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Hydroxylation mediates chromatin demethylation

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Methylation of DNA and histones in chromatin has been implicated in numerous biological processes. For many years, methylation has been recognized as static and stable modification, as compared with other covalent modifications of chromatin. Recently, however, several mechanisms have been demonstrated to be involved in demethylation of chromatin, suggesting that chromatin methylation is more dynamically regulated. One chemical reaction that mediates demethylation of both DNA and histones is hydroxylation, catalysed by Fe(II) and α-ketoglutarate (KG)-dependent hydroxylase/dioxygenase. Given that methylation of chromatin is an important epigenetic mark involved in fundamental biological processes such as cell fate determination, understanding how chromatin methylation is dynamically regulated has implications for human diseases and regenerative medicine.

Keywords: JmjC/TET/demethylation/5-hydroxymethylcytosine/5-methylcytosine.

Abbreviations: 5caC, 5-carboxylcytosine; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; AID, activation-induced cytidine deaminase; AML, acute myeloid leukaemia; AP, apurinic/apyrimidinic; APE1, AP endonuclease 1; APOBEC1, apolipoprotein B mRNA editing enzyme, catalytic peptide 1; ARID, AT-rich interactive domain; ARID5, ARID-containing protein 5; BER, base excision repair; C5-MTases, DNA cytosine-5-methyltransferases; CGI, CpG islands; CKO, conditional knockout; CMML, chronic myelomonocytic leukaemia; CMV, cytomegalovirus; CTCF, CCCTC-binding factor; CYP27B1, cytochrome p450 27B1; ChIP, Chromatin immunoprecipitation; DME, DEMETER; DML, DEMETER-like protein; DNMT, DNA methyltrasferase; EGFP, enhanced green fluorescent protein; ESC, embryonic stem cell; FAD, flavin adenine dinucleotide; GADD45, growth arrest- and DNA damage-inducible 45; HKMT, histone lysine methyltransferase; hmeDIP-seq, hydroxymethylated DNA immunoprecipitation followed by next-generation sequencing; IAP, intracisternal A-particle; JBP, J-binding protein; JHDM, JmjC domain-containing histone demethylase; JmjC, Jumonji-like domain C terminus; KDM, lysine demethylase; KG, ketoglutarate; Kme1, monomethyllysine; Kme2, dimethyllysine; Kme3, trimethyllysine; LSD1, lysine-specific demethylase 1; MBD, methyl-CpG-binding domain (protein); MBT, malignant brain tumour; MDS, myelodysplastic syndromes; MEF, mouse embryonic fibroblast; MLL, mixed lineage leukaemia; MPN, myeloproliferative neoplasm; NER, nucleotide excision repair; PARP1, poly (ADP-ribose) polymerase 1; PGC, primordial germ cell; PHD, plant homeo domain; PHF2, PHD finger 2; PRC2, polycomb repressive complex 2; PRMT, protein arginine methyltransferase; RBP2, retinoblastoma binding protein 2; ROS1, repressor of silencing 1; Rme1, monomethylarginine; Rme2as, dimethylarginine (asymmetric); Rme2s, dimethylarginine (symmetric); SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; sAML, secondary AML; SET, Su(var)3-9/Enhancer of zeste/Trithorax; SRA, SET and RING-associated; SSB, single-strand break; SUMG1, single strand uracil DNA glycosylase; SUMO, small ubiquitin-like modifier; TDG, thymine-DNA glycosylase; TET, Ten-Eleven-Translocation; TPR, tetratricopeptide repeat; UHRF1, ubiquitin-like protein containing PHD and RING finger domains 1; UTX, ubiquitously transcribed TPR gene on X chromosome; UV, ultraviolet; WDR5, WD repeat-containing protein 5; XPG, xeroderma pigmentosum group G-complementing protein; XRCC1, X-ray repair complementing defective repair in Chinese hamster cells 1; ZF, zinc finger.

The term 'epigenetics' has historically been used to describe events that could not be explained by genetic principles (*1*). More recently, the term has come to refer to a collection of phenomena and mechanisms that define stably heritable phenotypes that result from changes to chromosomes without alterations in DNA sequence (*1*, *2*). One phenomenon that illustrates the importance of epigenetics is cellular differentiation. A multicellular organism consists of diverse types of cells that share an identical genotype (with some exceptions such as immune system cells). Despite their identical genotype, the cells of the body have distinct cellular phenotypes and functions that are attributable to the differences between their gene-expression profiles. The molecular mechanisms underlying these epigenetic phenomena involve a range of chromatin modifications including covalent modification of chromatin, non-covalent mechanisms

Featured Article

such as chromatin remodelling and the incorporation of histone variants, non-coding RNA and higher order chromatin reorganization. Chromatin is a complex of DNA, histones and non-histone proteins. The basic building block of chromatin is the nucleosome, a structure consisting of an octamer of four histone proteins (two copies each of H2A, H2B, H3 and H4) around which 147 bp of DNA is wrapped in 1.75 superhelical turns (*3*). The covalent modifications of chromatin include DNA methylation and post-translational modifications of histones (acetylation, phosphorylation, ubiquitination, small ubiquitin-like modifier (SUMO)-ylation and methylation); all of these can influence overall chromatin structure. Among these covalent modifications of chromatin, methylation has been considered static and enzymatically irreversible, although most other modifications are controlled by a balance between enzymes that catalyse the addition and removal of a given modification. Nonetheless, several potential mechanisms of chromatin demethylation have been suggested. One such mechanism, hydroxylation of methyl groups, turns out to be utilized in both histone and DNA demethylation; a number of enzymes involved in this mechanism have been identified. In this review, I survey the mechanisms of regulation of chromatin methylation, specifically the mechanisms of hydroxylation-mediated chromatin demethylation, and describe recent advances in our understanding of these processes.

Chromatin methylation

Chromatin methylation involves the covalent addition of a methyl group to histones and DNA. It is a common epigenetic modification in most eukaryotes, and plays a fundamental role in epigenetic phenomena, in concert with other chromatin modifications.

