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Roles and regulation of histone methylation in animal development

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Abstract

Histone methylation can occur at various sites in histone proteins, primarily on lysine and arginine residues, and it can be governed by multiple positive and negative regulators, even at a single site, to either activate or repress transcription. It is now apparent that histone methylation is critical for almost all stages of development, and its proper regulation is essential for ensuring the coordinated expression of gene networks that govern pluripotency, body patterning and differentiation along appropriate lineages and organogenesis. Notably, developmental histone methylation is highly dynamic. Early embryonic systems display unique histone methylation patterns, prominently including the presence of bivalent (both gene-activating and gene-repressive) marks at lineage-specific genes that resolve to monovalent marks during differentiation, which ensures that appropriate genes are expressed in each tissue type. Studies of the effects of methylation on embryonic stem cell pluripotency and differentiation have helped to elucidate the developmental roles of histone methylation. It has been revealed that methylation and demethylation of both activating and repressive marks are essential for establishing embryonic and extra-embryonic lineages, for ensuring gene dosage compensation via genomic imprinting and for establishing body patterning via HOX gene regulation. Not surprisingly, aberrant methylation during embryogenesis can lead to defects in body patterning and in the development of specific organs. Human genetic disorders arising from mutations in histone methylation regulators have revealed their important roles in the developing skeletal and nervous systems, and they highlight the overlapping and unique roles of different patterns of methylation in ensuring proper development.

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All authors made substantial contributions to the discussion of content, wrote the manuscript and edited it before submission. A.J. and A.D. also researched data for the article.

Competing interests

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DNA is packaged inside eukaryotic nuclei by being wrapped around histone proteins, and this assembly of DNA and histones, together with associated non-histone proteins and RNA, comprises chromatin. DNA and histones can be modified by the attachment or removal of small chemical groups such as methyl or acetyl, which can regulate gene activation or repression. During development, these modification marks control the recruitment of transcription factors and/or RNA polymerase to ensure the proper expression of a highly orchestrated set of gene networks, as cells transition from the pluripotent state through multiple progenitor states to their final differentiated cell fate. Errors in establishing or maintaining proper chromatin modifications are often lethal during embryogenesis. Histone methylation has emerged as a particularly important modification during development, one involved in both gene activation and repression. Although much progress has been made in understanding the multiple roles of histone methylation during development, many of the precise mechanisms by which histone methylation regulates developmental events in response to intracellular and extracellular signals remain incompletely understood.

In this Review, we discuss the effects of histone methylation on gene activity and the factors that regulate histone methylation during development (for a discussion of DNA methylation, see REF.[1]). The overall effects of histone methylation regulators on different stages of embryogenesis and their roles in promoting the development of specific organ systems are reviewed. We also cover how histone methylation affects genomic imprinting and the regulation of HOX genes. Embryonic stem cells (ESCs) have provided valuable models for studying the effects of histone methylation on development, and the roles of different histone methylation regulators in promoting the maintenance of pluripotency and in driving differentiation are discussed. Finally, we discuss human genetic disorders caused by mutations in histone methylation regulators, which provide further insight into the critical roles of histone methylation in organismal development.

Histone methylation and gene activity

Early models of chromatin function hypothesized a histone ‘code’ or ‘language’ whereby combinations of different histone modifications — occurring either sequentially or simultaneously — would determine the activity of the associated gene[2]. Genomic techniques held promise for cracking the code by surveying a large number of modifications in a large number of contexts. Although a one-to-one correspondence between modifications and gene expression did not emerge, such studies[3–6] established general themes for the effects of histone modifications on gene expression.

The methylation of proteins involves the attachment of a methyl group to nitrogen atoms in amino acid side chains and/or at the amino termini. In histones, lysine (Lys or K) and arginine (Arg or R) residues serve as the most common acceptor sites of methylation marks, which have varying effects on gene activity depending on the specific residues that are modified, the degree and pattern of methylation, and the genomic context in which the methylation occurs (that is, the exact location of the modified nucleosome in the genome) (FIG. 1). Histone H3 is the primary site of histone methylation, although the other core histones display methylations as well.

Histone Lys methylation can exist in one of three states: mono-, di- or tri-methylation. Di- and tri-methylation at H3K4, H3K36 and H3K79 are typically gene-activating, with H3K4 tri-methylation (H3K4me3) marking promoters[3,7,8], and H3K36 and H3K79 methylations occurring primarily over gene bodies[9,10]. Mono-methylation of H3K4 is an activating mark unique to enhancers[11]. H3K9 and H3K27 methylations are generally gene-repressive[3,8] but serve unique functions. H3K27me3 is considered easily reversible[12] and marks dynamically regulated genes, and thus is especially important in development, when genes need to be switched on and off in a highly dynamic fashion in accordance with developmental signals. H3K9me3 is characteristic of heterochromatin[13–15], whereas H3K9me2 is found more commonly at silent or lowly expressed genes in euchromatin[13,15].

Generally, methylations at Arg show a greater complexity than do those at Lys, owing to the multiple nitrogen atoms in Arg. Three major forms of methyl-Arg have been identified in mammals, of which omega-N^G,N'^G-symmetric di-methyl-Arg (SDMA) is found on a small percentage of nuclear and cytoplasmic proteins, whereas omega-N^G-mono-methyl-Arg (MMA) and asymmetric di-methyl-Arg (ADMA) are more ubiquitous[16,17] (for an expanded discussion of Arg protein methylation, see REF.[18]). On mammalian histones, MMA and ADMA are the prevailing Arg methylations at sites H3R2, H3R17, H3R26 and H4R3 (REF. [17]). The association of histone Arg methylation marks with gene expression is poorly understood. Symmetric H3R8me2 (H3R8me2s) and H4R3me2s are generally associated with transcription repression[19], but emerging evidence suggests that these modifications might influence certain genomic loci differentially, with both activating and neutral effects of these methylations in specific settings[20,21].

Although many other aspects of chromatin structure contribute to its cumulative gene-activating or gene-repressive properties[22–24], the genomic studies mentioned above have established histone methylations as important activating and repressing chromatin marks (FIG. 1).

Histone methylation regulators

Histone modifications are regulated by chromatin ‘writers’ (methyltransferases, for methylation), which add modifications; ‘erasers’ (demethylases, for methylation), which remove modifications; and ‘readers’ (for example, chromodomain and bromodomain proteins for methylation), which recognize modifications and influence gene expression; all of these have important roles in governing the modifications present at each genetic locus and for their translation into gene-activating or gene-repressing events (FIG. 1). For a comprehensive review of all known histone modifications and their regulators, see REF.[25].

Multiple histone methyltransferases with varying activities have now been identified. Some generate multiple degrees or types of methylation, whereas others catalyse a single species. The catalytic activity of histone lysine methyltransferases often resides in the SET domain, first identified in the human and mouse H3K9 methyltransferase SUV39H1 (REF.[14]), and subsequently in many other histone Lys methyltransferases[26–30]. Notably, the H3K79 methyltransferase DOT1 and its homologues are the only histone Lys methyltransferases to lack a SET domain[31–33]. Most methyltransferases show a strong preference for specific

sites; for example, the SETD1/MLL family places gene-activating H3K4 methylation marks[34], whereas the PRC2 complex[35,36] places repressive H3K27 methylation marks[37,38]. The H3K9 methyltransferases show varying specificities for genomic regions: SUV39H1 and SUV39H2 regulate H3K9me3 at pericentric heterochromatin, whereas G9a (also known as EHMT2) and GLP (also known as EHMT1) regulate H3K9me1 and H3K9me2 in euchromatin[15,39]. Arg methyltransferases, in addition to showing site specificity, also show specificity for the type of methylation. Protein arginine methyltransferase 1 (PRMT1), the founding member of the class, ubiquitously catalyses the formation of MMA and ADMA in mouse tissues on histone and non-histone proteins[16,40] and accounts for the majority of mammalian Arg methylation[17]. SDMA is found on a small percentage of nuclear and cytoplasmic proteins and is catalysed by PRMT5 (REF.[41]). The third and least understood family of PRMTs is thought to catalyse the formation of only MMA and, so far, has only one putative member, PRMT7.

The specificities of many methyltransferases have been determined using antibody-based methods. Although in many cases the results agree with mass spectrometry data, in some cases contradictory results have emerged, possibly arising from nonspecific antibody binding[42–44]. Even then, antibody-based methods are preferred, due to their relative ease of use, ability to be used in single-cell imaging studies and ability to probe modification sites missed during trypsin cleavage for mass spectrometry-based detection of Arg and Lys methylation. The varying specificities of enzymes for different substrates, as well as the possible nonspecific binding of antibodies that recognize histone modifications, have made it challenging to decipher the precise functions of these enzymes and their products — the specific patterns of histone methylation — in vivo.

Histone methylation was considered stable until the report of lysine-specific demethylase 1 (LSD1) in 2004. LSD1 was initially found to demethylate mono-methylated and di-methylated H3K4 (REF.[45]), and later to act on mono-methylated and di-methylated H3K9, as well[46] (because of the chemical mechanism LSD1 employs, it cannot act on tri-methylated Lys). Soon after the report of LSD1, the jumonji domain class of demethylases was found to act on mono-methylated, di-methylated and tri-methylated Lys[47–50]. Eighteen demethylases of this class have now been identified[51]. Like methyltransferases, demethylases show specificity for the Lys residues they act on, although several show activity towards two or more substrates; for example, LSD1 demethylates H3K4 and H3K9 (REFS[45,46]), and KDM4A demethylates H3K9 and H3K36 (REFS[48,50]). To date, no Arg demethylases have been conclusively identified.

