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Bivalent Histone Modifications In Early Embryogenesis

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Abstract

Histone modifications influence the interactions of transcriptional regulators with chromatin. Studies in embryos and embryonic stem (ES) cells have uncovered histone modification patterns that are diagnostic for different cell types and developmental stages. For example, bivalent domains consisting of regions of H3 lysine 27 trimethylation (H3K27me3) and H3 lysine 4 trimethylation (H3K4me3) mark lineage control genes in ES cells and zebrafish blastomeres. Such bivalent domains have garnered attention because the H3K27me3 mark might help repress lineage regulatory genes during pluripotency while the H3K4me3 mark could poise genes for activation upon differentiation. Despite the prominence of the bivalent domain concept, studies in other model organisms have questioned its universal nature and the function of bivalent domains has remained unclear. Histone marks are also associated with developmental regulatory genes in sperm. These observations have raised the possibility that specific histone modification patterns might persist from parent to offspring, but it is unclear whether histone marks are inherited or formed *de novo*. Here, we review the potential roles of H3K4me3 and H3K27me3 marks in embryos and ES cells and discuss how histone marks might be established, maintained and resolved during embryonic development.

Introduction

Histones are subject to various modifications, including methylation, acetylation, phosphorylation, ubiquitination and ribosylation [1]. These modifications alter protein-DNA and protein-protein interactions and regulate the interaction of transcriptional regulators with chromatin [2,3] (see Box 1 for more information about chromatin and specific histone modifications). Immunofluorescence studies have revealed that global patterns of histone modifications and chromatin architecture change during the early stages of development [4–8]. Genome-wide Chromatin Immuno Precipitation (ChIP) analyses have suggested that specific combinations of histone marks at promoters and enhancers correlate with the developmental potential and fate of cells [9–24]. For example, embryonic stem cells have a

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different histone modification landscape than cells with restricted fates [9–19,21–24]. The importance of these modifications in embryogenesis is highlighted by the severe phenotypes caused by mutations in histone modifying complexes (see Table 1 for a summary of mouse and ES cell phenotypes [25–74][75]). Here we review the potential roles of histone modifications during embryonic development with a focus on H3 lysine 27 trimethylation (H3K27me3) and H3 lysine 4 trimethylation (H3K4me3) marks at promoters in vertebrate embryos and embryonic stem cells.

Bivalent Promoters in Embryonic Stem Cells

Pluripotent cells from the inner cell mass of mammalian blastocysts can generate embryonic stem (ES) cells [76]. These cells are self-renewing and can give rise to all lineages of the developing organism (Figure 1). Pluripotency is maintained by the activity of a set of transcriptional regulators that include Nanog, Oct4 and Sox2 [77]. In contrast, transcriptional regulators that determine specific cell lineages are not expressed at significant levels in pluripotent cells. During differentiation, these lineage regulators are activated and pluripotency genes are repressed (Figure 1).

The analysis of histone modifications in embryonic stem cells has generated genome-wide location maps of H3K27me3 and H3K4me3 [9–14], catalyzed by Polycomb and Trithorax group proteins, respectively [78]. These studies indicate that many promoters are associated with both H3K4me3 and H3K27me3 [9–14]. The apparent co-localization of H3K4me3 and H3K27me3 might be due to population averaging and reflect heterogeneity within the ES cell population. In such cases, H3K4me3 marks occupy a given promoter in only a subset of cells, whereas H3K27me3 marks are present in a different subpopulation [24]. However, sequential Chromatin Immuno Precipitation (ChIP) has shown that H3K4me3 and H3K27me3 can co-occupy some promoters in ES cells [9,13]. Interestingly, these ‘bivalent’ chromatin domains often mark lineage regulatory genes.

Bivalent domains have garnered wide attention, because they might contribute to the precise unfolding of gene expression programs during pluripotency and differentiation. In particular, it has been proposed that bivalent domains might repress lineage control genes (H3K27me3) during pluripotency while keeping them poised for activation upon differentiation (H3K4me3) (Figure 2). In this model, H3K27me3-mediated repression of developmental control genes might protect cells from the aberrant expression of lineage regulators and thus help maintain pluripotency (Figure 2A). During the differentiation into specific cell types, continued association with H3K27me3 might maintain the repression of the majority of developmental control genes while only a specific subset of regulators is activated in a given lineage (Figure 2B). Conversely, it has been proposed that H3K4me3 might poise developmental regulators for activation upon differentiation. In this scenario, H3K4me3 might make the induction of developmental genes more efficient (Figure 2C) or more synchronous [79] (Figure 2D). H3K4me3 might also protect genes from permanent silencing, for example by repelling transcriptional repressors or blocking DNA methylation [80]. Thus, it is possible that bivalent domains convey temporal and spatial precision to the expression of lineage control genes during pluripotency and differentiation. In the following sections we review the evidence for the postulated roles of bivalent domains in ES cells and their potential occurrence in embryonic cells *in vivo*.