Histone methylation

Histone methylation occurs on both lysine and arginine residues. Lysine methylation involves the covalent addition of methyl group(s) to the nitrogen atom of the lysine ε-amino group. The first report of the occurrence of methyllysine in histone proteins was made 50 years ago, in a study demonstrating the presence of ε-*N*-methyl-lysine in calf thymus histones (*4*). The methylated histones were revealed to be a product of a post-translational side-chain modification reaction involving S-adenosylmethionine (SAM) (*5*, *6*). Subsequent studies established the presence of ε-*N*-dimethyllysine (Kme2) and ε-*N*-trimethyllysine (Kme3) in histones, in addition to ε-*N*-monomethyllysine (Kme1) (*7*, *8*). Thus, by 1968, it was established that lysine methylation can assume three states (mono-, di- and tri-methylation) (*9*).

The first histone lysine methyltransferase (HKMT) to be identified was SUV39H1, which targets histone H3 lysine 9 (H3K9) (*10*). Since that discovery, a number of HKMT have been identified. These enzymes belong to two classes of methyltransferase families: the Su(var)3-9/Enhancer of zeste/Trithorax (SET) domain–containing family, and the non-SET domain proteins DOT1/DOT1L (*11*–*13*). Both families of enzymes catalyse the transfer of a methyl group from SAM to the ε-amino group of lysine, resulting in S-adenosylhomocysteine (SAH) and methyllysine. The majority of HKMTs belongs to the SET family; these proteins have in common the SET domain as their catalytic core. The SET domain-containing family of enzymes has very specific substrate specificity with respect to methylation site and state, and acts almost exclusively near the amino-termini of histone proteins. On the other hand, DOT1/DOT1L do not contain a SET domain, and methylate H3K79, which falls within the globular domain of histone H3. The structural differences between these two types of enzymes might reflect differences in the accessibility of their substrates.

In general, protein arginine methylation involves the addition of methyl group(s) to the terminal nitrogen atoms of the arginine guanidino group. The first report of chromatin arginine methylation was made in 1970, in a study demonstrating the presence of ω-*N*-methylarginine (N^G-methylarginine) in histone proteins (*14*); earlier work in the late 1960s had set a precedent for this result by demonstrating that proteins (including histones) could be methylated on arginine by enzymes *in vitro*. Three main forms of methylated arginine have been identified in eukaryotes: N^G-monomethylarginine (Rme1), N^G,N^G-dimethylarginine (asymmetric, Rme2as) and N^G,N'^G-dimethylarginine (symmetric, Rme2s). Histone arginine methylation takes these forms as well (*15*).

Since the discovery of the first mammalian protein arginine methyltransferase (PRMT) (*16*), 11 proteins have been suggested to be a PRMT. These proteins fall into two classes: PRMT1–9, which share a common binding motif for the SAM co-factor, a seven-strand twisted β-sheet; and Fbox-only proteins, which do not harbour this signature motif (*15*, *17*). PRMTs transfer a methyl group from SAM to a terminal guanidino nitrogen of arginine, producing SAH and methylarginine. According to their methylation products, the PRMT1–8 enzymes have been further classified into two subclasses, type I and type II. The type-I enzymes generate Rme1 and Rme2as, whereas the type-II enzymes generate Rme1 and Rme2s. PRMT9(4q31) and two Fbox-only family members, FBXO10 and FBXO11, have been suggested to be PRMTs, but further study is necessary in order to conclusively determine whether these proteins possess arginine methyltransferase activity. With respect to histones, the relevant enzymes are PRMT1, 2, 4, 5, 6 and 7, which target multiple arginine residues on histones; however, their substrate specificity has not been as well characterized as that of lysine methyltransferases.

Histone methylation participates in a diverse range of biological processes including heterochromatin formation, X-chromosome inactivation, and transcriptional regulation (*12*, *13*, *18*). The most well-studied histone lysine methylation marks are on H3K4, K9, K27, K36, K79 and H4K20. Histone lysine

methylation can signal either activation or repression of gene expression, depending on the particular lysine residues that are methylated (*11*, *13*, *19*). Even within the same lysine residue, the biological consequences can differ depending on the methylation state, i.e. whether it is mono-, di-, or tri-methylated (*20*, *21*). To complicate matters, the same methylation state can result in opposite functional outcomes depending on how the modification is read and translated in a specific context (*22*). Histone arginine methylation occurs on H3 arginine 2 (R2), R8, R17, R26 and H4R3, and plays roles in defining both active and repressed chromatin states as well as lysine methylation (*23*).

Covalent histone modifications can influence chromatin structure, either directly or indirectly. Acetylation and phosphorylation alter the electrostatic properties of modified residues and structural interactions within the nucleosome, thereby leading to changes in chromatin structure (*24–26*). In contrast, methylation of lysine and arginine residues does not alter their charge, and there is no evidence that lysine methylation directly affects chromatin dynamics. However, effector-mediated functions have been well documented. Histone methylation recruits or stabilizes the localization of effector proteins that elicit functional outcomes. Thus far, proteins containing specific motifs (chromodomain, tudor domain, malignant brain tumour (MBT) domain, PWWP domain, WD40-repeat domain, plant homeo domain (PHD) and ankyrin repeats) have been demonstrated to interact with methylated histones, and to mediate their downstream effects through alteration of chromatin structure (*27*). Histone methylation also prevents the binding of proteins to chromatin. H3K4me and H3R2me2as block the binding of DNA methyltransferase (DNMT) 3A/3L to H3 and that of WD repeat containing protein 5 (WDR5) to H3, respectively, suggesting that histone methylation mediates downstream effects by the negative regulation of protein interactions with chromatin (*28–30*).

