## REVIEW

# Histone lysine demethylases in mammalian embryonic development

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Post-translational modifications, such as methylation, acetylation and phosphorylation, of histone proteins play important roles in regulating dynamic chromatin structure. Histone demethylation has become one of the most active research areas of epigenetics in the past decade. To date, with the exception of histone H3 lysine 79 methylation, the demethylases for all major lysine methylation sites have been discovered. These enzymes have been shown to be involved in various biological processes, with embryonic development being an exciting emerging area. This review will primarily discuss the involvement of these demethylases in the regulation of mammalian embryonic development, including their roles in embryonic stem cell pluripotency, primordial germ cell (PGC) formation and maternal-to-zygotic transition.

## INTRODUCTION

In eukaryotic cells, DNA is packed together with histone proteins in a highly organized form called chromatin. Post-translational modifications of histones, such as acetylation, methylation, phosphorylation and ubiquitination, have been shown to influence chromatin structure and thus play critical roles in regulating gene transcription and genome integrity.[1] Of the different histone modifications, methyl groups have been found to have the lowest turn-over rate; therefore, histone methylation was originally thought to be a permanent mark.[2] The discovery of the first histone demethylase, KDM1A/LSD1, remarkably changed our view on the dynamic nature of lysine methylation. So far, ~20 histone lysine demethylases have been discovered, and these cover most of the major lysine methylation sites, including H3K4, H3K9, H3K27, H3K36 and H4K20; however, the demethylase for H3K79 has not yet been identified (Box 1).

One of the major features of histone lysine methylation is that its regulation and function is highly site dependent. For example, H3K4me3 and H3K36me3 positively correlate with transcription and are usually restricted to the promoters and gene bodies of actively transcribed genes, respectively. H3K4me1 alone is the hallmark of primed enhancers, when presents with H3K27me3 or H3K27Ac, the combinatorial modifications are regarded as the marks of poised (H3K4me1/H3K27me3) and active (H3K4me1/H3K27Ac) enhancers, respectively.[3] Several genome-wide studies have demonstrated the dynamic quality of histone lysine methylation during various processes in normal development and disease, suggesting critical roles for the corresponding methyltransferases and demethylases during these processes.[4,5]

Specifically, embryonic development is a highly dynamic process, coupled with an orchestrated re-organization of the epigenome that shapes the chromatin environment to prepare for the subsequent development stage. In this review, we will first focus on the regulatory actions of histone demethylases on specific chromatin elements, including promoters, enhancers and repetitive elements, and then discuss their involvement in the regulation of mammalian embryonic development, including embryonic stem cell (ESC) pluripotency, primordial germ cell (PGC) formation and maternal-to-zygotic transition.

## REGULATION OF HISTONE DEMETHYLATION AT REGULATORY REGIONS

Recent epigenomic studies of human and mouse embryonic stem cells (hESCs and mESCs) have revealed certain characteristic features that are in contrast to differentiated cells, especially at regulatory regions, such as bivalent promoters,[6]

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**Box 1 Histone demethylases and their substrate specificities**

| Name | Substrates |
| --- | --- |
| KDM1A/LSD1/AOF2 | H3K4me1/me2; H3K9me1/me2; H4K20me1/me2 |
| KDM1B/LSD2/AOF1 | H3K4me1/me2 |
| KDM2A/JHDM1A/FBXL11/Ndy2 | H3K36me1/me2 |
| KDM2B/JHDM1B/FBXL10/Ndy1 | H3K36me1/me2; H3K4me3 |
| KDM3A/JHDM2A/JMJD1A/TSGA | H3K9me1/me2 |
| KDM3B/JHDM2B/JMJD1B | H3K9me1/me2 |
| JMJD1C/JHDM2C/TRIP8 | H3K9me1/me2 |
| KDM4A/JHDM3A/JMJD2A | H3K9me2/3; H3K36me2/3 |
| KDM4B/JHDM3B/JMJD2B | H3K9me2/3 |
| KDM4C/JHDM3C/JMJD2C/GASC1 | H3K9me2/3; H3K36me2/3 |
| KDM4D/JHDM3D/JMJD2D | H3K9me2/3; H1.4K26me1/me2 |
| KDM5A/JARID1A/RBP2 | H3K4me2/me3 |
| KDM5B/JARID1B/PLU-1 | H3K4me2/me3 |
| KDM5C/JARID1C/SMCX | H3K4me2/me3 |
| KDM5D/JARID1D/SMCY | H3K4me2/me3 |
| KDM6A/UTX | H3K27me2/me3 |
| KDM6B/JMJD3 | H3K27me2/me3 |
| KDM6C/UTY | H3K27me2/me3 |
| KDM7A/JHDM1D/KIAA1718 | H3K9me1/me2; H4K27me1/me2 |
| KDM7B/PHF8/JHDM1F/KIAA1111 | H3K9me1/me2; H4K20me1 |
| KDM7C/PHF2 | H3K9me2 |