Bivalent Promoters in Embryonic Cells?

The identification of bivalent domains in permanently pluripotent ES cells (and potentially in differentiated cell types [11,13,81–83]) raises the question how relevant these findings are to transiently pluripotent cells in the embryo. Direct evidence for bivalent domains *in vivo* comes from studies in zebrafish: sequential ChIP has established H3K4me3/H3K27me3 co-

occupancy of promoters in zebrafish blastomeres [84]. A study of mouse epiblast cells has also found putative bivalent domains but did not assess the simultaneous association of both chromatin marks in the same cell [85]. As in ES cells, H3K4me3 and H3K27me3 marks are enriched at the promoters of lineage regulators in mouse and zebrafish [84–86]. Surprisingly, however, H3K27me3 and bivalent domains have not been found in *Xenopus* blastomeres [87,88], and studies in *Drosophila* embryos have been unable to identify bivalent domains [89]. These observations do not exclude the possibility that these domains arise later during development [11,13,18,81–83], but it remains unclear how universal bivalent domains are across species.

The Function of H3K27me3 in Bivalent Chromatin Domains – Repression

It has been postulated that bivalently marked lineage-specific genes in ES cells are kept transcriptionally inactive by H3K27me3 [9,10] (Figure 2A). Indeed, loss of components of Polycomb Repressive Complex 2 (PRC2) results in a loss of H3K27me3 and a partial derepression of genes that are normally bivalent and repressed [36,45,46,51]. It was initially proposed that H3K27me3-mediated repression of lineage regulators was essential for maintenance of ES cell pluripotency [45,90]. However, despite the ectopic expression of transcription factors involved in lineage specification and a higher propensity to differentiate, ES cells can be derived from PRC2-deficient blastocysts and maintained in culture for many generations [36,44–46,51,69]. During differentiation, however, mutant cells display multiple phenotypes. While mutant ES cells can differentiate into ectoderm, mesoderm and endoderm [36,44,46,51], lineage regulators are not properly activated [36,51] (Figure 2B). This defect might seem paradoxical, because lineage regulators are prematurely activated in PRC2-deficient ES cells and the loss of PRC2 and H3K27me3 should promote gene activation. It is possible that the ectopic activation of genes from alternative lineages interferes with the execution of the proper developmental programs (Figure 2B). Furthermore, the failure to extinguish the expression of pluripotency genes may also affect proper differentiation and the activation of lineage specific gene expression programs [36].

Deficiencies in subunits of PRC2 also cause severe developmental defects *in vivo* (Table 1). In agreement with the differentiation problems observed *in vitro*, mutant mouse embryos form all three germ layers, but display severe gastrulation and patterning defects and die around implantation [33,37–40,47,48,52]. Similarly, interfering with PRC2 activity in *C. elegans* and *Xenopus* results in the prolonged activity of early-expressed genes [6] and the reduced activation of differentiation genes [6,49]. Together, these studies are consistent with the idea that H3K27me3 in bivalent chromatin domains is important for the repression of developmental genes and suggest that H3K27me3 is essential for lineage specification *in vivo*.

The Function of H3K4me3 in Bivalent Chromatin Domains –Poising?

It has been postulated that bivalently marked lineage-specific genes in ES cells are kept transcriptionally poised by H3K4me3 *i.e.* the association of H3K4me3 with an inactive gene facilitates the future activation of that gene [9,10] (Figure 2C). This putative function of H3K4me3 might extend beyond bivalent domains. For example, in *Xenopus* and zebrafish embryos and in ES cells, many inactive genes are marked with H3K4me3 in the absence of H3K27me3 [84,86,87].

Despite its prominence, evidence for the poising model is sparse, and the function of H3K4me3 is complex, as exemplified by two recent studies in ES cells [26,58]. Jiang *et al.* found that depletion of Dpy-30, a core subunit of MLL histone methyltransferase complexes, results in a partial reduction of H3K4me3. Consistent with the poising model, some lineage-associated genes are not properly activated upon differentiation [26]. In

contrast, ES cell specific genes are expressed normally. These results suggest that the function of H3K4me3 in ES cells is to allow for the proper activation of lineage regulators upon differentiation. However, it is also conceivable that Dpy-30 and normal levels of H3K4me3 associated with lineage-regulatory genes are only required upon differentiation and not in ES cells.