DNA methylation

DNA methylation, as an epigenetic mark of chromatin, involves the covalent addition of a methyl group to the C-5 position of cytosine (C); however, other forms of methylation also exist (N-4 position of cytosine and N-6 position of adenine, etc.). The first descriptions of this 'fifth base' (5-methylcytosine, 5mC) in DNA were by Hotchkiss in 1948 (*31*) and Wyatt in 1951 (*32*). Hotchkiss noted that DNA of calf thymus contains a cytosine-like component ('epicytosine') whose ultraviolet (UV) spectrum and chromatographic behaviour led him to suggest that it might be 5mC. Wyatt isolated 5mC from calf thymus DNA and identified its structure by comparison with synthetic 5mC. In mammalian cells, 5mC accounts for ~1% of all DNA bases (*33*). DNA methylation in mammals occurs predominantly in the context of symmetric CpG dinucleotides (~80% of all CpG dinucleotides are methylated) (*33*); a certain amount of non-CpG methylation is detected in embryonic stem cells (ESCs) (~20% of total 5mC) and in oocytes (*34–37*). In contrast, DNA methylation in plants can occur at cytosine in diverse sequence contexts (*38*). Genomic DNA methylation is found throughout the genome; in mammalian cells, short regions enriched in unmethylated CpG dinucleotides are termed 'CpG islands' (CGI) (*35*).

The enzymes responsible for this post-synthetic modification are the DNMTs, conserved and well characterized in mammals and plants. After the discovery of first DNMT, DNMT1 (*39*), five mammalian DNMTs (DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L) have been identified; though DNMT2 and DNMT3L have not been demonstrated to possess significant DNMT activity. DNMTs fall into two general classes: *de novo* and maintenance DNMTs (*40*). Both classes of enzymes catalyse the transfer of a methyl group from SAM to the C-5 position of C in DNA, yielding SAH and 5mC. The DNMT3 family of *de novo* methyltransferases establishes DNA methylation patterns during early development, whereas the maintenance methyltransferase DNMT1 preserves methylation patterns during cell division by specific methylation of hemimethylated CpG dinucleotides through an interaction with ubiquitin-like protein containing PHD and RING finger domains 1 (UHRF1) that recognizes hemimethylated sites (*41–44*). *De novo* methylation by the DNMT3 family of enzymes also contributes to the maintenance of DNA methylation patterns (*45*, *46*).

In general, DNA methylation functions to stably maintain the transcriptionally silent state of chromatin; it is involved in fundamental processes such as genomic imprinting, X chromosome inactivation and repression of retrotransposons. Thus, DNA methylation is essential for development, and its dysregulation is associated with cancer. DNA methylation interferes with transcription in at least two distinct ways that are likely to be biologically relevant. One mechanism involves direct interference of the C-5 methyl group with binding of proteins to their cognate DNA sequences. A number of factors appear to bind CpG-containing DNA sequences; some of these, such as CCCTC-binding factor (CTCF) and lysine demethylase (KDM) 2A, no longer bind to DNA sequences when CpG is methylated (*35*, *47*). The other mechanism involves indirect interference via recruitment of methyl-CpG binding proteins. Proteins containing methyl-CpG-binding domains (MBD) interact with components of complexes that establish a repressive chromatin environment. To date, five methyl-CpG binding proteins that contain MBD domain have been identified: methyl-CpG-binding protein 2 (MeCP2), MBD 1, MBD2, MBD3 (mammalian MBD3 lacks the capacity to selectively recognize methylated DNA) and MBD4. In addition, Kaiso/ZBTB33, ZBTB4 and ZBTB38 are able to bind preferentially or specifically to methyl-CpG, although they use zinc-finger (ZF) domains to bind methylated DNA.

Hydroxylation and α-KG–dependent dioxygenases

Hydroxylation involves the introduction of hydroxyl group(s) into various substrates, and is catalysed by multiple types of hydroxylases (*48*). The source of oxygen in the hydroxyl group can be derived from molecular oxygen, water, or possibly some other compound. When the oxygen atom is derived from molecular oxygen, the reaction is catalysed by oxygenases. A number of enzymes found in nature are able to catalyse the activation of molecular oxygen from the atmosphere, and use it to effect a wide variety of reactions. These enzymes are divided into two classes: oxidases and oxygenases (*49*). While oxidases use oxygen as an oxidant and reduce molecular oxygen to hydrogen peroxide or water, oxygenases incorporate oxygen atoms from molecular oxygen directly into the product(s). Oxygenases that catalyse the incorporation of hydroxyl group(s) into substrates as a result of addition of oxygen are also known as hydroxylases. There are two classes of oxygenases: monooxygenases and dioxygenases. Monooxygenases catalyse an addition of a single oxygen atom into the substrate, whereas dioxygenases catalyse addition of two oxygen atoms into the substrate or substrates (*49*). Dioxygenases catalyse two distinct types of reactions: the incorporation of both oxygen atoms from molecular oxygen into single substrate, and the separate incorporation of these two oxygen atoms into both substrate and co-factor such as α-KG (*49*). Recently, the latter type of reaction has been demonstrated to be involved in demethylation of histones and DNA within chromatin (*50–54*).

Hydroxylation of both methylated histones and DNA is facilitated by α-KG–dependent dioxygenases. Sequence analyses have suggested that there are more than 60 α-KG dioxygenases. These enzymes share a 'jellyroll' structural fold comprised of eight β-strands forming two four-stranded sides; they catalyse hydroxylation as well as desaturation, cyclization, ring expansion, epimerization and other chemical transformations (*49*, *55*). After the first discovery of α-KG–dependent hydroxylase/dioxygenase, prolyl 4-hydroxylase, a number of enzymes have been identified (*56*). These enzymes catalyse the incorporation of one oxygen atom from molecular oxygen into substrate and another oxygen atom into α-KG, yielding the hydroxylated product, succinate and CO_2 (*49*, *57*). In addition to its role in chromatin demethylation, hydroxylation catalysed by α-KG–dependent hydroxylase participates in a diverse range of biological functions, including protein modifications, hypoxic signalling, lipid metabolism and repair of alkylated DNA/RNA.