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**Box 2 Histone demethylases and embryonic stem cell fate**

| Name | Roles in embryonic stem cell fate |
| --- | --- |
| KDM1A | Regulate the balance between self-renewal and differentiation[9–17] |
| KDM2B | Necessary for proper differentiation[18,19] |
| KDM3A | Regulate ESC self-renewal[20] and essential in terminal endoderm differentiation[21] |
| KDM4A | Induce endothelial cell differentiation from ESCs[22] |
| KDM4B | Regulate ESC self-renewal[23] |
| KDM4C | Regulate ESC self-renewal,[20,23] induce endothelial cell differentiation from ESCs[22] |
| KDM5A | Regulate ESC self-renewal[24] |
| KDM5B | Regulate ESC self-renewal[25] and block terminal differentiation[26] |
| KDM6A | Required for ESCs to differentiate into a cardiac lineage[27] and for proper induction of ectoderm and mesoderm[28,29] |
| KDM6B | Induce mesodermal and cardiovascular differentiation from ESCs[30] |
| KDM7B | Regulate mesodermal lineage commitment[31] |

poised enhancers,[7] and super enhancers[8] as well as at some repetitive regions. Various demethylases have been reported to be involved in regulating these chromatin elements (Box 2).

**Regulation of bivalent promoters**

Promoters are regulatory DNA elements that initiate gene transcription. Two of the most studied lysine methylations at promoters are histone H3 lysine 4 tri-methylation (H3K4me3) and H3 lysine 27 tri-methylation (H3K27me3), which generally represent active and repressed transcriptional states, respectively. In 2006, the Beinstein group identified thousands of promoters in mESCs that contain both H3K4me3 and H3K27me3 marks and called them 'bivalent promoters'.[6]

Though bivalent promoters have recently been identified in differentiated cells, ESCs have a much larger number of bivalent promoters,[32] suggesting the unique role of these promoters in regulating ESC function. Consistently, many bivalent promoters were found to be associated with developmental genes in ESCs and are believed to keep these genes in a poised state, which is ready for gene activation or repression upon differentiation by the removal of either H3K4me3 or H3K27me3, respectively.[33,34] The resolution of the bivalent state is believed to involve active demethylation processes.

Of the H3K4me3 demethylases, KDM5B is the most studied due to its regulation of bivalent promoters. Loss of KDM5B causes a number of bivalent genes, including Hox

genes, to show elevated H3K4me3 levels during differentiation, resulting in enhanced self-renewal and compromised differentiation.[35–37] On the other hand, during lineage specification, H3K27me3 demethylases are recruited to resolve H3K27me3-mediated repression and activate lineage-specific genes. For example, upon Nodal signaling, KDM6B is recruited to the bivalent promoter of Smad2, resulting in demethylation of H3K27me3 and gene activation.[38] Another demethylase, KDM6A, has also been shown to be required for the activation of bivalent genes, such as Hox genes, during mESC differentiation.[39]

Interestingly, mammalian sperm also contain thousands of bivalent promoters.[40] Despite the occurrence of global H3K4me3 and H3K27me3 demethylation at the two-cell stage after fertilization (also discussed in the section 'Histone demethylation in fertilized oocytes'), after re-establishment, half of the sperm bivalent promoters regain the bivalent state in mESCs, but show paternal and maternal asymmetry.[40–42] Further investigations are required to understand how bivalent promoters are regulated in gametic and zygotic cells and whether the bivalent promoters in the gametic genome have a preset unique chromatin environment for later stages after fertilization.

### Regulation of enhancers

Enhancers are distinct genomic regions required for proper activation of their target gene(s), especially lineage-specific genes. Therefore, their activities need to be differentially regulated in each cell type. In fact, recent studies have revealed that enhancer activities are very dynamic, even in a given cell type.[43,44] Enhancers are generally considered to have silent, primed, poised and activated states, which are marked by different kinds of histone modifications. For instance, primed enhancers are marked by H3K4me1, active enhancers are marked by both H3K4me1 and H3K27Ac, and poised enhancers are marked by H3K4me1 and H3K27me3.[45] Interestingly, in ESCs, many highly expressed pluripotency factors are controlled by a stretch of active enhancers in close proximity, which are also called super enhancers.[8] During differentiation, enhancers of pluripotency genes need to be silenced, and in contrast, enhancers of differentiation genes need to be activated. In these processes, demethylases of H3K4me1, H3K4me3 and H3K27me3 play critical roles in regulating enhancer activities.