In apparent contradiction to Jiang *et al.*, a related study found that reduction of H3K4me3 levels upon depletion of Wdr5, another subunit of MLL histone methyltransferase complexes, results in severe defects in ES cell maintenance [58]. For example, Wdr5 depletion reduces the expression levels of key pluripotency genes [58]. The early effects of Wdr5-depleted ES cells precluded the detailed analysis of differentiation and suggest an earlier role for H3K4me3 than found in Dpy-30-depleted cells. The observed differences in these two studies may be due to different levels of H3K4me3 depletion and / or pleiotropic functions of Wdr5 and Dpy-30. Both studies establish essential roles of H3K4me3 in the regulation of developmental control genes (pluripotency factors and lineage regulators, respectively), but it remains unclear whether H3K4me3 has a function in poising the expression of embryonic genes.

Mutant and knockdown studies support an *in vivo* role for H3K4me3 in transcription regulation and lineage specification (Table 1) but have not addressed the poising model. For example, knockdown of Wdr5 in *Xenopus* results in a reduction of H3K4me3 levels and Hox gene expression [57]. Furthermore, deletion of Mll1, one of the H3K4 methyltransferases in mammals, results in an absence of Hox gene expression in mouse embryos [25,32]. Mll2 mutant mouse embryos display several developmental defects and embryonic lethality [29,30], and Mll2 mutant oocytes give rise to embryos that may be impaired in the activation of zygotic transcription [28]. While these studies are in agreement with a role for H3K4me3 in transcription regulation and lineage specification, further studies are required to determine precisely when H3K4me3 is required and whether H3K4me3 poises genes for activation during embryogenesis.

Establishing H3K4me3 and H3K27me3 marks

How is the positioning of H3K4me3 and H3K27me3 marks directed? Several mechanisms could guide the *de novo* methylation of histones. For example, long noncoding RNAs can provide sequence specificity to Polycomb and Trithorax proteins [91–95], or DNA binding proteins can recruit methyltransferases to specific sequence elements. Such elements might include Polycomb and Trithorax response elements [96–98] or CpG islands (genomic regions that contain a high frequency of mostly unmethylated CpG sites) [9,11,14,99,100].

Studies in ES cells suggest that pluripotency factors might play a role in the positioning of H3K4me3 and H3K27me3 [9,58,90,101]: bivalent chromatin domains and the position of core subunits of MLL and PRC2 histone methyltransferase complexes often coincide with the binding sites of pluripotency transcription factors [9,58,90]. Moreover, Oct4 has been shown to interact with components of MLL and PRC protein complexes [101]. While these observations are correlative, a recent study revealed that depletion of Oct4 in ES cells results in a reduction of H3K4me3 levels on selected genes, providing evidence for a causal relationship between the pluripotency network and H3K4me3 levels [58]. It remains unclear, however, whether Oct4 and other pluripotency factors are required for the establishment or maintenance of bivalent and monovalent chromatin domains and what other factors play a role.

Inheritance from Sperm?

In embryos, it is not only unclear how H3K4me3 and H3K27me3 marks are established but also controversial when they first appear. Studies in human, mouse and zebrafish have shown that some developmental regulatory genes are already marked by H3K4me3 and H3K27me3 in sperm [102–104]. It has been proposed that some of these marks are inherited after fertilization [86,102,103], but other studies have suggested that H3K4me3 and H3K27me3 marks are erased after fertilization and re-established during early embryogenesis [84,86]. For example, studies in zebrafish indicate that the majority of bivalent and monovalent marks are established when the embryo transitions from a stage when the genome is inactive to a stage when pluripotent blastomeres are transcriptionally active [84,86]. Interestingly, a small subset of genes (e.g. Hox genes) have H3K4me3 and H3K27me3 marks both in sperm and in early embryos, but it is not yet clear if these marks are permanently associated with specific genomic regions through cleavage stages or established *de novo* after fertilization [86,102,103]. Doubts about the inheritance of histone marks are also raised by studies in *Xenopus* embryos where H3K4me3 marks are established only during genome activation and H3K27me3 marks appear even later [87,88]. Similarly, studies in *Drosophila* embryos identified H3K27me3 later during development than H3K4me3 [105]. It thus remains unclear whether histone marks are established *de novo* or are inherited from sperm (or oocytes; their chromatin landscape has not yet been analyzed due to technical challenges).