In vivo, histone methyltransferases and demethylases often operate in large protein complexes, and their genomic targets are often influenced by the presence of reader proteins or domains, which recognize various histone modifications. Although the members of a family of readers can recognize different degrees of methylation at different sites, each individual reader typically recognizes a single or a few closely related methylation marks. The plant homeodomain (PHD) fingers represent the largest class of readers, recognizing unmethylated or methylated Lys residues[52–56]. The Tudor domain shows greater versatility, binding both methylated Lys and Arg residues[57]. Reader domains can occur in writers and erasers or in their binding partners, and they can recognize the products or substrates of the

enzymes present together in the complex or marks generated by other enzymes that help target the complexes to appropriate genomic locations. For instance, BHC80 (also known as PHF21A), a member of the LSD1 demethylase complex, recognizes the reaction product of LSD1, unmethylated H3K4 (H3K4me0), and helps maintain LSD1 at H3K4me0 sites to protect H3K4 from re-methylation[52]. The SETD1 family recognizes its target sites, unmethylated Cpg islands, via the binding partner CFP1 (REF.[58]). Some chromatin regulatory complexes contain multiple readers that facilitate the integration of multiple histone marks[59–61]. In other cases, a single reader domain provides the desired specificity[62,63]. Thus, readers are essential for ensuring that initial chromatin modifications are translated into additional structural changes, and eventually into the activity of functional effectors to mediate and reinforce the appropriate gene expression networks[25].

Histone methylation in development

Histone methylation orchestrates developmental gene expression programmes beginning before fertilization and continuing into the postnatal period. Defects in histone methylation affect various developmental processes and can result in developmental arrest and lethality at different stages or lead to specific deficits in organ function in mature animals, depending on the nature and cell-type specificity of the methylation defect. Histone methylation regulators are generally ubiquitously expressed during development[64,65]. However, knockout experiments in mice and expression profiling studies have revealed that there are certain cell-type-specific and tissue-specific differences in the activity of histone methylation regulators: the tissues that are most affected in knockouts often show moderate to high expression of the regulator during normal development.

Analysis of individual methylation marks in early embryonic systems has revealed their unique distribution and functions in gene regulation. Notably, H3K4 methylation was widely present at transcription start sites in human ESCs[66], zebrafish[67] and mouse embryos[68], but it showed varying degrees of correlation with gene expression. In human ESCs, 80% of H3K4me3-marked genes were expressed[66], whereas in early zebrafish embryos (before the mid-blastula transition (MBT)), minimal expression of H3K4-methylated genes was observed[67] (likely because embryonic transcription is globally repressed by other mechanisms in zebrafish embryos at this stage). However, the H3K4me3-marked genes in zebrafish were enriched among the set of genes expressed following the MBT, suggesting that early deposition of H3K4me3 poised these genes for later expression[67]. Histone methylation patterns are clearly associated with the earliest stage of differentiation: the formation of the trophectoderm and the inner cell mass. In mouse embryos, H3K27 methylation was found at distinct sets of genes in these tissues, whereas H3K4 methylation sites were largely common between the trophectoderm and inner cell mass[68]. Neither the H3K4 nor H3K27 methylation patterns correlated especially well with gene expression, suggesting the presence of more complex regulatory mechanisms in vivo than those emerging from studies of differentiated cells[68].

During development, cells commit to specific lineages and must silence genes that promote pluripotency as well as those that determine alternative fates. Both H3K27 and H3K9 methylations contribute to this silencing. Comparison of H3K27me3 in ESCs and in

differentiated cells revealed a broadening of H3K27me3 domains in the differentiated cells[69,70]. Genes silenced in this manner included those encoding pluripotency factors, developmental factors and lineage-specific transcription factors[69]. Similarly, differentiated liver and brain cells harboured expanded H3K9me3 tracts relative to ESCs[71]. Genes silenced by H3K9 methylation in differentiated cells were additionally maintained in an inactive state by physical association with the nuclear lamina[70], which was shown to depend on H3K9 methyltransferase G9a in *Caenorhabditis elegans* embryos[72]. These data suggest the occurrence of progressive heterochromatinization during development and lineage specification. However, a recent study using mouse embryos showed few changes in the overall numbers of H3K9me3-marked genes when comparing the inner cell mass, the three germ layers at gastrulation and differentiated cells[73]. Instead, H3K9me3 was dynamically altered during development, such that lineage-specific genes became activated by the loss of H3K9me3, while pluripotency genes and those pertaining to other lineages gained this mark[73]. This study indicates that developmentally programmed heterochromatin reorganization, rather than an overall increase in heterochromatin, accompanies the progression of development. Although the exact mechanisms that govern the acquisition and readout of these methylation patterns remain to be fully determined, these studies cumulatively demonstrate the importance of repressive H3K9 and H3K27 methylations in promoting differentiation and lineage specification during development.

A unique aspect of developmental systems related to histone methylation is the presence of genes bivalently marked with both activating H3K4me3 and repressive H3K27me3 marks, which partially explains the weak correlations between gene expression and single H3K4 or H3K27 methylation marks discussed above. These marks typically occur in the promoters of lowly expressed genes in early embryos — before lineage commitment — that often encode developmental transcription factors such as the SOX, PAX and POU families[4,5]. During differentiation and lineage specification, cells lose one of the two marks in specific regions, resulting in gene activation or repression that is appropriate for the fate the cell will acquire. For example, neuronal differentiation led to the loss of H3K27me3 from neuronal gene promoters[4], whereas mouse embryonic fibroblasts retained this mark at these promoters but lost the activating H3K4me3 modification[5]. Genomic studies have revealed enrichments of bivalent marks at developmental genes in zebrafish[67], human ESCs[66,74] and mouse embryos[68]. By contrast, the genes with reduced H3K4me3 and H3K27me3 marks encoded proteins with functions in physiological responses, such as receptors and other proteins that respond to environmental stimuli[68,74]. Surprisingly, bivalently marked genes were not found in very early embryogenesis (in pre-MBT zebrafish and in pre-implantation mouse embryos)[75,76]. In mouse embryos, H3K27 marks, which were initially present in gametes, disappeared after fertilization and were re-established only post-implantation[77]. By contrast, maternal chromosomes in pre-implantation embryos contained broad, non-canonical tracts of H3K4me3 (REF.[77]), which in humans were correlated with open chromatin[78]. The non-canonical H3K4me3 tracts in mouse embryos were paradoxically associated with gene silencing[77]. These studies hint at the existence of a mechanism separate from bivalency to suppress gene expression in early embryos, but how such a mechanism operates and possibly interacts with bivalent marks remains elusive. Bivalent histone marks have also been documented at enhancers. Active enhancers display H3K4me1 together with H3K27

acetylation, whereas poised enhancers harbour H3K27me3 and H3K4me1 (primed enhancers lack H3K27 modifications but retain H3K4me1)[79,80].

Beyond the regulation of lineage fate decisions in early embryos, bivalent methylation marks also have other specialized roles during development. For example, several pluripotency-associated genes expressed in ESCs were silenced during differentiation by acquiring bivalent marks associated with their promoters[66]. Similarly, most bivalently marked genes in haematopoietic progenitor cells were shown to lose H3K4me3 and to become silenced upon differentiation. However, in progenitors destined to become erythrocytes, some bivalently labelled genes lost H3K27me3 and were activated upon differentiation, which correlated with the presence of additional histone marks: H3K9me1, H3K27me1 and H4K20me1 at the promoters and gene bodies. This led the authors to conclude that these marks confer activation potential to bivalent genes and that cell fate was predetermined before the onset of differentiation and could be predicted on the basis of the histone modification patterns present in progenitor cells[81]. Similar predictions about future gene expression could be made on the basis of histone methylation patterns in zebrafish embryos before zygotic gene activation[67,82]. Thus, although bivalently marked genes were initially thought to be a unique feature of early embryonic systems, it is likely that they play special roles in later stages of development and in specialized stem cell populations in adults.

Importance for animal development

The importance of histone methylation is conserved across animal development. Accordingly, as has been indicated by many studies throughout the years, the removal of various histone methylation regulators has profound effects on early embryogenesis, body patterning and organ development.

Histone methylation in whole-body development and body patterning.

The first indications of the importance of histone methylation in embryonic development came from studies in *Drosophila melanogaster*. Early genetic screens in *D. melanogaster* identified numerous genes required for embryo development — in particular, body patterning — many of which were later found to regulate histone methylation. Mutations in these components typically led to homeotic transformations (changes of one body segment into another). For example, mutations in Trithorax (Trx) — later shown to be an H3K4 methyltransferase[83] — resulted in transformations of the first and third thoracic segments towards the second[84], whereas heterozygous mutants of the Polycomb group (PcG) complex — the fly orthologue of the H3K27 methyltransferase complex PRC2 — developed extra sex combs on the limbs[85], which was linked to aberrant homeotic gene regulation[86,87]. Various PcG components were later identified in screens for body pattern regulation[88–90]. Surprisingly, demethylases were not found in these genetic screens, possibly because demethylases function redundantly (for example, KDM4A can substitute for KDM4B, and vice versa[91]) and/or because their depletion has a less pronounced impact on early development, owing to maternal contributions of RNA or protein (as seen, for example, for LSD1 (REF.[92])), thereby resulting in milder phenotypes. Such maternal effects have also been observed for histone demethylases in mammals[93–95]. It is also worth noting that not all

histone methylation regulators are required for *D. melanogaster* development. For example, loss of Su(var)3–9, an H3K9 methyltransferase[14,96], had minimal effects on fertility and embryogenesis, but it was required for position effect variegation[97], a process known to depend on chromatin structure.