Histone demethylation

A brief history of histone demethylation

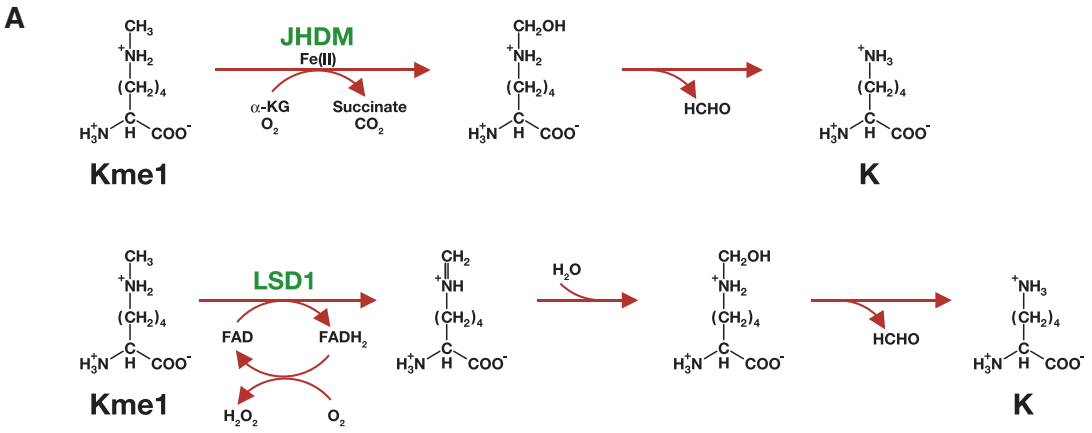
Prior to the discovery of histone demethylases, histone methylation was widely considered to be an irreversible process. This notion largely arose from studies demonstrating that histones and their methyllysine residues have almost the same half-life (*58*, *59*), although there was some evidence that active turnover of methyl group does take place at low levels (1–2%/h) (*60*). The search for histone demethylases started in 1964 when Kim *et al*. reported an enzyme capable of demethylating free mono- and di-*N*-methyllysine (*61*). In 1973, the same group reported an enzymatic activity that could demethylate histones, and subsequently achieved a partial purification, although this enzymatic activity was not characterized at the molecular level (*62*, *63*). These early studies implied the existence of histone demethylases, yet the molecular identities of these enzymes remained elusive the next three decades; consequently histone methylation had been considered as a 'permanent' epigenetic mark until the discovery of a *bona fide* histone demethylase.

The first mechanism to be proposed for lysine demethylation was amine oxidation. At the end of 2004, using a candidate approach, Shi *et al*. demonstrated that the flavin monoamine oxidase KDM1A/lysine-specific demethylase 1 (LSD1) specifically demethylates H3K4me1/2 (*64*). KDM1A/LSD1 is a flavin adenine dinucleotide (FAD)-dependent amine oxidase, and as such can activate dioxygen, use it as an oxidant, and then reduce it to hydrogen peroxide. This discovery revealed that histone lysine methylation is a reversible modification, like other covalent modifications such as acetylation and phosphorylation. KDM1/LSD1 family proteins catalyse the amine oxidation of the methylated lysine, transferring two hydrogen atoms from amine to FAD in order to form the imine intermediate. This reaction reduces co-factor FAD to $FADH_2$, which is then reoxidized by molecular oxygen, producing H_2O_2 (Fig. 1A). The imine intermediate is hydrolysed via non-enzymatic process to produce an unstable carbinolamine intermediate; subsequent spontaneous release of formaldehyde generates demethylated lysine (Fig. 1A).

However, this reaction mechanism is not compatible with a trimethyllysine substrate, since the formation of an imine intermediate via transfer of two hydrogen atoms to FAD requires protonated nitrogen on the ε-amino group of lysine. Therefore, the reversibility of trimethyllysine in histones remained unclear. In addition, the number of KDM1/LSD1 family proteins is limited, making it unlikely that these family proteins would satisfy the enzymatic requirements to demethylate the diverse range of histone methylation status. These observations raised the possibility that additional demethylases may exist that use a different reaction mechanism, leading to the discovery of another demethylase family that employs distinct chemical reactions.

JmjC domain-containing proteins catalyse histone demethylation via hydroxylation of the methyl group

Hydroxylation of the methyl group was the second mechanism to be proposed for lysine demethylation. At the end of 2005, using an unbiased activity-based biochemical purification, Tsukada *et al*. identified a Jumonji-like domain C terminus (JmjC) domain-



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Protein domains	Name	Synonyms	Specificity H3 K4	H3 K9	H3 K27	H3 K36	H3 R2	H4 K20	H4 R3	*D. rerio*	*M. musculus*
	KDM2A	JHDM1B/FBXL10				●●					**KDM2B:** About half of embryos exhibit failure of neural tube closure, resulting in exencephaly and die shortly after birth (77)
	KDM2B	JHDM1B/FBXL10	●●●			●●					
	HR										**KDM3A:** Infertility in males (germ cell apoptosis and spermatogenesis defects), adult-onset obesity, and metabolic syndrome (78-81)
	KDM3A	JHDM2A/JMJD1A									
	KDM3B	JHDM2B		●●							
	KDM3C	JHDM2C/JMJD1C									
	KDM4A	JHDM3A/JMJD2A									
	KDM4B	JHDM3B/JMJD2B		●●●		●●●					
	KDM4C	JHDM3C/JMJD2C		●●							
	KDM4D	JHDM3D/JMJD2D									
	KDM5A	JARID1A/RBP2								**Kdm5c:** Brain patterning and neuronal developmental defects with neuronal cell death (83)	**KDM5A:** Behavioral abnormalities when held upside down by the tail, and some hematological abnormalities (82)
	KDM5B	JARID1B/PLU1	●●●								
	KDM5C	JARID1C/SMCX	●●								
	KDM5D	JARID1D/SMCY									
	JARID2										
	KDM6A	UTX			●●●					**Kdm6al:** Posterior developmental defect (84)	
	KDM6B	JMJD3			●●						
	UTY										
	KDM7A	KIAA1718/JHDM1D		●●	●●					**Kdm7a(a)+Kdm7a(b):** Simultaneous depletion: Loss of neurons from the tectum region and curly tail (85) **Kdm7b:** Apoptosis in the developing brain and craniofacial developmental abnormality (86)	
	KDM7B	PHF8/JHDM1F		●●	●●	●●		●			
	KDM7C	PHF2/JHDM1E		●●							
	JMJD4									**Jmjd6:** Sequentially disrupted development of the somite, brain, heart and notochord (90)	**JMJD6:** Severe anemia and perinatal lethality with developmental defects in various organs including brain, eyes, lungs, heart, thymus, liver, kidney and intestines (87-89)
	JMJD5					●●					
	JMJD6	PSR/PTDSR					●●		●●		
	JMJD7										
	JMJD8										
	HIF1AN										
	HSPBAP1										
	MINA										
	NO66		●●●			●●●					