How might histone marks that are established in the parent be transmitted to offspring? During replication, parental histones re-associate locally with newly synthesized DNA [106]. Histone modifications could thus be re-established by complexes that recognize a specific modification on an inherited parental histone and catalyze the same type of modification on adjacent, newly deposited nucleosomes. For example, H3K27me3 might recruit PRC2 to maintain the mark through replication [107,108]. Similarly, the histone methyltransferase MES-4 might recognize and maintain H3K36me3 domains from the parental germ line to offspring in *C. elegans* [109]. Notably, however, a mechanism by which histone modifications alone are sufficient to direct their own inheritance has not been established unequivocally in any system. Rather, specificity factors such as sequence elements or RNA scaffolds are thought to cooperatively contribute to the reestablishment of the parental chromatin state [110]. Functional analyses of non-coding RNAs, sequence-specific transcription factors, and histone marks during early embryonic stages might help to determine if chromatin states are inherited or re-established after fertilization [84,86–88,105,111–113].

Activation of Lineage Specific Genes

How do lineage regulators transition from an inactive state in ES cells to an active state during differentiation? In ES cells, many lineage regulators are inactive, associated with bivalent domains [9–14] and occupied by pluripotency factors [9,114–118]. It is thought that these factors recruit signal transducers [119], which then overcome H3K27me3-mediated repression and activate lineage regulatory genes [9–11,113,120–124]. For example, upon Nodal signaling, Smad2 binds to its target sites and recruits the histone demethylase Jmjd3, resulting in demethylation of H3K27me3 and gene activation [121]. Interestingly, H3K27me3-mediated repression can also be overcome without demethylating H3K27. One study reported that phosphorylation of Serine 28 in the tail of Histone 3 (the neighbor of Lysine 27) in response to stress signaling results in the displacement of PRC2, relieving transcriptional repression [122]. This mechanism might allow for the transient activation of PRC2-regulated genes until dephosphorylation of S28 reestablishes PRC2 binding and repression. While these *in vitro* studies have started to reveal how signaling pathways can

overcome H3K27me3-mediated repression, the interaction between developmental signaling and chromatin during the transition from pluripotency to cell fate specification remains unclear.

Perspectives

There have been impressive advances in the genome-wide mapping of histone modifications and the phenotypic analysis of mutants that affect histone modifications. Novel concepts such as the bivalent poising of lineage regulators and the epigenetic inheritance from sperm have garnered wide attention. However, it remains poorly understood whether bivalency is a universally conserved principle across species, whether H3K4me3 truly poises genes for activation, and how parental histone marks can be transmitted to offspring.

It also remains largely unclear how embryonic histone marks act at smaller scales and higher-order dimensions *i.e.* how histone marks regulate the assembly of the transcriptional machinery and affect genome folding, respectively. For example, how does H3K27me3 repress transcription at the molecular level? Polycomb repressed chromatin can prevent RNA polymerase from accumulating at promoters [125,126], potentially by compacting chromatin and rendering it inaccessible for RNA polymerase II [127]. It has also been suggested that H3K27me3 and Polycomb group proteins can prevent the release of paused polymerases into the elongation phase of transcription via the ubiquitination of H2A [128,129]. The poising model predicts that H3K4me3 positively influences the recruitment or activity of RNA polymerase II. Although it has been assumed that H3K4 trimethylation follows the binding of RNA polymerase II [130–132], recent work has suggested that H3K4me3 marks can be established independently of RNA polymerase II association [84,100,126] and that H3K4me3 may facilitate RNA polymerase II recruitment [133,134]. Understanding the molecular function of bivalent domains in the regulation of transcription will be essential to understand their role during embryogenesis.

In the broader context of transcription regulation, it is important to note that the concept of bivalency has recently been extended from promoters to enhancers. Analogously to H3K4me3/H3K27me3 bivalent promoters, H3K4me1/H3K27me3 bivalent enhancers are thought to be associated with repressed but poised genes [19,21–23]. It will be interesting to determine the roles of bivalent marks on enhancers and to uncover the relationship between bivalent promoters and bivalent enhancers.

Finally, we also need to consider histone modifications within the larger context of chromatin structure and nuclear organization. For example, PRC2 has been shown to promote the compaction of chromatin and repress gene expression during differentiation in *C. elegans* [6]. Furthermore, Polycomb-repressed domains interact with each other over long distances in PcG bodies, stabilizing their silencing [135,136]. It will be achallenge for the future to integrate the role of histone modifications with long-range chromatin interactions [137,138], higher order chromatin structures [139], and the spatial organization of genes in the nucleus [140,141].