Similar to their roles in *D. melanogaster*, many histone methylation regulators have critical roles in mammalian development (FIG. 2a), as revealed by extensive knockout studies in mice. These studies have largely focused on analysing the effects of embryonically expressed regulators; fewer studies have analysed the maternal contributions of these regulators, but those that have suggest that many regulators are indispensable for very early stages of development[94,98]. Notably, the timing, nature and extent of defects associated with embryonic knockouts are largely uncorrelated with the affected methylation site or whether the mark is activating or repressive. Overall, the effects of the removal of histone methylation regulators have complex aetiologies and likely arise from misregulation of specific sets of developmental genes and/or perturbations of other aspects of chromatin regulation (BOX 1).

Loss-of-function mutation of the methyltransferase *Setdb1* responsible for H3K9 tri-methylation was associated with the earliest embryonic lethality of all methyltransferase knockouts, occurring before embryo implantation (between embryonic day 3.5 (E.3.5) and E5.5 (REF.[99]). Loss of many other methyltransferases led to lethality at various stages of organogenesis (E6–E15), suggesting that these methyltransferases may have critical roles in promoting the development of specific organs (see also below). Notably, the loss of enzymes that regulate the same mark can have differential consequences. For example, in contrast to the early embryonic lethality associated with *Setdb1* mutations, double knockouts of a pair of related H3K9 methyltransferases, SUV39H1 and SUV39H2, did not display a fully penetrant requirement for unperturbed embryonic development, and these mice generated viable progeny at sub-Mendelian ratios[39] (TABLE 1). These results suggest that the SUV39 and SETDB1 methyltransferases regulate distinct sets of genes during development, but the nature of these differences has not been elucidated. Several other histone methylation regulators have also been shown to be dispensable for normal development (TABLE 1). Nevertheless, loss of these regulators still led to reduced viability after birth or to tissue-specific defects, suggesting tissue-context-specific functions of the different regulators and distinct roles for histone methylation marks in different tissues.

The roles of histone Arg methylation during mammalian development have been more challenging to decipher than those of Lys methylation, largely because of the broad specificity of PRMTs, which target histone and non-histone proteins (BOX 2) (see also REF. [18]). Some embryonic lethality was observed around E18.5 and E19.5 in *Prmt4*$^{-/-}$ embryos, which were significantly smaller than their wild-type counterparts[100]. At birth, all the *Prmt4*$^{-/-}$ pups showed reduced levels of H3R17 and p300 methylation and died due to a failure to breathe[101]. *Prmt5* (REF.[102]) and *Prmt1* (REF.[103]) homozygous mutant mice showed much earlier embryonic lethality, at E6.5. However, in *Prmt1* mutant cells, global protein methylation was markedly reduced[103], obscuring any contributions of histone methylation to this phenotype. Direct examination of changes in histone Arg methylation,

rather than relying on the analysis of methyltransferase mutants, will thus be required to unravel the chromatin-specific effects of Arg methylation in development.

Histone methylation in organogenesis.

Histone methylation regulators have many established roles in the development of specific organs (FIG. 2b). These roles have been identified by observing mouse full-body knockouts or tissue-specific knockouts designed to circumvent embryonic lethality at earlier stages (see above). Full-body knockouts revealed important roles for H3K4 methylation in embryonic haematopoiesis. Reports of MLL1 loss have documented a plethora of effects, including specific deficiencies in myeloid and lymphoid lineages[104–107], reduced overall haematopoiesis[104] and reduced haematopoietic stem cell (HSC) function[105]. LSD1 was also reported to be required at multiple stages of haematopoietic differentiation, and its activity was shown to repress critical targets of the haematopoietic transcription factors GFI-1 and GFI-1b[108,109]. Proposed functions of LSD1 include promoting granulocytic differentiation[108], impairing both the self-renewal and differentiation of HSCs[110] and enhancing production of precursor cell populations for specific lineages[111]. Additionally, H3K9 methylation mediated by G9a was shown to be involved in silencing pluripotency genes to promote HSC differentiation to mature lineages[112], consistent with the observed roles for H3K9 in inactivating pluripotency-associated gene expression programmes during lineage specification (see above). Although the H3K27 methyltransferase EZH2 was required for embryogenesis at a very early stage (FIG. 2a), a role for H3K27 methylation in haematopoiesis was suggested by knockouts of two PRC2 complex members: BMI1 in embryos, which were viable until birth but showed reduced haematopoietic cells[113], and EED in adults, which led to bone marrow failure[114]. At later stages of haematopoiesis, the recombinase RAG2, which contains a PHD finger reader domain recognizing the H3K4me3 and H3R2me2s marks[62,63], is essential for DNA recombination and the maturation of adaptive immune cells[115]. Although the exact roles of H3K4 methylation regulators are yet to be elucidated, it is clear that dynamic H3K4 methylation is required for successful haematopoiesis.

Dynamic regulation of H3K27 methylation has a role in cardiac development. Cardiomyocyte-specific knockouts of PRC2 components, which circumvented the requirement for PRC2 in haematopoiesis[113,114], led to multiple defects in cardiac morphology, including defects in separation of the heart chambers and myocardial hypoplasia, and were accompanied by the expression of non-cardiac genes[116,117]. Just as opposing H3K4 methylation and demethylation were required for haematopoiesis, the H3K27 demethylase KDM6B was required along with methyltransferase PRC2 for cardiac development, as shown in zebrafish, where the loss of KDM6B led to a lack of cardiomyocyte proliferation late in development[118]. Interestingly, loss of KDM6A led to much earlier defects in embryogenesis, during embryo patterning, suggesting non-redundant roles for these related H3K27 demethylases in organismal development[119]. Cardiac development was also highly sensitive to changes in H3K4 methylation, with the H3K4 mono-methyltransferase SETD7 being required for proper cardiac morphology[64] and the expression of cardiac-specific genes[65], suggesting roles for both activating and repressive methylation marks in forming cardiac structures.

Roles for multiple types of histone methylation have been observed in neurodevelopment. KDM6B promoted the differentiation of neuronal precursors in both the cerebellum[120] and olfactory bulb[121], and PRMT1 was required in neural crest cells for palate development[122]. Full knockout of the H3K4me3 demethylase KDM5C in some genetic backgrounds resulted in mice with neuro-developmental deficits and impaired cortical development[123,124]. Deletion of MLL1 in sub-ventricular zone neural stem cells (NSCs) did not affect embryonic development but did lead to postnatal lethality resulting from a reduced numbers of neurons[125]. NSC-specific knockout of both *Prmt1* and *Prmt5* also led to postnatal lethality[126,127], although the underlying mechanisms of their action differed greatly. PRMT5 was shown to control the differentiation and proliferation of NSCs by generating H4R3me2s to downregulate specific pro-mitotic genes[128] and promote proper mRNA splicing[126], resulting in neural progenitor cell depletion and decreased neuronal numbers, whereas *Prmt1* loss caused a large reduction in the number of mature oligodendrocytes, resulting in severe hypomyelination in the central nervous system[127]. Brain-specific knockout of *Mll1* early in development led to changes in H3K4 methylation patterns at superenhancers, resulting in increased proliferation of cells in the cerebellum and increased susceptibility to the development of medulloblastoma, which was traced to the role of MLL4 in activating superenhancers to stimulate the expression of tumour suppressor genes[129]. Thus, neurodevelopment is governed by various histone methylations that employ both transcriptional and post-transcriptional mechanisms to ensure the expression of appropriate developmental programmes.

The development of the reproductive system is also regulated by histone methylation. Knockout of the H3K9 demethylase *Kdm3a* in mice showed no effects on mortality[130,131] but caused a fraction of XY mice to develop into females[132], which can be linked to defects in spermatogenesis arising from misexpression of the sex determination gene *Sry* upon KDM3A loss of funcion[133]. Similarly, *Suv39h1* and *Suv39h2* double knockout mice also displayed deficits in spermatogenesis[39]. Finally, the founding member of the Tudor family of readers (encoded by *tud*), which recognizes H3R2me2s marks, was first identified in *D. melanogaster* more than three decades ago, on the basis of defective germ cell development in the progeny of mutant mothers[134].

Overall, it is now well established that haematopoiesis, cardiac development, neurodevelopment and reproduction are all importantly controlled by histone methylation regulators. This indicates that the establishment of precise patterns of histone methylation is required for the development of these different tissues, and it is likely that histone methylation is important for nearly all aspects of organogenesis. The molecular mechanisms by which the different histone methylations act and their functional outcomes are poorly conserved across tissues. Essentially, the same methylation type or enzyme can have vastly different effects depending on the context, which precludes predictions of the roles of each regulator and its associated mark in development. Recently, meta-analyses combined with machine-learning algorithms have provided a new approach for predicting the developmental functions of histone methylation marks[135].

