consequences of disrupting different heterochromatin-associated proteins and complexes in various developmental and reprogramming contexts to establish a more unifying principle that governs heterochromatin functions (see Outstanding questions). Finally, understanding how pioneering factors interact with silenced chromatin will also inspire novel designs for synthetic reprogramming factors that combine the chromatin-binding capacities of pioneer factors with chromatin effector domains that modulate repressive heterochromatin environments to improve reprogramming.

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Declaration of interests

No competing interests are declared.

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