Box 1. Chromatin at a glance

Chromatin

Chromatin refers to DNA and its associated proteins. The basic subunit of chromatin is the nucleosome, an octamer of four core histone proteins; two copies each of H2A, H2B, H3 and H4, around which ~147 bp of DNA is wrapped [142]. Five major types of changes in chromatin structure that affect gene expression have been characterized: (i) DNA methylation. The methyl group that is added to the cytosine of CG dinucleotides

(as well as cytosines in other contexts) is thought to alter chromatin density and accessibility of DNA, thereby modulating the transcriptional potential of the underlying DNA sequence [143]. (ii) Histone variants can replace canonical histones [144]. (iii) Histones can be modified post-translationally [1,144]. Histone variants and modifications can affect transcription either *in cis* (by sterically hindering DNA-protein interactions, by changing the charge of chromatin, or by changing the stability of the nucleosome) or *in trans* (by creating binding platforms for downstream effectors). (iv) Chromatin can be remodeled and compacted by ATP-dependent chromatin remodelers [145]. These chromatin remodelers can be recruited to specific locations in the genome by modified histones or by proteins with sequence specificity. (v) Long-range interactions can affect higher order chromatin structure and transcription by bridging distant sites in the genome [137,139]. This review focuses on two specific histone modifications, H3K4me3 and H3K27me3.

Histone modifications

Technological advances have allowed researchers to map histone modifications throughout the genome by combining chromatin immunoprecipitation (ChIP) with DNA microarray (ChIP-chip) or deep sequencing (ChIP-Seq). These studies have revealed that modifications can mark large chromatin domains or regulatory elements such as promoters or enhancers. They have also associated specific histone modifications with transcriptional output [20,81,146–148]. For example, histone acetylation increases the accessibility of DNA by weakening the interaction between histones and DNA and by binding chromatin-remodeling complexes that contain bromodomains. Acetylated lysines are generally associated with genes that are actively transcribed [1]. Histone methylation is more complex as lysines may be mono-, di- or trimethylated (me1, me2, me3). These modifications can provide binding sites for both positive and negative transcriptional regulators [1]. Lysine trimethylation (H3K4me3, laid down by Trithorax (Trx)/Mixed lineage leukemia (Mll) proteins) is often found at promoters. H3K4me3 binds chromatin remodelers that contain a chromodomain or a PHD finger [149,150]. H3K27me3 (laid down by Polycomb group proteins) is associated with genes that are repressed. Transcriptional repression by Polycomb group proteins is mediated by the action of two complexes: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). Ezh2, a component of PRC2, catalyzes trimethylation of H3K27. A chromodomain protein in PRC1 specifically recognizes H3K27me3. Together, PRC1 and PRC2 repress transcription. While it was initially suggested that Polycomb-repressed chromatin restrains RNA polymerase II from entering the elongation phase via ubiquitination of H2A [128,129], it was recently shown that H3K27me3 marked genes have reduced levels of RNA polymerase II [125,126], perhaps due to the compaction of chromatin [127].

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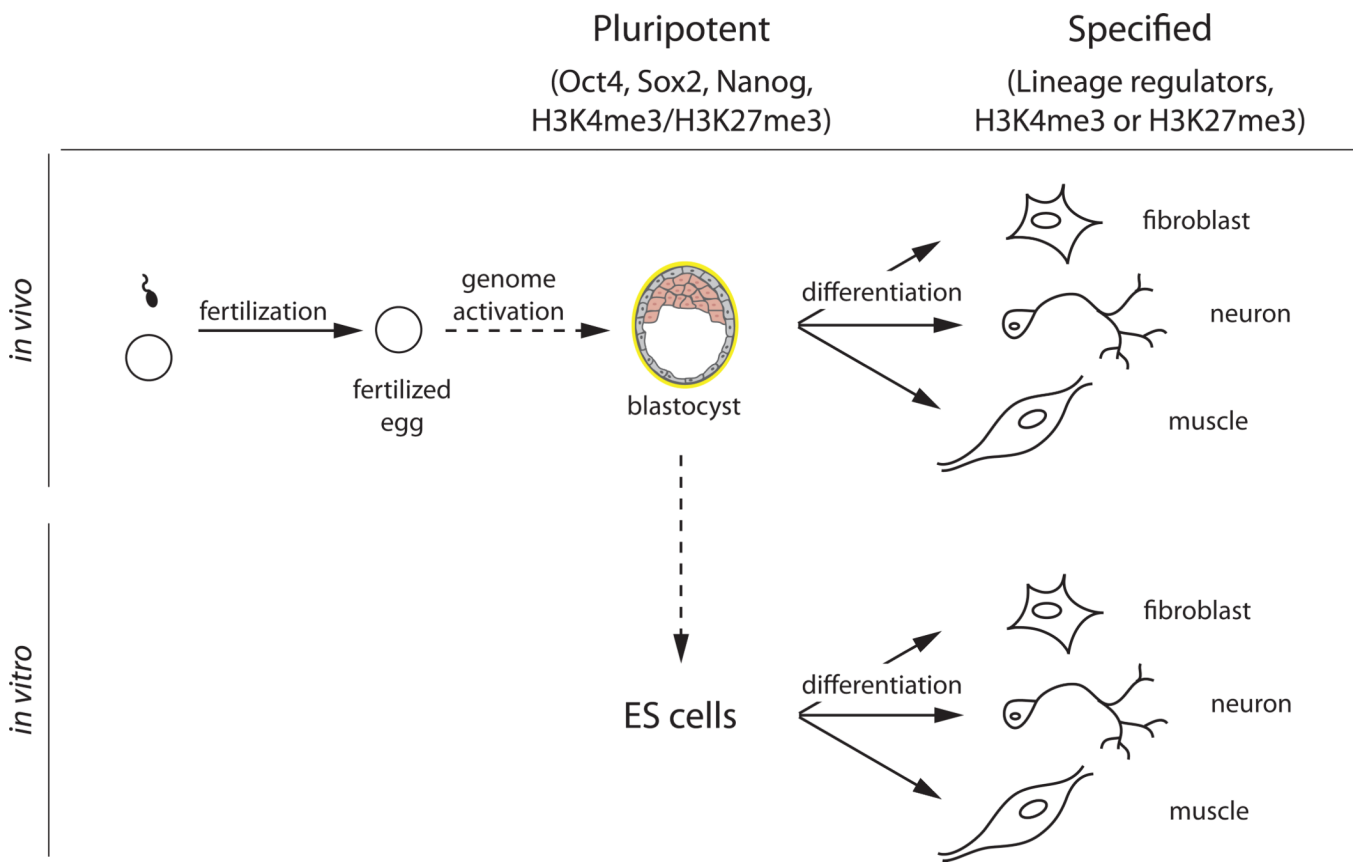