Roles in developmental processes

It is now clear that histone methylation has a vast impact on animal development, but in many cases the exact underlying mechanisms are elusive. Here we will discuss three notable examples of developmental processes controlled by histone methylation in early embryonic systems: genomic imprinting, HOX gene expression and the regulation of pluripotency and differentiation programmes.

Genomic imprinting.

One of the earliest steps in development is the establishment of embryonic and extra-embryonic lineages. Genomic imprinting, which leads to mono-allelic expression of a gene by silencing one of the parental copies, is critical for this stage, by ensuring proper dosage of each gene product. This process is especially important for genes on the X chromosome, which exist in two copies in females and one in males. In females, gene expression from one copy of the X chromosome is silenced in *cis* by the long non-coding RNA *Xist*, which is expressed from the otherwise silenced X chromosome and recruits PRC2 to deposit gene-inactivating H3K27me3 marks[136]. On the active chromosome, *Xist* itself is silenced by PRC2-mediated H3K27me3 deposition[137]. Consequently, mice with mutated *Eed* — which encodes the essential PcG protein EED — were defective in X inactivation in the extra-embryonic lineages[138] and in pre-implantation embryos[139]. Genes on other chromosomes are also imprinted, and the prevailing model has been that one allele is silenced by DNA methylation[1], with or without co-occurring H3K9 methylation. DNA methylation and H3K27 methylation are mutually exclusive at one imprinted gene, *Rasgrf1* (REF.[140]). However, H3K27 methylation was recently shown to mediate imprinting at sites with low levels of DNA methylation[141], demonstrating the existence of two independent imprinting pathways (FIG. 3a). Approximately half of the active chromatin on the paternal DNA showed DNA hypomethylation on the maternal allele and contained H3K27me3 tracts[141]. Genes imprinted by this mechanism included *Sfmbt2* (REFS[141,142]), a PRC2 component required for the development of extra-embryonic lineages[143]. In the future, it will be important to understand how H3K27-mediated imprinting contributes to organ development and lineage specification in the embryo.

Regulation of HOX genes.

One critical and well-conserved role for histone methylation in development is in regulating expression of HOX genes. These genes are arranged in linear arrays, where the position of the gene controls its spatiotemporal expression patterns: genes at the distal ends of the clusters are typically expressed later in development and are restricted to caudal regions of the embryo[144,145]. Progressive activation of HOX genes corresponds to removal of H3K27me3 and appearance of H3K4me3 (REF.[146]). Later in development, HOX genes are silenced by methylation at H3K27 and H3K9 (REF.[69]) (FIG. 3b). Interestingly, recent studies have suggested that HOX genes are briefly devoid of H3K27 methylation after fertilization, but it is not known how they are kept inactive during this stage[76].

The effects of many genes discovered in the early *D. melanogaster* embryogenesis screens for developmental defects were traced to their roles in regulating HOX gene expression. *Trx*

mutant embryos showed reduced expression of the HOX gene *Ultrabithorax* (*Ubx*) concomitant with thoracic transformations. Overexpression of *Ubx* rescued these transformations[84], leading to the conclusion that Trx was a Hox activator[84,147]. *PcG* mutants, by contrast, led to expanded expression of Hox genes in embryonic regions in which they are normally not expressed[148,149], indicating a negative role for PcG in restricting Hox expression to specific body regions[149]. Combined mutations of *PcG* and *Trx* restored some of the body patterning defects seen in the single mutants, suggesting that the two regulators act in opposite ways[147]. Of note, later studies uncovered more complex interactions between Trx and PcG group proteins, in which the double mutants failed to completely rescue Hox expression in all regions of the embryo. The wing discs of double mutants expressed *Ubx* at elevated levels comparable to those in single PcG mutants, leading the authors to conclude that Trx proteins act as 'anti-repressors' to suppress PcG-mediated repression of gene expression, and that Trx is not otherwise required for Hox gene activation in all contexts[150]. Although the biochemical functions of Trx and PcG proteins were not understood at the time, these studies have demonstrated essential roles for both gene-activating (H3K4) and gene-repressing (H3K27) methylation in ensuring that appropriate Hox genes are expressed in each body segment.

Despite the variable effects of different types of histone methylation on mammalian development, their effects on HOX gene expression typically align with the activating or repressive features of each mark. Mouse embryos lacking the H3K4 methyltransferase MLL1, the homologue of *D. melanogaster* Trx, displayed embryonic lethality between E10.5 and E15 (REFS[104,106,151]), with defects in body patterning — abnormal branchial arches, mixed rostral–caudal neural patterning[152] and caudalization of Hox gene expression[153] — similar to its effects in flies[154]. Knockout of LSD1, the H3K4 demethylase, showed effects opposite to those of MLL1 at the molecular level, leading to premature or excessive expression of *Hoxb7* and *Hoxd8* (REF.[155]) and embryonic lethality at E7.5 (REF.[95]). Thus, it is clear that a balance of histone methylation and demethylation is also required for proper Hox gene expression and successful embryogenesis in mammals. However, in mammals, more complex roles for H3K4 methylation have been seen, owing to the presence of six H3K4 methyltransferases: SETD1A, SETD1B and MLL1–MLL4. These enzymes were individually required at various stages of embryogenesis, resulting in lethality between E8 and E15 in the individual knockouts[99,104–106,151,156–158]. Their distinct embryonic phenotypes supported their non-overlapping methylation patterns, including global H3K4 tri-methylation (SETD1A and SETD1B[159]), promoter-specific methylation (MLL1 (REF.[160])) and mono-methylation at enhancers (MLL4 (REF.[161])). Currently, the detailed mechanisms by which these locus-specific methylations ultimately govern HOX genes and other developmental regulators remain poorly understood. As in *D. melanogaster*, methylation dynamics at H3K27 are also required in mammals for successful embryogenesis and HOX gene regulation. Accordingly, mutations in the components of the PRC2 complex — which is the mammalian PcG homologue, consisting of the methyltransferases EZH1 and EZH2, along with the other regulatory components BMI1, EED, SUZ12, RBAP46 and RBAP48 (REF.[35]) — resulted in body-patterning defects in mice. Conditional knockout of *Ezh2* in specific cell types later in development — to circumvent early embryonic lethality in *Ezh2*-null embryos[162,163] by E6.5 (before body patterning) — led to mispatterned

(anteriorized)[164] or generally increased[165] expression of Hox genes. Both *Eed* and *Suz12* knockout mutations were lethal by E8.5 and E7.5, respectively, with major body-patterning defects such as reduced mesoderm and misexpression of key developmental regulators (brachyury, Evx1)[166–168]. *Bmi1* knockout mice, despite clear alterations in the proper establishment of Hox gene expression boundaries[169], were viable until birth, but died shortly afterwards with impaired haematopoiesis and subtle skeletal, neurological and haematopoietic defects[113]. These variable results suggest that PRC2 subunits may perform some functions independently of their roles in the methyltransferase complex, and/or that the complex may retain some functions during development in the absence of some subunits. As expected, the H3K27 demethylases KDM6A and KDM6B displayed effects opposite to PRC2 on Hox gene expression[170]. In zebrafish, loss of KDM6A resulted in posterior patterning defects and reduced expression of multiple Hox genes[119]. In mice, female embryos lacking KDM6A died before birth with defects in cardiac development and neural tube closure; however, the requirement for KDM6A for unperturbed development was partially compensated for in males by KDM6C (also known as UTY)[171], a catalytically inactive homologue of the enzyme present on the Y chromosome[119,172], which allowed survival until birth. The critical roles of KDM6A may involve the recruitment of other chromatin regulators instead of direct H3K27 demethylation (BOX 1), a function that KDM6C is expected to be able to fulfil. Loss of the catalytic activity of KDM6B delayed expression of posterior Hox genes and led to the anteriorization of Hox boundaries, with resulting skeletal abnormalities; however, the embryos were viable until birth, albeit with postnatal loss of viability[173]. Overall, the distinct phenotypes resulting from loss of biochemically equivalent H3K27 methylation regulators suggest that they likely have distinct genomic targets, and therefore act on non-overlapping sets of essential developmental genes.

Regulation of cell fate and specification.