Bright/Arid | C5HC2-ZF | CXXC-ZF | FBOX | JmjC | JmjN | LRR | PHD | TPR | Tudor | ZF-like

Fig. 1 Overview of JmjC domain-containing histone demethylase family. (**A**) Hydroxylation-mediated histone demethylation catalysed by JHDMs (top panel) and oxidation-mediated histone demethylation catalysed by LSD1 family proteins (bottom panel). For simplicity, only Kme1 is illustrated. While the JHDM-mediated mechanism can be applied to Kme2/3, the LSD1 family-mediated mechanism cannot be used for demethylation of Kme3. (**B**) Schematic representation of the mammalian members of the JmjC domain-containing family. Proteins whose demethylase activity has been reported are shown in red. Synonyms, substrate specificity of demethylase activity, and phenotype of enzyme-deficient mutants in vertebrates are indicated. Green dots represent three methylation states: mono-, di- and tri-methylation. Reference numbers are in parentheses. CXXC, CXXC ZF domain; LRR, leucine-rich repeat domain, JmjN, Jumonji-like domain N terminus; C5HC2, C5HC2 ZF domain.

containing protein, KDM2A/JmjC domain-containing histone demethylase (JHDM) 1A, as a H3K36me1/2-specific demethylase (*50*, *65*, *66*). KDM2/JHDM1 is an α-KG–dependent hydroxylase/dioxygenase; as noted above, such enzymes can activate molecular oxygen and incorporate oxygen atoms into both substrate and a co-factor, α-KG. KDM2/JHDM1 catalyses the direct hydroxylation of the methyl moiety of methyllysine in a reaction that requires Fe(II) and α-KG as co-factors (Fig. 1A). Reaction

products are succinate, CO_2 and an unstable carbinolamine; subsequent release of formaldehyde from the carbinolamine produces demethylated lysine (Fig. 1A). In this reaction, the co-factor–bound JmjC domain contains the catalytic domain, and is thought to produce a highly reactive oxoferryl species that is required for hydroxylation of substrate (Fig. 1B). The JmjC domain was first noted in Jarid2 (Jumonji), Jarid1C (Smcx) and Jarid1A (retinoblastoma-binding protein 2 (RBP2)), and defined as a domain shared by a group of eukaryotic transcription factors, now known as the KDM4 and KDM5 subfamilies (*67*). Subsequently, another group found the JmjC domain in a much wider set of proteins, and revealed that JmjC domain-containing protein family is a branch of the cupin metalloenzyme family (*68*). JmjC domain adopts a 'jellyroll' fold consisting entirely of β-sheet, similar to other members of the cupin superfamily of metalloenzymes; the residues of the active pocket in the interior of this jellyroll structural motif are coordinated with Fe(II) and α-KG.

The human and mouse genomes each have 30 different proteins that contain a JmjC domain. Information on the domain architecture of the full-length protein and JmjC domain-based phylogeny has defined seven subfamilies of these proteins (*51*). So far a series of studies aimed at identifying histone demethylases through sequence homology have shown that 21 of these 30 proteins possess demethylase activity toward H3K4, K9, K27, K36, R2, H4K20, or R3, and that all subfamilies contain histone demethylase; thus, JmjC-domain proteins constitute the largest family of histone demethylases. Most JHDMs, though not all, are able to demethylate trimethyllysine in histones; the hydroxylation-mediated mechanism was initially proposed to be compatible with a Kme3 substrate. Therefore, the discovery of JmjC domain-containing demethylases revealed that Kme3 can be demethylated; in other words, all states of lysine methylation (mono, di, tri) are reversible modifications.

Substrate specificity, functions and regulation of JmjC domain-containing histone demethylases

A series of studies have shown that JHDMs possess demethylase activity toward H3K4, K9, K27, K36, R2, H4K20 and R3, although the activity toward methylarginine still remains to be established (*69*, *70*). JHDMs exhibit exquisite substrate specificity (site and state). Such stringent specificity provides significant insight into the function of histone demethylases. JHDMs seem to rely on both the JmjC domain and auxiliary domains found within each enzyme (such as PHD domains) for their substrate specificity. The determination of the JmjC-domain structure of four JHDMs (KDM2A/JHDM1A, KDM4A/JHDM3A, KDM7A/KIAA1718 and KDM7B/PHD finger 8) has revealed that JHDMs use distinct strategies to achieve state- and site-specificity in the demethylation reactions (*71*, *72*). JmjC domain adopts a jellyroll-like, all β-strand fold; the enzymatically active pocket is buried in the interior of this structural motif, where it is coordinated with Fe(II) and α-KG. With regard to state-specificity, JHDMs can be divided into two classes: type-I enzymes have substrate specificity for me1/2, and type-II enzymes are specific for me2/3. These two distinct state-specificities arise from steric hindrance in the catalytic active pocket, and the accessibility of substrate methyl groups to the Fe(II) at the active site. Type-I enzymes simply do not have sufficient space in their active pocket to accommodate a third methyl group; thus, they are unable to demethylate trimethyllysine. In the type II-enzymes, a network of hydrogen bonds (C–H . . . O type of hydrogen bond that occurs in the active site of SET domain (*73*)) between the methyl group of the substrate and oxygen atoms of active pocket residues places one of the three methyl groups of the trimethyllysine close to the Fe(II), in an ideal position for catalysis. When the degree of methylation decreased to di and mono, the methyl group positioning becomes less optimal. This model explains the state-specificity of type-II enzymes, which do not demethylate monomethyllysine. It is interesting to note that, among the JHDMs identified thus far, type-I enzymes have aspartate as Fe(II)-binding site, whereas glutamate is present at this position in type-II enzymes (*74*). Important determinants of substrate site-specificity include the local sequence at methylation sites, the stability of interaction between JHDMs and the peptide side chains of the substrate, and the auxiliary functional domains of JHDMs.