Figure 1. Pluripotency and differentiation of embryonic cells

The embryonic genome is initially transcriptionally inactive after fertilization. At the blastocyst stage, cells are pluripotent and transcriptionally active. Pluripotent cells from the inner cell mass of mammalian blastocysts can be used to generate embryonic stem (ES) cells. Pluripotent blastomeres and ES cells can give rise to all lineages of the developing organism. Pluripotency is characterized by the presence of the pluripotency factors (Oct4, Sox2 and Nanog). Transcriptional regulators that determine specific cell lineages are not expressed at significant levels in pluripotent cells, and they are often marked by bivalent chromatin domains (H3K4me3/H3K27me3). Recently, it has been shown that a subset of bivalent genes is also marked and repressed by the presence of H3K9me3 marks, adding another layer of repression to this subset of lineage regulators [60]. During differentiation, specific sets of lineage regulators are activated.

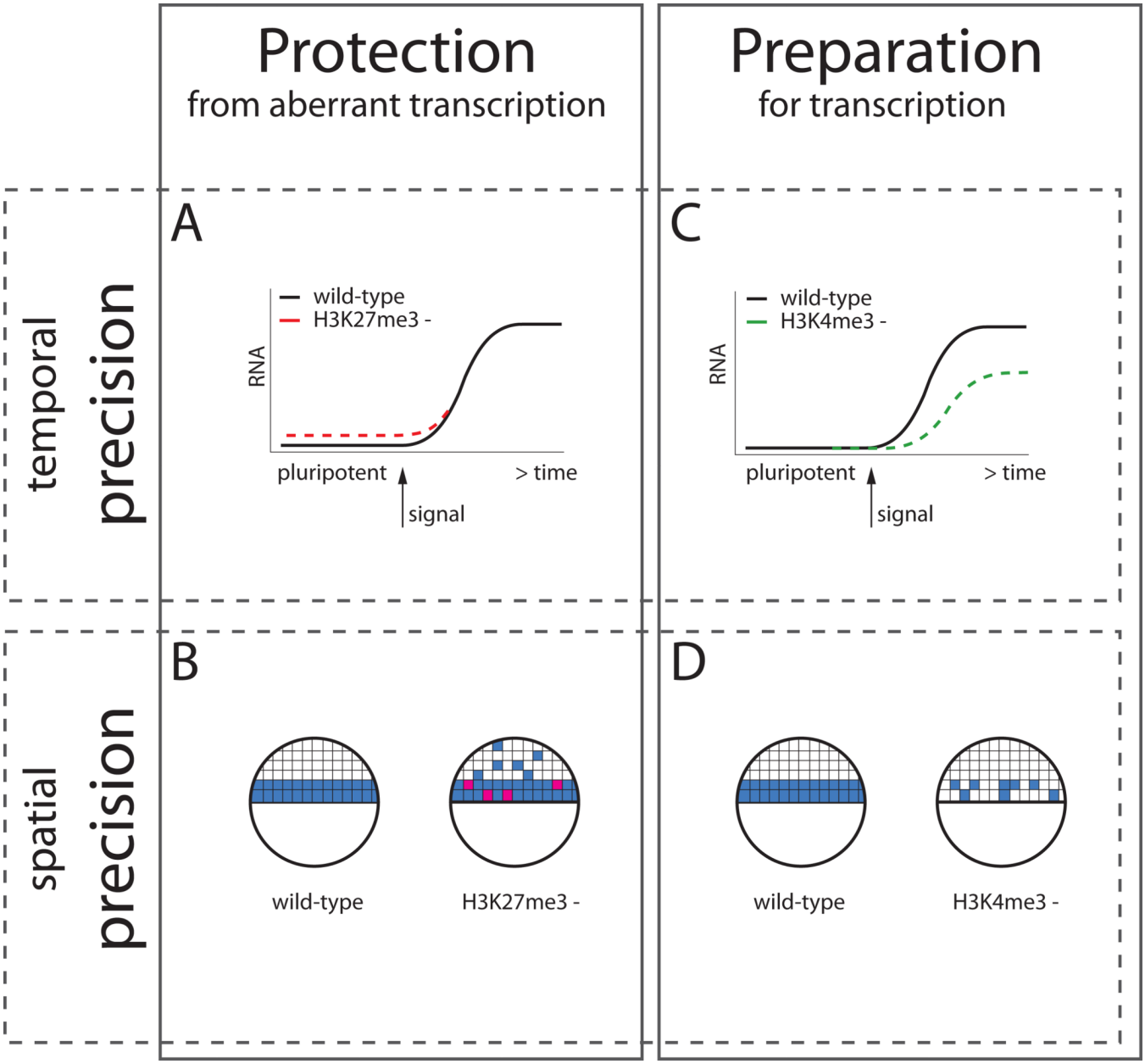

Figure 2. Potential roles of H3K27me3 and H3K4me3 in developmental gene expression

(A) Loss of H3K27me3 might result in the derepression of developmental genes that are normally not expressed in pluripotent cells. (B) During lineage specification, the loss of H3K27me3 might result in both the derepression of developmental control genes (ectopic blue cells) and a failure to properly activate genes (pink cells), perhaps due to the misexpression of pluripotency genes or other lineage genes. (C) Loss of H3K4me3 might result in a less efficient induction of gene expression during differentiation. (D) Loss of H3K4me3 might result in loss or stochastic activation of gene expression.

Table 1