The embryonic defects arising at the onset of organogenesis from aberrant histone methylation suggest that histone methylation potently affects cell fate decisions. To circumvent the obstacles of embryonic lethality and to study cell fate decisions directly, many studies have analysed the self-renewal or differentiation of ESCs in vitro. An emerging theme from these studies is that the regulators that are required early in development have roles in maintaining ESC pluripotency, whereas those acting during organogenesis or later tend to suppress or enhance ESC differentiation (FIG. 3c; see also FIG. 2). For example, SETDB1, an H3K9 methyltransferase that shows the earliest requirement during embryogenesis, was required for the maintenance of pluripotency in ESCs[99]. By contrast, regulators that are required later during embryonic development, such as the methyltransferases MLL1 (REFS[174,175]), MLL2 (REFS[174,176,177]), SETD1A[178], G9a[179] and PRC2 (REF.[180]), or demethylases such as KDM6A and KDM6B[181], were largely not required for the maintenance of pluripotency, but their loss compromised differentiation in vitro[174,177,179,182]. Loss of the demethylase LSD1 (REF.[183]) or KDM3A[184] promoted ESC differentiation, in accordance with their antagonistic relationships to the H3K4 and H3K9 methyltransferases. These results suggest that the stage at which a regulator acts during embryogenesis may be inferred from in vitro models of ESC maintenance and differentiation. Notably, however, not all methylation regulators showed this direct

correspondence between embryonic and ESC phenotypes. For example, PRMT4 (REF.[100]) supported pluripotency maintenance in human and mouse ESCs, likely by directly promoting ESC self-renewal, rather than by regulating pluripotency versus differentiation programmes as such[100,185,186]. A study separate from REF.[178] reported a similar role for SETD1A[187] in supporting ESC pluripotency. Also included in this group are regulators that promote ESC differentiation when they are lost (for example, LSD1 (REF.[183])). These regulators shift the cell fate decision away from self-renewal, and thus appear to be required for the maintenance of pluripotency via affecting differentiation programmes, which recapitulates their embryonic roles. KDM4C, whose embryonic phenotype has not been investigated, was reported in separate studies to both promote[188] and inhibit[184] differentiation, a discrepancy that has not been resolved. Thus, although ESC-based studies have been very powerful in elucidating the developmental functions of chromatin regulators, such exceptions, along with discrepancies in histone methylation patterns between ESCs and pre-implantation embryos[76], highlight the imperfect correspondence between in vitro and in vivo studies.

Another complication in studying the role of histone methylation in cell fate determination stems from the fact that individual histone marks and their regulatory enzymes often show distinct effects on ESC differentiation towards different lineages. For example, MLL4 loss led to deficits in differentiation towards endoderm, an increase in mesodermal markers, but relatively few effects on differentiation into embryoid bodies and ectoderm[189]. As another example, PRMT6 overexpression induced endodermal markers but had varying effects on mesodermal and ectodermal markers, whereas its depletion led to varying increases in markers of all three lineages[190]. Furthermore, these effects on differentiation due to changes in the expression of methylation regulators in many cases do not correspond to the effects observed for other related and/or seemingly functionally equivalent enzymes. For example, both KDM4C and KDM3A remove repressive H3K9 methylations (tri-methylation and di-methylation, respectively), yet the gene expression programmes in knockout ESCs showed distinct differences, which corresponded to the differentiation towards different lineages: endodermal in *Kdm4c* knockout cells, and mesodermal in *Kdm3a* knockout cells[184]. In addition, an independent study showed no deficits at all in self-renewal of *Kdm3a* knockout ESCs[191]. These differences may arise from localization of the KDM4C and KDM3A substrates to different loci, with H3K9me3 localizing to pericentric heterochromatin and H3K9me2 localizing to euchromatin[13,15]. The details of how these distinct marks lead to differences in lineage specification remain to be elucidated. Similarly, SETD1A and MLL2 both methylate H3K4, but only SETD1A appeared to promote methylation during ESC differentiation, in part by regulating the *Hoxa* locus[178] via the methylation of two enhancers[187]. The loss of WDR5, which forms a complex with SET1D1A (and SET1D1B), showed effects opposite to those of MLL2 loss, leading to impaired ESC self-renewal[192] in contrast to impaired differentiation. Finally, MLL2 was required for establishing methylation at bivalent genes in ESCs, whereas the highly related MLL1 showed a weaker requirement[174]. Although the finding is in contrast to the late-stage lethality of MLL2 knockout embryos (FIG. 2a), the involvement of MLL2 in promoting bivalent methylation marks in ESCs is consistent with observations that embryos lacking maternal MLL2 arrest by the two-cell stage[98], whereas MLL1 is required for development only much later, at the

onset of haematopoiesis[104,151]. In summary, owing to these complex effects of methylation marks and the enzymes that regulate them, a description of a unifying developmental histone code has been so far inaccessible.

Relevance to human genetic disorders

Human disease-associated mutations in genes encoding histone methylation regulators (TABlE 2) have highlighted the importance of dynamic regulation of histone methylation in skeletal and neurodevelopment. Mutations in the H3K4 methyltransferase genes *SETD1B* and *MLL1–4* have all been implicated in syndromes characterized by skeletal and facial abnormalities and intellectual disability, typically involving reduced body size and microcephaly[193–199]. Mutations in the H3K4 demethylase-encoding genes *KDM5C*[200–202] and *LSD1* (REFS[203,204]) or in the gene encoding the LSD1 binding partner PHD finger protein 21A (*BHC80*; also known as *PHF21A*)[52,205] resulted in similar syndromes, characterized by intellectual disability and skeletal defects. Notably, mutations in both *MLL4* and its antagonist *LSD1* have been associated with Kabuki syndrome[197,198,203,204], revealing that opposing activities at a molecular level can cause similar outcomes at an organismal level. Dynamic methylation at H3K9 is also required for proper skeletal and neural development. Mutations in both the H3K9 demethylase-encoding *PHF8* (REFS[206,207]) and the methyltransferase-encoding *GLP* were associated with craniofacial defects and intellectual disability in both humans[208–211] and animal models[212,213].

In contrast to the above mutations that cause reduced skeletal and brain size, mutations in other histone methylation regulators can lead to overgrowth syndromes. For example, mutations in *EZH2* and *NSD1* — which encode H3K27 and H3K36 methyltransferases, respectively — lead to overgrowth pathologies typical of Weaver, Sotos, and Beckwith–Wiedemann syndromes[214–217]. However, mutations in *NSD2*, another H3K36 methyltransferase-encoding gene, led to growth delays, indicating non-redundant, or even opposing, roles for these related enzymes. Beyond the differences between the enzymes, it should also be considered that germline and somatic mutations can result in divergent phenotypes. This seems to be the case for EZH2 and NSD1, which are often overexpressed in cancers[218–220], despite their loss of function in embryos being associated with overgrowth as discussed above, and even in cancer predisposition[221].

In the only known example of a clinical phenotype associated with genetic disruption of Arg methyltransferases, loss-of-function mutations in *PRMT7* were associated with mild intellectual disability along with obesity and poor development of bones[222]. A recent large-scale sequencing study showed many mutations in histone methyltransferases and demethylases linked to developmental delays[223], suggesting that the full scope of involvement of these enzymes in embryonic and postnatal development in humans has yet to be explored.

Analyses of disease-associated mutations in histone methylation regulators have provided limited insights into the molecular mechanisms underlying the associated pathologies. Many of the reported mutations disrupt or reduce the activity of the corresponding enzymes[193,194,197,205,214,224], interfere with their binding to complex members or to

DNA[194,214], or reduce their expression at the RNA or protein level[205,225]. It is not known whether truncated or catalytically inactive forms of the mutant proteins have functional roles, although the variations in disease presentation caused by different mutations (for example, in *NSD1* (REFS[215–217])) suggest that these mutant proteins may have non-catalytic effects. In some cases, phenotypes have been linked to misregulation of a few critical genes. For example, *MLL2* mutations led to reduced expression of intellectual disability-associated genes such as *THAP1* and *TOR1* (REF.[194]). A mouse model of Kleefstra syndrome, driven by *Glp* mutation, showed increased rather than decreased H3K9me3, as would be expected on the basis of the function of GLP as an H3K9 methyltransferase. It was then proposed that a concomitant reduction in expression of protocadherins underlies some of the phenotypes of *Glp* mutants[213]. Although mouse knockouts have served as faithful models of disease in some cases[213,226], many human genetic disorders are not recapitulated in mouse models. For example, the loss of H3K4 methyltransferases in mouse results in haematopoietic rather than skeletal or neural disorders, although one report showed that heterozygous *Mll4* knockouts display smaller body size, similar to human patients with *MLL* mutations[198]. Further characterization of the functions of histone methylation regulators in various organs and cell types in humans — for example, using organoid systems — may begin to uncover the molecular mechanisms by which their deregulation causes disease.

Concluding remarks

Studies in embryonic model systems laid the foundational basis for the field of epigenetics five decades ago, yet many mysteries still remain regarding the roles and regulation of epigenetic marks during embryonic development. It is clear that histone methylations have important functions throughout development in nearly all cell and tissue types. Key roles for histone methylation in promoting body patterning via HOX gene regulation are well-conserved across species, and histone methylations additionally regulate other developmental gene networks. However, different chromatin regulators often have distinct roles during development even when acting on the same modification; thus, obtaining a full understanding of their functions will require integrating their effects at different genomic loci — for example, promoters, enhancers, heterochromatin and individual genes — and in different cellular contexts with the phenotypic effects on development. Analyses of chromatin in specific embryonic tissues have begun to shed light on some of these differences. In addition, ESCs have provided powerful alternative models for studying the effects of chromatin regulators on maintaining pluripotency and promoting differentiation along diverse lineages.

Although genomic studies have defined overarching histone methylation patterns during development, direct causal relationships between methylation events and the formation of embryonic structures or mature organs have been difficult to define. In many cases, methylation has critical effects at multiple genetic loci, making classical reverse genetic studies focused on the regulation of individual genes difficult to interpret. Furthermore, bulk analyses can mask the heterogeneity of methylation patterns and their outcomes observed across cell types and developmental stages. Whereas imaging analysis can provide useful single-cell information, further advances in molecular techniques will be needed in order to define how methylation patterns influence gene expression in a tissue-specific manner.