Earlier studies of JHDMs focused on their activity *in vitro*; more recently, however, the focus has shifted to their cellular and physiological functions. Histone methylation participates in the control of transcription and chromatin architecture, as with other covalent modifications of histones. Furthermore, the degree of lysine methylation on nucleosomes and their relative locations throughout the genome are related to different functional outcomes. Therefore, histone methyltransferases and demethylases play roles in balancing methylation dynamics. In particular, given that JHDMs have exquisite substrate specificity (with respect to both site and state); JHDMs may provide a mechanism for fine-tuning of the histone methylation level. Since the discovery of the first JHDM, we have learned that a number of JHDMs fulfil cellular and physiological functions via control of gene-expression programmes. Growing evidence from studies of mammalian cell culture systems suggests that JHDMs function in context-dependent tumour-promotion and suppression, cell differentiation and stem cell self-renewal (*75*, *76*). Furthermore, studies of the physiological function of JHDMs in model vertebrates showed that JHDMs are emerging as important players in developmental processes such as germline and neuronal development, and implicated them in diseases such as metabolic and neurological disorders and cancer (*77–90*).

How are JHDMs regulated in the nucleus? Though a number of JHDMs have been identified, the mechanisms that regulate their activities are largely unknown; however, a few regulatory mechanisms have been suggested: regulation of expression level, association with chromatin and recruitment to target genes. In

S. cerevisiae, the expression of the H3K4me3 demethylase Jhd2 is tightly regulated by polyubiquitination/proteasome-mediated degradation (*91*). Given that a mammalian counterpart of Jhd2, KDM5C/SMCX/JARID5C, is also targeted for polyubiquitination, this mechanism seems to be conserved in higher organisms (*91*). KDM7C/PHD finger 2 (PHF2), in which the putative binding site of the Fe(II) co-factor contains tyrosine instead of histidine, acquires the ability to associate with chromatin upon phosphorylation by protein kinase A; once on chromatin, PNF2 exhibits demethylase activity toward H3K9me2 (*92*). The association of JHDMs with auxiliary factors also regulates their activity by influencing enzyme recruitment to target genes. KDM7C/PHF2 is recruited to its target genes by association with AT-rich interactive domain (ARID)-containing protein 5 (ARID5), which resides in the co-activator complex for farnesoid X receptor (*92*). Furthermore, KDM6A/ubiquitously transcribed tetratricopeptide repeat (TPR) gene on the X chromosome (UTX) is associated with the mixed lineage leukaemia (MLL) HKMT complex; KDM5A/RBP2 is associated with polycomb repressive complex 2 (PRC2) (*93*, *94*). These enzymes may be recruited to target genes by forming complexes with other factors. Auxiliary factors are not restricted to proteins but may also include non-coding RNAs.

The discovery of JHDMs revealed that histone methylation is a more dynamic process than previously recognized, thereby providing an important conceptual framework for understanding the regulation of histone methylation in the cell. However, there are more than 24 methylation sites on histones, and the reversibility of large portions of them, including H3K79me1/2/3, H4K20me2/3 and arginine methylation, has yet to be demonstrated (*95*). Determination of the reversibility of each methylation sites will contribute to our understanding of the biological function of methylation, both individually and overall. The study of JHDMs, which began with the identification of enzymes, has been making a shift toward the elucidation of the regulatory mechanisms and biological functions of these enzymes. Still, our knowledge regarding them remains sporadic. Further study of these fascinating enzymes in animal models will be required in order to better understand their *in vivo* roles, and the roles of histone methylation.

DNA demethylation

Potential mechanisms of DNA demethylation in mammals

DNA methylation has been considered to be a stable and mitotically heritable epigenetic modification. However, DNA demethylation can arise either by a passive mechanism (absence of maintenance DNA methylation executed by DNMT1 during DNA replication) or by an active mechanism (DNA replication-independent processes that produce unmethylated DNA). The loss of 5mC has been observed both genome-wide and at specific loci. Genome-wide DNA demethylation occurs at two different stages of mammalian development: the migration of primordial germ cells (PGCs) towards the genital ridge (*96*, *97*), and during early development before preimplantation (*96–98*). In the former case, when germ cell fate is established at E7.25, levels of genome-wide epigenetic marks including DNA methylation are similar to those in surrounding somatic cells. However, genome-wide loss of 5mC has been observed in PGC by the time they arrived at genital ridge (*99–101*). The erasure of imprints, which is reflected by DNA demethylation at the imprinted loci, occurs concomitantly with demthylation of other regions (*99*). Given that DNA demethylation of imprinted loci is a rapid process that is completed within 1 day, and that PGCs have undergone several cell cycles in the presence of DNMT1, this demethylation is considered to be an active process. The earliest phase of genome-wide loss of 5mC during early development before implantation is confined to the paternal pronucleus of the zygote, beginning with sperm decondensation and is marked in extent before first replication of DNA (*102*, *103*). Some genomic regions, such as imprinting control regions (*104*), intracisternal A-particle (IAP) retrotransposons (*105*), and centric and pericentric heterochromatin (*106*, *107*), are resistant to this loss of 5mC in the paternal pronucleus. After the completion of the first cell cycle and until the morula stage, there is a stepwise decline in methylation in the early embryo, due to the absence of the maintenance methyltransferase, DNMT1 in nucleus (*96*, *106–109*). These two phases of DNA demethylation, in the paternal DNA before first DNA replication and in the early embryo after the first cell cycle, were initially termed 'active demethylation' and 'passive demethylation', respectively (*102*, *106*, *107*). On the other hand, locus-specific DNA demethylation has been observed in somatic cells, including neurons and T cells, that respond to certain stimuli (*110*, *111*). Locus-specific DNA demethylation also participates in the nuclear hormone-regulated activation of specific genes, such *pS2* and cytochrome p450 27B1 (*CYP27B1*) (*112–114*).