Representative examples that illustrate mouse and ES cell phenotypes associated with the loss of histone modifiers. Please note that the reported defects have not been causally linked to the loss of histone modifications and could thus be due to other functions of the histone modifiers. ko: knock-out, kd: knock-down. dko: double knockout.

gene	domains	Phenotype in vitro (mESC)	Phenotype in vivo (mouse)	Key references
MLL				
mll	SET domain	Embryoid bodies display reduced hematopietic potential. Failure to activate hox genes.	Embryonic lethal (E11.5–14.5), impaired segmental identity, reduction in hematopoietic precursors.	[25, 31, 32]
mll2	SET domain	ES cells pluripotent. Proliferation defects due to increased rates of apoptosis. Compromised timing and coordination of lineage commitment.	Conditional ko: genome-wide reduction in H3K4me3 in oocyte, oocyte death. Full ko: embryonic failure before E11.5. Slowed growth, increased apoptosis, retarded development, male sterility, failure to maintain hox gene expression.	[27–30]
dpy-30 *		Self renewal unaffected but failure to properly differentiate. Lineage-associated genes are not properly activated. Global downregulation of H3K4me3.		[26]
wdr5 *	WD repeats	Severe defects in ES cell maintenance, reduced expression of key pluripotency genes. Global downregulation of H3K4me3.		[57, 58]
ash2l	SPRY domain	Failure to derive ES cells from mutant blastocyst.	Embryonic lethal early during gestation.	[56]
men1			Embryonic lethal (E11.5–12.5). Heterozygotes develop endocrine tumors.	[53–55]
PRC2				
ezh2	SET domain	ES cells fail to undergo mesendoderm differentiation, but phenotype	Depletion of maternal Ezh2: Eed localisation, H3K27me3 and H3K9me3 patterns affected. Severe growth retardation of neonates. Full	51, 52, 74]