Determining the relative contributions of promoter and enhancer regulation by methylation, as well as how histone methylations interact with other aspects of chromatin structure, such as nucleosome positioning and 3D genome architecture, will help to further define the molecular mechanisms by which histone methylations operate in development. Studies of early embryos in mouse and zebrafish have suggested the existence of unique mechanisms of gene regulation. For example, zygotic transcription in zebrafish is suppressed despite the presence of activating marks at some genes[67], and mouse Hox genes transiently lose repressive H3K27me3 marks after fertilization yet maintain gene silencing[76]. Determining how these alternative modes of gene repression operate and how they interact with histone methylations will be important for fully understanding the regulatory mechanisms of gene expression in embryos. Finally, although much progress has been made in understanding the roles of histone Lys methylation, our understanding of histone Arg methylation lags behind. Comprehensive mapping of Arg methylation across developmental stages and tissue types will be an essential first step towards understanding its role in development.

Overall, dissecting the roles of various histone methylation regulators at each stage of embryogenesis, using conventional genetics combined with conditional knockout studies and analyses of ESCs, will help us understand the overlapping and unique features of human genetic disorders involving aberrant histone methylation, and eventually will allow the developmental histone code to be cracked.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 |

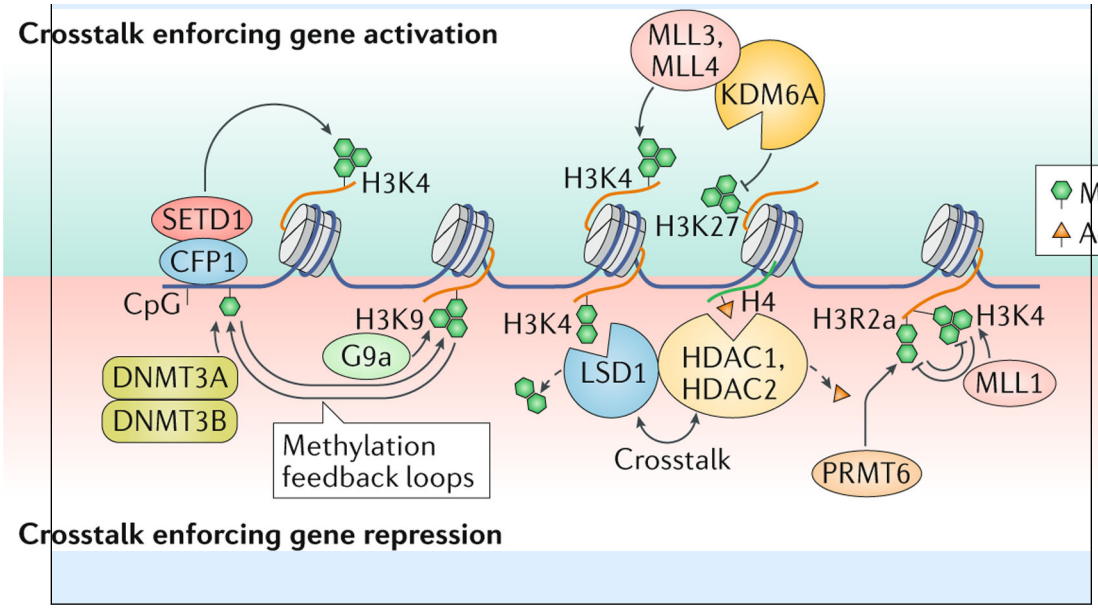
crosstalk between chromatin marks

Chromatin is typically marked by multiple modifications, and it is essential that these marks work in concert to achieve a coordinated cellular response (see figure). in some cases, the presence or absence of one modification can stimulate or inhibit deposition of another. For example, during neuronal differentiation, PRMt6-mediated catalysis of asymmetric arginine di-methylation at histone H3 (H3r2me2a) — by affecting recruitment of the relevant methyltransferases — precluded H3 methylation at lysine 4 (H3K4) at promoters, but stimulated it at enhancers, which was required for proper induction of neuronal gene networks[227]. reciprocally, H3K4 tri-methylation (H3K4me3) precluded PRMt6-mediated H3r2 methylation[228]. a similar interplay was observed with histone methylation (Me) and acetylation (ac): demethylation of H3K4 by LSD1 was stimulated by the removal of acetylation by histone deacetylase 1 (HDaC1) and HDaC2, which are LSD1 binding partners[229,230]; reciprocally, deacetylation was stimulated by H3K4 demethylation[229]. this positive feedback loop was shown to be required for maintaining pluripotency in embryonic stem cells (ESCs)[231].

several chromatin regulators have important catalytically independent roles in setting chromatin modifications, often by recruiting other enzymes. PRC2 can be recruited by both PRMt6 (REF.[232]) and SETDB1 (REF.[233]) to appropriately govern cell-type-specific gene repression programmes. similarly, LSD1 was recruited to enhancers by MLL4 in a catalytically independent manner to suppress pluripotency genes[234]. the H3K27 demethylase KDM6a forms important associations with multiple chromatin regulators. For example, KDM6a–MLL4 complex[235,236] led to a coordinated increase in H3K4 methylation and decrease in H3K27me3 at certain HOX loci[237]. Notably, H3K4me3 preceded the H3K27 demethylation, suggesting the importance of temporal coordination of these events237. in other cases, KDM6a had a purely scaffolding role, recruiting MLL4 and p300 to activate enhancers in ESCs with H3K4me1 and H3K27 acetylation[238], or recruiting chromatin remodellers to cardiac enhancers in order to drive cardiac differentiation of ESCs[119,172]. the latter function could be partially supplied by the H3K27 demethylase KDM6C[182]. similar roles in recruiting chromatin remodellers were reported for KDM6a and KDM6B, which were required for proper gene expression in mature T cells[239].

Histone methylations are also highly coordinated with DNA methylation through multiple feedback loops. in ESCs, unmethylated H3K4 acted to promote gene repression by providing a binding site for the recruitment of DNA methyltransferase 3a2 (DNMt3A2)[240]. Conversely, unmethylated DNA at CpG islands activated genes via the recruitment of CFP1, a member of the SETD1 complexes that deposit gene-activating H3K4 methylation[58]. similarly, H3K9 and DNA methylation show strong cooperation. in ESCs, the H3K9me3 reader HP1 (REF.[241]) recruited DNMT3B[242], while MBD1, a DNA methylation reader, recruited the H3K9 methyltransferase SETDB1 (REF.[243]), thereby coordinating the two methylation marks to enforce gene silencing. accordingly, proper DNA methylation and the suppression of aberrant gene expression programmes were

shown to require the H3K9 methyltransferases SUV39H1, SUV39H2 (REF.[242]) and G9a[244].



Box 2 |

Non-histone protein methylation in development

Although rich in lysine (Lys) and arginine (Arg) residues, histone proteins form a small fraction of the known protein methylome. soon after the discovery of histone Lys methylation[245], numerous groups reported the presence of methylated Lys and Arg residues in non-histone proteins involved in transcription regulation, signalling pathways, DNA damage response and RNA processing[246,247]. in the first example of methylation-dependent regulation of non-histone proteins, SET7 was shown to mono-methylate transcription initiation factor TFIID subunit 10 (TAF10) and p53, with transcription-stimulatory effects[248,249]. the p53 protein is required for the proper development of organs in the renal, musculoskeletal and nervous systems[250–254], but how methylation regulates the activity of p53 in the development of these systems has not been investigated. since then, multiple transcription factors have been shown to be regulated through methylation, with some having direct effects on mammalian development. EZH2-mediated methylation of GATA4 at Lys299 attenuates the activity of GATA4 as a transcription factor and is essential for proper cardiogenesis[255]. similarly, SMYD1 methylates the putative kinase TRB3, which activates its function as a transcription co-repressor to downregulate anti-proliferative and autophagy-related genes in embryonic cardiomyocytes[256].

Importantly, complete knockout of methylation regulators often fails to delineate the contributions of histone versus non-histone protein methylation towards developmental programmes. in analysing histone versus non-histone modifications, it has to be considered that many chromatin readers also recognize non-histone substrates, which could have important roles in development. For example, tudor domain proteins are involved in many aspects of cytoplasmic and nuclear RNA regulation, and they are critical for gamete formation and other stages of development (reviewed in REF.[257]). recent genetic studies in *Drosophila melanogaster* revealed that mutations in some histone residues that are targeted by methyltransferases, such as histone H3 Lys36 (H3K36) and H3K27, closely mimicked the phenotypes caused by mutations in their methyltransferases[258], pointing to a pivotal role of these methylation sites in embryogenesis. By contrast, other sites, such as H4K20, were shown to be dispensable for fly development, unlike the H4K20 methyltransferase PR-set7 (SET8)[259], suggesting essential non-histone targets of this enzyme. in a different approach, introducing mutated elongation factor 1 alpha 1 (EF1α1) in which lysines were substituted for alanines in chick embryos elucidated the importance of EF1α1 methylation in neural crest development[260].