The observation of active DNA demethylation—combined with the notion that if there are DNA methyltransferases that add a methyl group to cytosine, there should also exist DNA demethylases that remove this methyl group—prompted researchers to search for DNA demethylases (*115*). The search for DNA demethylases began in 1982 when Gjerset and Martin reported an enzymatic activity that removed methyl groups from DNA in murine erythroleukaemia cells (*116*). More than a decade later, Weiss *et al*. also detected DNA demethylase activity in rat myoblasts (*117*). However no further characterization of these putative enzymes has been reported. MBD2 was the first protein reported to catalyse removal of a methyl group by breaking a stable carbon–carbon bond (*118*). This reaction is mediated by hydrolysis, does not require any co-factor other than water, and produces a methanol as its release product. However, this observation has not been replicated by other groups. Furthermore, *Mbd2*-null mice are viable and fertile, and have normal pattern of genomic methylation (*119*). Importantly, MBD2 is not required for global

demethylation of the paternal genome in zygotes (*106*). Although most of the early reports of DNA demethylases are controversial, studies performed over the past 5 years have proposed several potential mechanisms for active demethylation, including a DNA repair-based mechanism, a radical SAM-based mechanism, and a hydroxylation-mediated mechanism.

It has been proposed that active DNA demethylation might be accomplished through DNA repair mechanisms, which involve two major pathways: nucleotide excision repair (NER) and base excision repair (BER). NER is generally used to repair a bulky lesion in DNA. The nuclear protein growth arrest- and DNA damage-inducible 45 alpha (GADD45A), previously implicated in this process, promotes global DNA demethylation, in conjunction with its binding partner, NER endonuclease xeroderma pigmentosum group G-complementing protein (XPG) (*120*). However, this finding could not be reproduced by another group, and *Gadd45a*-null mice exhibit no change in either locus-specific or global methylation levels (*121*, *122*), although some reports have supported a role for GADD45 family proteins in locus-specific DNA demethylation (*123*, *124*).

BER is the most versatile among excision repair pathways, and is responsible for repairing most endogenous base lesions, lesions generated by environmental agents, and abnormal bases. Active DNA demethylation can be achieved by BER pathways either via initiation by direct excision or via deamination of 5mC. The direct excision of 5mC is well established in DNA demethylation in plants. 5mC-specific DNA glycosylases (DEMETER (DME), repressor of silencing 1 (ROS1), DEMETER-like protein (DML) 2 and DML3) excise 5mC directly and initiate BER (*125*). In contrast, no mammalian orthologue of the ROS1 family of 5mC glycosylases has been found, and only weak 5mC glycosylase activity can be detected for thymine-DNA glycosylase (TDG) and MBD4 (*126*, *127*). However, their glycosylase activity against 5mC is stimulated by the presence of both RNA and RNA helicase (TDG), and by PKC-mediated phosphorylation (MBD4) (*114*, *126*). In contrast to other DNA glycosylase-null mice, which generally have mild phenotypes, *Tdg*-null mice are embryonic lethal around E11.5, indicating that TDG is essential for embryonic development (*128*, *129*). *Tdg*-null lineage–committed cells (mouse embryonic fibroblast (MEF) cells and neuronal progenitor cells) misregulate the expression of genes that control developmental functions; most of these genes have CGIs in their promoters and are targets of the polycomb repressive system. The methylation level of CGIs in the promoters of these genes is increased in *Tdg*-null lineage-committed cells, concomitant with the enrichment of H3K27me3. Therefore, TDG seems to keep *de novo* DNMT activities in check in order to avoid erroneous methylation; the engagement of X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) and apurinic/apyrimidinic (AP) endonuclease 1 (APE1), and the interaction of TDG with activation-induced cytidine deaminase (AID) and GADD45A, suggest that this enzyme operates through base excision repair. On the other hand, *Mbd4*-null mice are viable and fertile (*130*). The paternal pronucleus of *Mbd4*-null zygotes exhibits normal global DNA demethylation (*131*), although MBD4 carries out active DNA demethylation of the *CYP27B1* promoter in response to parathyroid hormone (*114*).

The deamination of 5mC to thymine initiates BER, as T-G mismatch repair. Both cytidine deaminases and DNMTs have been proposed to contribute to the conversion of 5mC into thymine. AID and apolipoprotein B mRNA editing enzyme, catalytic peptide 1 (APOBEC1) can deaminate 5mC with a strong preference for single-stranded DNA (*132*). *Aid*-null embryos exhibit some increase in the level of DNA methylation in PGC, although the absence of AID still elicits considerable DNA demethylation in PGC (*133*). AID is required for the active DNA demethylation of pluripotent genes during the artificial reprogramming of fibroblast genomes by cell fusion with mouse ESCs (*134*). However, neither *Aid*-null mice nor *Apobec1*-null mice have obvious developmental or reproductive defects (*135–138*). Surprisingly, DNMT3A and DNMT3B, which catalyse methylation of DNA, also possess deaminase activity, and participate in cyclical methylation and demethylation of estrogen receptor target genes (*113*). However, the deamination of 5mC can only occur when the concentration of SAM is very low, raising a question about whether such conditions could be physiologically relevant. In this pathway, thymine glycosylases such as TDG and MBD4 may function in T-G mismatch repair, but there is no evidence that they recognize (or use as substrate) the TG/GT double mismatches that are generated from deamination of symmetrically methylated CpG dinucleotides, repair of which would cause double strand breaks.

In addition to enzymes that initiate BER, BER components such as APE1, poly (ADP-ribose) polymerase 1 (PARP1) and XRCC1, which function in the process after base excision, are also involved in active DNA demethylation (*139*, *140*). Chromatin-bound XRCC1, which is a single-strand break (SSB) sensor protein, is detected only in the zygotic paternal pronucleus, concomitantly with the onset of zygotic paternal DNA demethylation. Another SSB sensor protein, PARP1, is also predominantly detected in the zygotic paternal pronucleus. Treatment of zygotes with inhibitors of key BER components, such as APE1 and PARP1, result in a zygotic paternal genome with significantly higher level of DNA methylation, suggesting that the BER pathway participates in active DNA demethylation. These BER components are also up-regulated in E11.5 PGCs, compared with neighboring somatic cells.