Among the H3K4 demethylases, KDM1A, a demethylase of H3K4me1 and H3K4me2, has been extensively studied. It is found to occupy ESC-specific enhancers and catalyze H3K4me1 demethylation at these enhancers upon differentiation. This process is required for the decommissioning of pluripotency-specific enhancers to facilitate differentiation,[9–13] while other groups have also reported that KDM1A is required for the maintenance of the ESC self-renewal ability.[14–17]

Moreover, recent studies have found that active enhancers are also regulated by the H3K4me3 demethylase KDM5C.[46–48] In Shen's study, active enhancers were demonstrated to be marked by H3K4me3 and H3K27Ac, and interestingly, these enhancers show higher activity than enhancers marked by H3K4me1 and H3K27Ac. They have termed such enhancers 'overly activated enhancers'. Importantly, a chromatin complex containing RACK7/ZMYND8 and KDM5C was identified to control the balance of H3K4me3 and H3K4me1 at these 'overly activated enhancers' to avoid inappropriate activation of downstream genes. This finding not only indicated that a subset of the H3K4me1 marked enhancers could be the demethylation products but also revealed a previously unknown enhancer state. Although the study was primarily done in a breast cancer cell line, RACK7 was also shown to bind thousands of active enhancers in mESCs in the same study; whether it and the KDM5C complex play a role in regulating ESC pluripotency is an interesting question for further study. Notably, KDM5B also binds and regulates H3K4 methylation at active enhancers in mESCs, and KDM5B loss leads to the spread of H3K4 methylation to enhancer shores,[36] which appears to indicate a different mechanism compared to KDM5C-mediated regulation.

### Regulation of repetitive elements

Retrotransposons, which encompass more than half of the human genome, are usually epigenetically silent and packed into heterochromatin, to avoid insertional mutagenesis or transcriptional perturbation. However, it has recently been reported that they could also be transcriptionally active in ESCs, and this mechanism may create genetic diversity at genic and regulatory regions. Conceivably, this mechanism needs to be tightly regulated during embryonic development to avoid the deleterious effects of potential retrotransposition.

Consistent with the fact that they are controlled at a low transcriptional state, the constitutive heterochromatin marks H3K9me3 and H4K20me3 are enriched at telomeric, satellite and long terminal repeats in mESCs,[32] with similar patterns. In addition, H3K9me3 has been reported to specifically repress intact LINE elements in mESCs.[49] The demethylation of H3K9me3 by the JmjC family of histone demethylases such as KDM4A and KDM4B is required to control heterochromatic organization.[50–52]

Moreover, H3K27me3, a facultative heterochromatin mark, has also been shown to be regulated at certain retrotransposons in mESCs. When cultured in serum and LIF conditions, mESCs display a great degree of intercellular heterogeneity, consisting of a 'ground' state with robust self-renewal ability and a more committed 'primed' state. Interestingly, repetitive elements are differentially regulated in these two states. Compared to the primed state, a lower level of H3K27me3 at retrotransposons, such as HERV-H, was observed in the ground state,[53] suggesting that H3K27 demethylases are potentially involved in regulating these repetitive elements in the transition between the ground and primed states.

As the long terminal repeat elements were originally retroviral promoters, it is conceivable that they may escape the repression mechanisms discussed above and regain transcriptional activities. Supporting this notion, the active histone mark H3K4 methylation has been found at MERV-L

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elements and correlates with their transcriptional activities. Importantly, KDM1A plays a role in safeguarding de-repressed MERV-L. This mechanism, when compromised, leads to development arrest at gastrulation.[54]

## REGULATION OF HISTONE DEMETHYLATION IN EMBRYONIC DEVELOPMENT

In this section, we will discuss the involvement of histone lysine demethylases in various embryonic development processes. We have primarily summarized the processes using ESCs and KO mice as models. In addition, due to the uniqueness of PGC formation and maternal-to-zygotic transition, we have discussed them separately at the end of this section.