Large-scale mass spectrometry studies have shown that the scope and complexity of Arg methylation is greater than that of Lys methylation, and that non-histone Arg methylation affects the activity, localization and protein–protein interactions of several signalling proteins involved in development[18,247,261]. the first example of the role of non-histone arg methylation in transcription regulation was provided in 2001, when it was shown that methylation of the transcription co-activator CBP by CARM1 reduces the association of CBP with its binding partner, caMP-dependent transcription factor CreB, which results in

a decrease in caMP-dependent gene expression[262]. so far, more than 100 substrates of CARM1 have been identified, many of which are involved in RNA processing[263]. For example, methylation of p54nrb (also known as NONO) by CARM1 is critical for decreasing its binding to mRNAs containing inverted-repeat Alu elements, thereby reducing their nuclear retention[264]. recent studies in mouse embryos suggest that this methylation event, along with CARM1-mediated methylation of H3r26, is critical for the establishment of the embryonic and extra-embryonic lineages during early embryogenesis[265]. these studies corroborate and provide mechanistic insights into the small size and perinatal death that was observed decades earlier in CARM1 knockout embryos or embryos containing a catalytically dead knock-in of CARM1 (REFS[101,266]).

Despite these insights, our understanding of the contributions of hundreds of non-histone protein methylation events to mammalian development still remains elementary. Future studies utilizing gene-editing techniques to install site-specific mutations targeting methylated residues should help elucidate the molecular basis of this versatile modification in mammalian development.

Genomic imprinting

Monoallelic expression of a gene specifically from either the maternal or paternal copy.

HOX genes

Genes encoding homeodomain-containing developmental transcription factors that are arranged in linear arrays and expressed in a spatially and temporally regulated manner corresponding to their one-dimensional arrangement along the chromosome.

Chromodomain

Protein domain that binds methylated lysine.

Bromodomain

Protein domain that binds acetylated lysine.

SET domain

Protein domain that typically harbours catalytic methyltransferase activity.

Plant homeodomain (PHD) fingers

Zinc-finger-containing domain involved in recognizing histone modifications.

Tudor domain

Protein domain that recognizes methylated lysines and arginines.

CpG islands

Regions of the genome with elevated frequency of Cpg dinucleotide, often occurring in gene regulatory regions and often displaying DNA hypomethylation on cytosine.

Mid-blastula transition

Embryonic stage at which cells in the blastula switch from rapid cycling between S and M phases to lengthened cell cycles including G1 and G2 phases.

Trophectoderm

Cells forming the outer layer of the mammalian embryo that later give rise to the placenta.

Inner cell mass

Cluster of undifferentiated cells in the mammalian embryo that give rise to the fetus.

Poised enhancers

enhancers that contain H3K4me1 and H3K27me3 marks and are unable to activate gene expression but that remain capable of activation in the future.

Primed enhancers

An enhancer state that is intermediate between poised and active states. Such enhancers are characterized by H3K4me1 marks and DNA hypomethylation but are unable to activate gene expression.

Zygotic gene activation

Activation of transcription from the genome of the embryo, accompanied by clearance of maternal transcripts.

Sex combs

In *Drosophila melanogaster*, a set of male-specific bristles on the leg.

Position effect variegation

Variation in gene expression levels based on the surrounding genomic context of the gene.

Neural crest cells

embryonic cells that arise from the ectoderm and give rise to multiple tissues, including craniofacial structures and peripheral nerves.

Sub-ventricular zone

Region of the brain lining the ventricles that generates neural and glial precursors.

Medulloblastoma

Paediatric brain tumour, believed to originate from primitive (undifferentiated) neuro-ectodermal cells, that most commonly arises in the cerebellum during the first decade of life and accounts for approximately 10% of primary brain tumours in children.

Alu elements

Short stretches of DNA containing the Alui restriction site that are repeated millions of times throughout the human genome.

Embryoid bodies

Aggregates of pluripotent cells that contain cells differentiating towards each of the three germ layers.

Microcephaly

Reduced head circumference.

Protocadherins

Family of cell adhesion proteins important for the development of neurons.

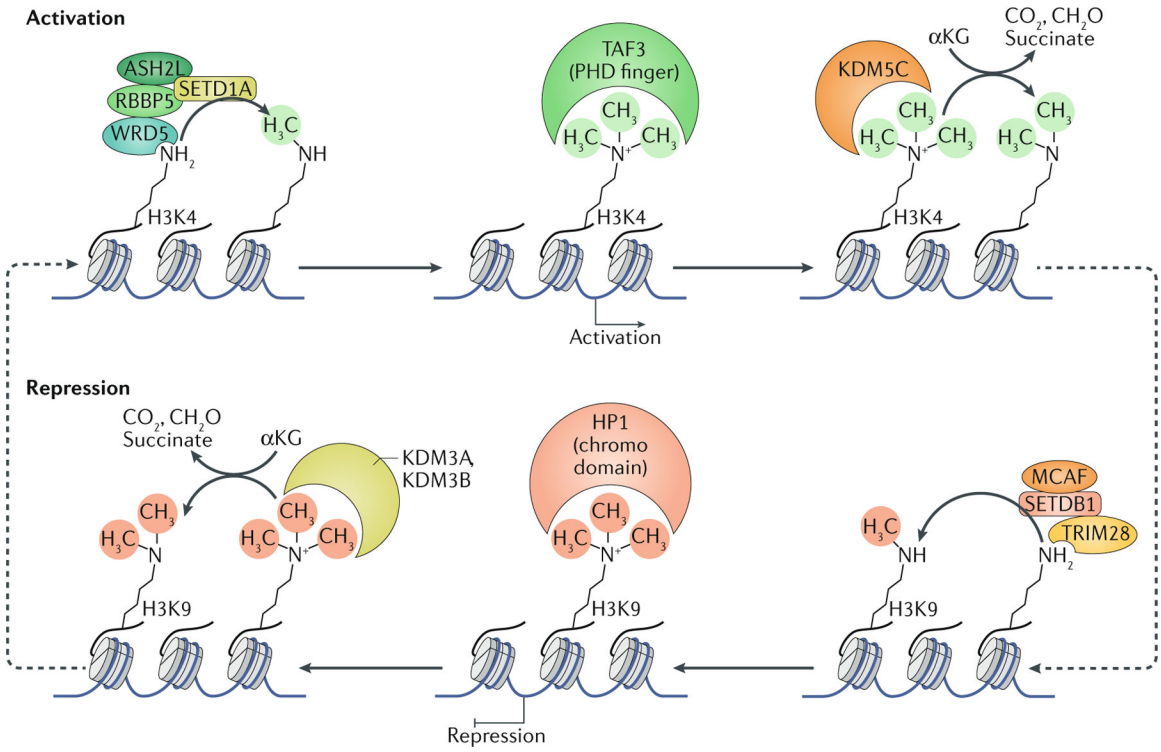


Fig. 1 |. Examples of regulation of gene expression by histone methyltransferases and demethylases.

SETD1A methyltransferase complex (comprising SETD1A (a catalytic SET-domain subunit), together with the binding partners ASH2L, RBBP5, WDR5 and other complex-specific subunits not shown) deposits the gene-activating H3 Lys4 tri-methyl (H3K4me3) mark at the promoters of various genes. H3K4me3 is recognized by PHD finger domains in proteins such as TAF3, which bind to methylated Lys. Gene activation can be reversed through the removal of this modification by the demethylase KDM5C, which utilizes α-ketoglutarate (αKG) as a cofactor. Gene-repressive states can be established by the deposition of H3K9me3 by the SETDB1 histone methyltransferase complex (including the catalytic subunit SETDB1 together with a regulator, MCAF (also known as ATF7IP) and a reader protein, TRIM28). H3K9me3 is recognized by the chromodomain in HP1 proteins and can be removed by the KDM3A and/or KDM3B demethylase in the presence of αKG as a cofactor, to allow for gene activation.