gene	domains	Phenotype in vitro (mESC)	Phenotype in vivo (mouse)	Key references
		is less severe than eed null, because of partial redundancy with Ezh1. Decreased levels of bulk H3K27me2 and me3, not me1. Specifically at developmental genes, H3K27me3 and me1 are still significantly enriched, because of partial redundancy with Ezh1.	ko: Early post-implantation lethality (E8.5). Gastrulation defects.	
ezh1*;ezh2	SET domain	Depletion of Ezh1 in Ezh2 null cells abolishes residual methylation on H3K27 and de-represses H3K27me3 target genes.		[51]
eed	WD40 repeats	ES cells are pluripotent but fail to differentiate properly. Genome-wide decrease in H3K27me1, me2 and me3. Target genes are de-repressed. Decrease in Ezh2 protein levels.	Embryonic lethal (~E8.5). Failure to properly gastrulate and to produce embryonic mesoderm. Disrupted axial patterning.	[42, 44–48, 51]
suz12	Zinc-finger domain	Failure to properly differentiate. Global loss of H3K27me2 and me3, de-repression of lineage-specific genes. Decrease in Ezh2 protein levels.	Early post-implantation lethality (~E7.5). Severe developmental (gastrulation) and proliferative defects.	[36, 40]
yy1	Zinc-finger domain		Peri-implantation lethality. Developmental and proliferative defects.	[39]
jarid2	JmjC domain	Failure to properly differentiate. Global levels of H3K27me3 unaffected but	Embryonic lethal (E10.5–15.5, depending on genetic background). Neural, cardiac, liver and hematopoietic defects.	[35, 37, 38, 41, 49, 50]

gene	domains	Phenotype in vitro (mESC)	Phenotype in vivo (mouse)	Key references
		H3K27me levels up on some target genes, down on others.		
pcl2/mtf2	PHD-finger domain	Failure to properly differentiate. Global levels of H3K27me3 unaffected. Upregulated pluripotency factors.	Viable, but growth defects. Low penetrance posterior homeotic transformation.	[33, 34]
PRC1				
ring1b (rnf2)	RING-finger domain	Embryoid body formation is abnormal. Global loss of H2Aub, not H3K27me3. Upregulation of target genes, *e.g.* lineage regulators. Decrease in Bmi1 levels.	Embryonic lethal by E10.5. Developmental arrest in early gastrulation, similar to PRC2 component mutants.	[43, 44, 71–73]
ring1a(ring1);ring1b(rnf2)	RING-finger domain	Loss of ES cell morphology. Developmental regulators are de-repressed.	Viable. Anterior transformation and other axial skeletal patterning abnormalities, both in heterozygous and homozygous mutants.	[69, 70]
bmi1	RING-finger domain		Viable. Posterior homeotic transformations. Neurological abnormalities. Hematopoietic defects. Bmi1/Mel18 dko mice display strongly exacerbated phenotypes.	[65, 67, 68]
mel18	RING-finger domain		Mice die 4 weeks after birth exhibiting strong growth retardation. Posterior homeotic transformations. Bmi1/Mel18 dko mice display strongly exacerbated phenotypes.	[65, 66]
m33	Chromodomain		Most mice die between birth and 4 weeks of age. Severe growth defects. Homeotic transformations.	[64]
rae28	Zinc finger SPM domain		Perinatal lethality. Posterior skeletal transformations. Various defects in neural-crest related tissues.	[75]
H3K9-methyltransferases				
glp/ehmt(euchromatin)	SET domain		Embryonic lethality (~E9.5). Severe growth retardation. Global loss of H3K9me1 and me2, H3K9me3 unaffected.	[62]
g9a/ehmt2 (euchromatin)	SET domain	ES cells can be maintained in culture but display growth defects during differentiation. Decreased levels of bulk H3K9me2.	Embryonic lethality (E9.5–12.5). Severe growth retardation. Global loss of H3K9me2.	[63]

gene	domains	Phenotype in vitro (mESC)	Phenotype in vivo (mouse)	Key references
eset/setdb1 (euchromatin)	SET domain	Defects in ICM outgrowth, no derivation of mutant ES cells possible. H3K9me2 and me3 levels largely unaffected. RNAi kd results in loss of ES cell morphology and upregulation of differentiation markers.	Peri-implantation lethality (E3.5–5.5)	[60, 61]
suv39h1;suv39h2 (pericentric heterochromatin)	SET domain		Reduced viability after E12.5. Growth retardation. Increased risk of tumorigenesis. Chromosomal instability. Loss of H3K9me3 from heterochromatin. (single mutants are normally viable and do not exhibit apparent phenotypes)	[59]

* genes that have only been analyzed in RNAi knockdown studies.