### Demethylases regulating ESC pluripotency and embryonic development

*H3K4 demethylases.* Of the four H3K4me3 demethylases (Box 1), KDM5B is the most studied in ESCs. As mentioned above, KDM5B acts as an important regulator at promoters and enhancers in mESCs and is required for proper differentiation. KDM5B expression has been reported in various tissues during mouse embryogenesis, whereas its expression becomes restricted to testis in adults, indicating a role in testis-related functions.[55,56] As mentioned above, KDM5B is essential for ESC pluripotency and is required for the resolution of the bivalent state of developmental genes during differentiation,[25,26] and its loss leads to early embryonic lethality between E4.5 and E7.5.[57] However, another group reported that the loss of KDM5B only resulted in post-natal lethality of the majority of pups.[58] This discrepancy could be due to the differences in genetic backgrounds and experimental approaches. In contrast, its closely related paralog, KDM5A, is not an essential gene in mice,[57] although KDM5A loss in mESCs leads to downregulation of stem cell markers and activation of differentiation genes.[24,59,60] A KDM5C gene trap mouse model was reported to exhibit abnormal social behavior, consistent with the observation that it is a frequently mutated gene in patients with mental retardation and autism.[61] The H3K4me1 and H3K4me2 demethylase KDM1A is important for enhancer and ERV silencing in ESCs and essential for mouse embryonic development beyond E6.5 and extra-embryonic tissue development.[54]

*H3K27 demethylases.* On the H3K27me3 side, both KDM6A and KDM6B have been found to be required for proper lineage specification. Loss of KDM6A was reported to compromise mESC differentiation but not the self-renewal ability.[27,28,62,63] Although still capable of retinoic acid-induced differentiation,[63] KDM6A KO mESCs displayed impaired cardiac lineage differentiation,[27] and improper ectoderm and mesoderm formation.[28,29] Interestingly, while KDM6A-deficient females develop complete embryonic lethality, males only display partial embryonic lethality.[27,29,63–65]

Regarding KDM6B, there are discrepancies among different studies. In some studies, KDM6B was required for blastocyst development[66] and essential for embryo development.[30]

However, in other studies, KDM6B KO fetuses could survive until birth but with several defects in lung development,[67–69] possibly due to compromised regulation of the WNT pathway.[70]

*H3K9 demethylases.* H3K9 demethylases have also been shown to regulate ESC function. For instance, KDM3A was reported to demethylate H3K9me2 at the promoter regions of several pluripotency-associated genes and positively regulate their expression.[20] It also acts as a key modulator of cell fate decisions in terminal endoderm differentiation.[21] KDM3A directly regulates the promoter H3K9 demethylation and the expression of Sry, causing XY sex reversal.[71] In another study, the Zhang group showed that KDM3A knockout mice exhibit spermatogenesis defects and develop obesity in adulthood.[72] In addition, it has been reported that the H3K9me3 demethylases KDM4A and KDM4C are both required for mESC differentiation into endothelial cells.[22] KDM4A initiates differentiation by targeting the *Flk1* promoter, whereas KDM4C promotes endothelial cell fate by targeting the *VE-cadherin* promoter.[22] In addition, KDM4B and KDM4C are both required for ESC self-renewal. Mechanically, KDM4C has been shown to regulate Nanog expression,[20] while KDM4B regulates a different set of target genes,[23] indicating non-overlapping functions between the two paralogs. Moreover, another H3K9 demethylase, KDM7B, has also been reported to regulate mesodermal lineage commitment and cardiomyocyte differentiation of mESCs.[31]

*H3K36 demethylases.* As an accessory factor of PRC1 (Polycomb repressive complex 1), KDM2B was reported to be enriched at PRC1-repressed genes related to embryo development, morphogenesis and cellular differentiation in ESCs. In addition, depletion of KDM2B induced expression of a subset of these genes and, similar to PRC1 loss, caused a defect in embryoid body (EB) formation.[18,19] Interestingly, the function of KDM2B in ESCs is dependent on the non-methylated CpG island binding ability through its CXXC domain, but not the H3K36me2 demethylase activity.[18,19] Consistently, KDM2B prevents DNA hypermethylation of target CpG islands, and its loss leads to increased embryonic lethality, especially in females.[73,74] This may be because KDM2B is required for the coordinated expression of Xist, Tsix and Xist RNA-associated factors.[74] On the other hand, KDM2A, a closely related paralog but without a CXXC domain, is required for proper regulation of cell-cycle genes, and its loss leads to embryonic lethality at E10.5–E12.5.[75]

Many demethylases have been shown to be required for ESC pluripotency and embryonic development, suggesting that active demethylation plays critical roles in these processes. Interestingly, despite a high level of sequence similarities, subfamily members usually possess non-redundant roles, and their KO mice develop different phenotypes (Boxes 2 and 3).