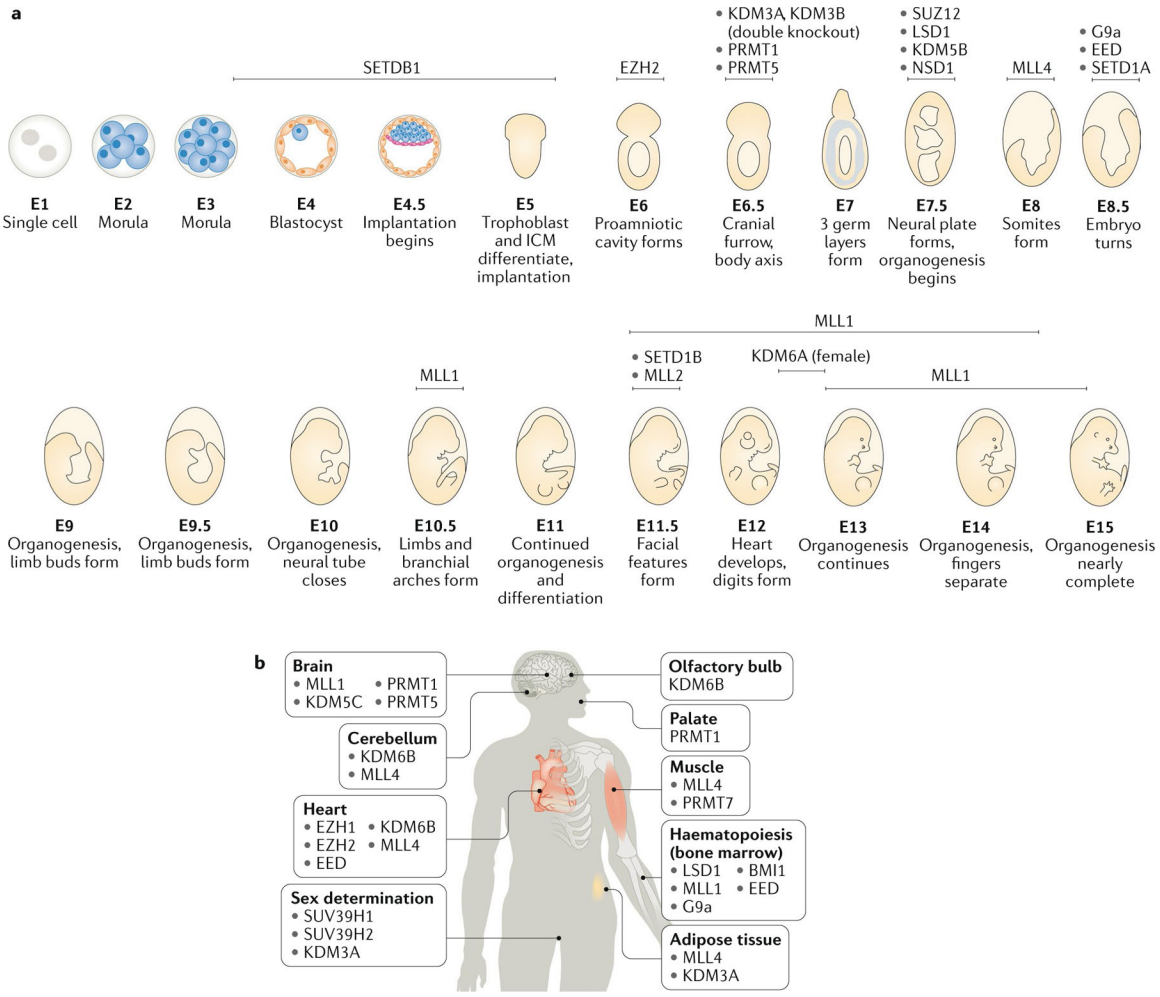


Fig. 2 |. The importance of histone methylation regulators in mammalian development and organogenesis.

a | Mouse developmental stages at which null alleles of the indicated histone methylation regulators exhibit embryonic lethality. Loss of some histone methylation regulators causes very early lethality, before or during implantation (for example, SETDB1), whereas other regulators are required at later stages of organogenesis, with the majority exhibiting lethality between embryonic day 7 (E7) and E12. For some regulators (MLL1, SETDB1), lethality was observed at different stages, depending on the report, in which case all reports are shown. For references to the relevant reports, see Supplementary Table 1. **b** | Tissue-specific sites of action of histone methylation regulators, as revealed by conditional knockout analysis or by analysis of viable systemic knockouts in mice. Many regulators are essential for neurodevelopment and cardiac development, whereas others regulate myogenesis, adipogenesis and haematopoiesis. For references to the relevant reports, see Supplementary Table 2. ICM, inner cell mass.

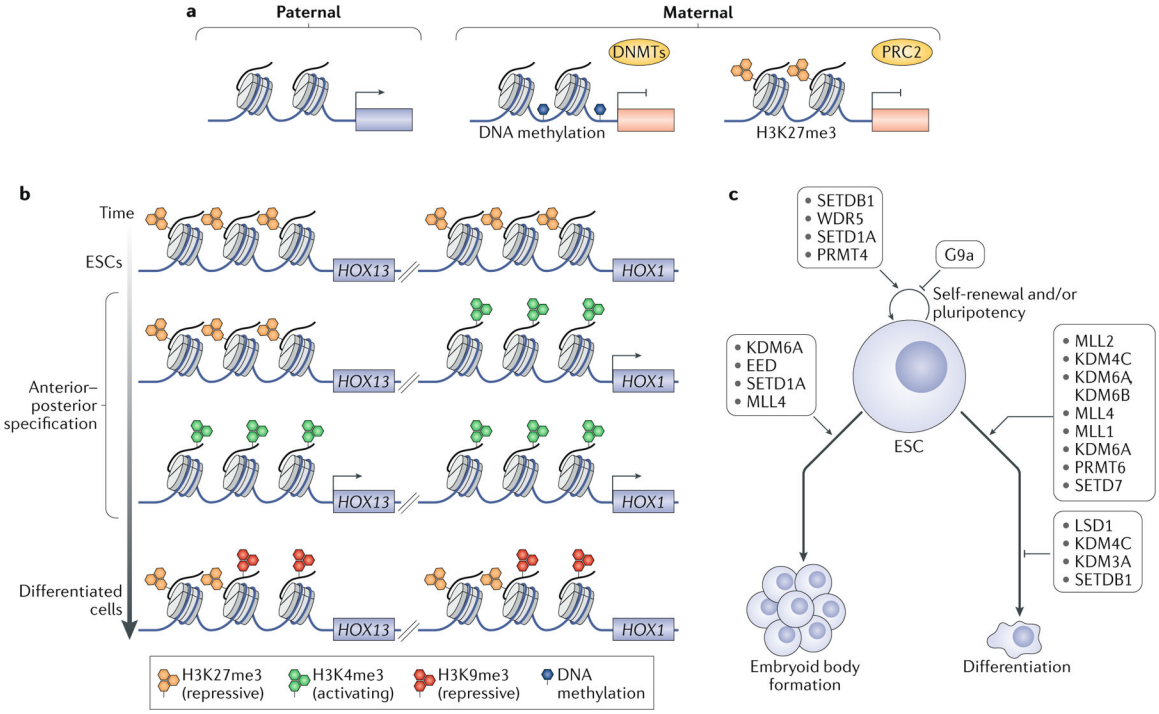


Fig. 3 |. Developmental processes regulated by histone methylation.

a | Genomic imprinting is mediated by both histone and DNA methylation. Paternally expressed genes display undetectable or very low levels of repressive DNA and H3K27 methylation marks. The maternal allele is silenced either by DNA methylation, introduced by DNA methyltransferases (DNMTs), or, in regions of hypomethylated DNA, by tri-methylation of Lys27 of histone H3 (H3K27me3), introduced by the PRC2 complex. The predominant mechanism for the silencing of imprinted genes varies by the locus. **b** | Regulation of HOX genes by histone methylation. In embryonic stem cells (ESCs), histone tri-methylation at H3K27 represses all HOX genes. At later stages of differentiation, early HOX genes are activated along the anterior–posterior axis by removal of H3K27me3 and addition of H3K4me3. Subsequently, late HOX genes are activated in caudal regions by a similar mechanism. In differentiated cells, HOX genes are again repressed by tri-methylation at H3K9 and H3K27. It is not known whether these methylation marks occur on the same or separate nucleosomes. **c** | Effects of histone methylation regulators on ESC self-renewal and differentiation. Although some regulators directly influence ESC self-renewal (SETDB1, WDR5, SETD1A, G9a), most affect the ability of ESCs to differentiate (as assessed by using embryoid body formation assays, which indicate the ability to form the three germ layers, or by various differentiation-inducing protocols (including those that direct ESCs towards a particular lineage)). In cases where different studies have indicated divergent roles, all outcomes are listed. For references to the relevant reports, see Supplementary Table 3.

Table 1 |

Histone methylation regulators not essential for development

Regulator gene	Phenotype	Refs
Nsd2	Lethal at P1	267
Kdm3b	Lethal at P1	268
Prmt4	Partial embryonic lethality, fully lethal at P1	100,101
Kdm6b	Lethal by P18	173
Bmi1 (encodes PRC2 complex component)	50% lethal between P1 and P3, with further lethality at 3–20 weeks	113
Kdm6A (males)	Reduced viability after birth, runted body	171
Suv39h1 and *Suv39h2* (double knockout)	Viable at sub-Mendelian ratios	39
Kdm3a	Viable, obese	131
Prmt7	Viable, reduced muscle-to-fat ratio	226
Prmt6	Viable	269
Kdm5c	Viable, neurological deficits	123
Ezh1 (encodes PRC2 catalytic subunit)	Viable	270

P, postnatal day.

Table 2 |

Histone methylation regulators associated with inherited genetic disorders in humans

Gene	Mutation	Syndrome	Refs
MLL1	Point mutations	Wiedemann–Steiner syndrome	193
MLL2	Point mutations	Dystonia, skeletal abnormalities, intellectual disability	194
MLL3	Point mutations	Kleefstra syndrome, autism spectrum disorder	195
MLL4	Point mutations	Kabuki syndrome	197,198
SETD1B	Point mutations	Seizures, developmental delay, intellectual disability	199
LSD1	Point mutations	Kabuki syndrome	203,204,224
BHC80 (also known as *PHF21A)*	Deletion[a], translocation	Potocki–Schaeffer	205
GLP (also known as *EHMT1)*	Translocation, microdeletion[a]	Kleefstra syndrome	211,271
KDM5C	Point mutations	Skeletal abnormalities, X-linked intellectual disability	200–202
PHF8	Point mutations	Craniofacial abnormality, X-linked intellectual disability	208–210
EZH2	Point mutations	Weaver syndrome	214
NSD1	Point mutations	Weaver syndrome	215,216
	Deletion[a], point mutation	Sotos syndrome	215,216
	Microinsertion or microdeletion[a]	Beckwith–Wiedemann syndrome	217
NSD2	Deletion[a]	Wolf–Hirschhorn syndrome	218
PRMT7	Point mutations	Skeletal abnormalities, intellectual disability	222

[a]Haploinsufficient deletions.