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**Box 3 The reported phenotypes of KDM knockout animals**

| Name | Roles in early embryonic development |
|---|---|
| KDM1A | KO embryos display reduced size[15] and die after E6.5[11,12,15] |
| KDM2A | KO embryos display embryonic lethality at E10.5–E12.5[75] |
| KDM2B | KO embryos display increased lethality at E10.5, especially in females[73,74] |
| KDM3A | KO embryos display male-to-female sex reversal[71] |
| KDM5B | KO embryos display homeotic transformations of the skeleton, several neural defects and post-natal lethality;[58] KO resulted in early embryonic lethality[57] |
| KDM5C | Gene-trapped embryos display embryonic lethality[76] |
| KDM6A | KO female embryos display embryonic lethality, while males show a partial embryonic lethality[27,29,63–65] |
| KDM6B | KO embryos display lethality[30,66] or perinatal lethality[67–69] |

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## Demethylases regulating PGC development

Reprogramming of histone methylation is a critical process during mammalian germline development. It has been shown that the epigenome of early gonadal human PGCs is characterized by low H3K9me2 and high H3K27me3 levels.[77] Accordingly, various histone demethylases have been reported to be involved in this process.

KDM1B, an H3K4me2 demethylase, is required to establish maternal genomic imprinting.[78] In the same study, conditional deletion of KDM1A in growing oocytes resulted in precocious resumption of meiosis and spindle, and chromosomal abnormalities.[78] The reprogramming of H3K27me3 in E10.5–E11 primordial germ cells is safeguarded by KDM6A.[62] This study also reported that KDM6A-deficient PGCs showed aberrant epigenetic reprogramming *in vivo*, which led to germline transmission failure in mouse chimaeras generated from KDM6A KO mESCs.[62] Moreover, it has also been reported that the H3K9me2 demethylase KDM3A is critical for Tnp1 and Prm1 transcription, and mouse spermatogenesis.[79,80] Last but not the least, KDM2B has been reported to regulate the proliferation of spermatogonia and to ensure long-term sustainable spermatogenesis in mice.[81]

## Histone demethylation in oocytes

Embryogenesis starts with a fertilized oocyte, which undergoes multiple mitotic divisions and cellular differentiation without growing in size, leading to the development of a multicellular embryo. Due to the limitation in obtaining sufficient material for epigenomic analyses, the dynamic regulation of histone lysine methylation in this process had remained unclear until recent progress in small-cell-number ChIP technology.[82–84] Importantly, these studies uncovered a highly dynamic nature of histone methylation from the fertilized oocyte to ICM.

In differentiated cells, trimethylation of histone H3 at lysine 4 (H3K4me3) primarily forms sharp peaks (~1000 bp) at transcriptional start sites (TSSs);[85] however, two different kinds of 'broad' H3K4me3 domains have recently been discovered, and the involvement of KDM5A and KDM5B in regulating these broad H3K4me3 domains has also been illustrated. Specifically, one kind of broad H3K4me3 domain (hereafter type A) mainly occurs at TSSs and is positively correlated with transcription level in two-cell embryos.[82,83]

The other kind (hereafter type B), however, is not restricted to promoters and exists at a large number of intergenic loci[84] and coincides with gene silencing in metaphase II (MII) oocytes. Type A broad H3K4me3 domains have been shown to be inversely correlated with DNA methylation but positively correlated with transcriptional activity.[82] In contrast, type B broad H3K4me3 domains overlap almost exclusively with partially methylated DNA domains,[84] and their removal leads to improper activation of the target genes. Despite the different features of these two kinds of broad H3K4me3 domains, active removal of H3K4me3 by the corresponding demethylases, KDM5A and KDM5B, has been shown to be required for the integrity of both kinds of broad H3K4me3 domains and normal early embryo development.[82–84]

In addition to H3K4me3, global demethylation of H3K27me3 was also found at the two-cell stage right after fertilization and was then re-established asymmetrically at the maternal and paternal bivalent genes at the ICM stage.[84] The underlying mechanisms and the purpose of such global erasure and re-establishment are interesting subjects for future investigations.

## CONCLUDING REMARKS

In this review, we first reviewed the dynamic regulation by histone demethylases at characteristic chromatin regions and then the contributions of these demethylases to mammalian embryonic development processes, including ESC pluripotency, PGC formation and maternal-to-zygotic transition. Insights into these processes have accumulated rapidly in recent years. Along with technological advances, we can expect that more epigenomic features and the involvement of demethylation process, such as broad H3K4 domains in the oocyte and two-cell stage after fertilization and the global H3K4me3 and H3K27me3 demethylation after fertilization, will be revealed.

Future investigations of histone demethylases are needed to advance our understanding of their chromatin recruitment mechanism, as well as the crosstalk with cellular metabolic states and their roles in regulating cell fate decisions. In addition, demethylases targeting H3K79, as well as histone arginine methylation, remain to be discovered.

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## CONFLICT OF INTEREST

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