Okada *et al.* have proposed a model involving radical SAM-based demethylation mediated by the elongator complex (*141*). To identify proteins involved in zygotic paternal DNA demethylation, they first developed a system to detect zygotic paternal DNA demethylation using a probe that consists of the Cys-X-X-Cys domain of MLL fused to enhanced green fluorescent protein (EGFP). Given that the

Cys-X-X-Cys domain of MLL has high affinity for unmethylated CpG, DNA demethylation can be detected as accumulation of the probe. Using this monitoring system combined with knockdown of candidate genes, they identified Elp3, a component of elongator complex, as a candidate. Knockdown of *Elp3* as well as that of other components of elongator complex, *Elp1* and *Elp4* impedes DNA demethylation in paternal genome. Furthermore, the requirement of Fe-S radical SAM domain for DNA demethylation suggests that radical SAM-based mechanism might catalyse DNA demethylation via formation of 5-hydroxymethylcytosine (5hmC) as an intermediate (*142*).

In addition to the mechanisms described above, enzymatic hydroxylation of 5mC and its reaction product, 5hmC, has been proposed to be involved in active DNA demethylation as described later in detail.

TETs catalyse hydroxylation of 5mC

5hmC was initially identified in DNA from T-even bacteriophages by the same group that first described 5mC in DNA (Fig. 2) (*143*). In the DNA of these viruses, C is completely replaced by 5hmC and >70% of 5hmC is glucosylated by three different glucosyltransferases (*144*, *145*). This DNA modification system probably evolved for the protection of T-even bacteriophage DNA from host restriction endonucleases as well as their own encoded enzymes that degrade host DNA (*145*). 5hmC exists in mammalian genomes (*146*), but it is unclear whether it is present at physiologically relevant levels (*147*). Therefore, not much attention was paid to mammalian 5hmC until two recent studies addressed this issue (*52*, *148*). These two studies showed that 5hmC is indeed present in mouse Purkinje neurons (0.6% of total nucleotides) and ESCs (0.03% of total nucleotides) as a 'sixth base'; the authors proposed that 5hmC could be an intermediate in active DNA demethylation. Developing more sensitive methods for the detection of 5hmC enabled its discovery in a wide spectrum of mammalian tissues in levels between 0.03% and 0.69% of deoxycytidine (*149–151*). Genome-wide distribution of 5hmC in both mouse and human ESCs, analysed by hydroxymethylated DNA immunoprecipitation followed by next-generation sequencing (hmeDIP-seq), has demonstrated that 5hmC is widely distributed across gene-rich chromosomal domains, and enriched in CpG-rich promoter regions, enhancers marked with H3K4me1 and acetylated H3K27, and binding sites for the insulator CTCF and transcription factors associated with pluripotency, such as OCT4 and NANOG (*152–158*).

The genome-wide distribution and relative stability of 5hmC suggested a potential role for 5hmC beyond that of intermediate in DNA demethylation. In a study of an *in vitro* transcription system using the cytomegalovirus (CMV) promoter and a generic gene body, the presence of 5hmC at the CMV promoter strongly inhibited transcription in human nuclear

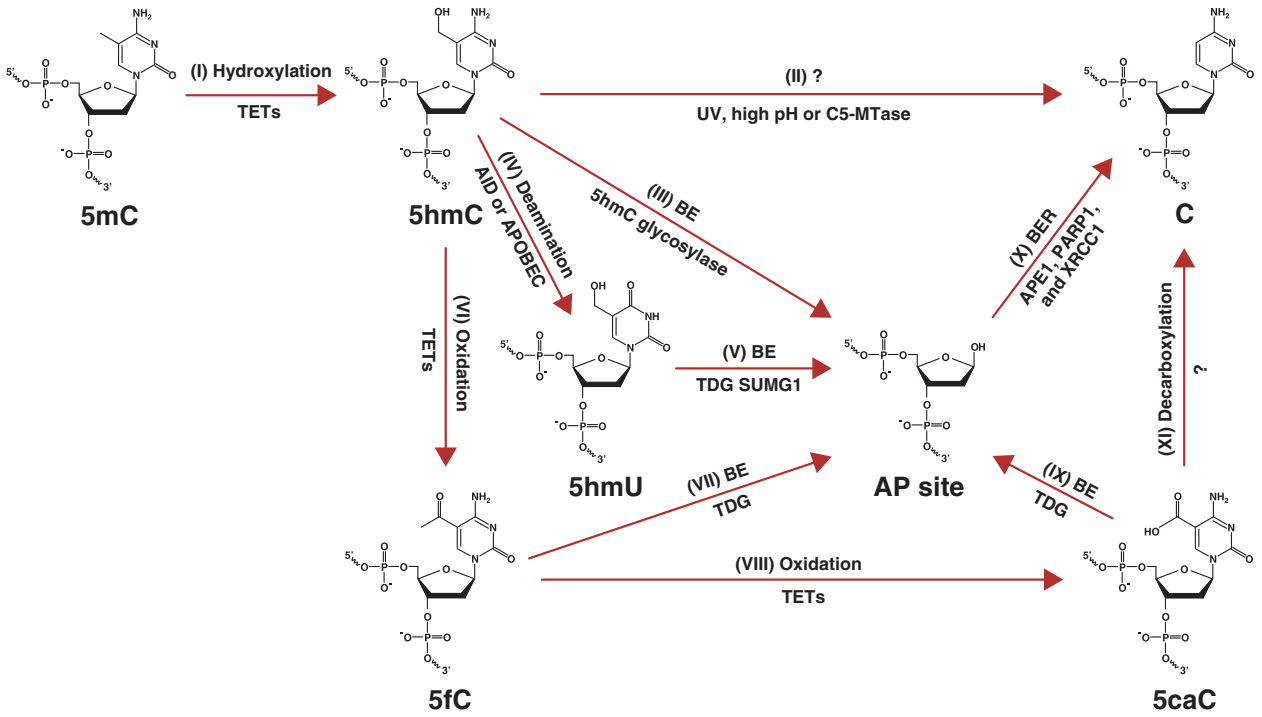

Fig. 2 DNA demethylation through conversion of 5mC into 5hmC. (**I**) TETs catalyse hydroxylation of 5mC into 5hmC using molecular oxygen, Fe(II), and α-KG as co-factors. (**II**) 5hmC could be directly converted into C through loss of formaldehyde upon UV light exposure or exposure to high pH, or by C5-MTases. (**III–IX**) Possible pathways for generation of AP sites: (**III**) 5hmC might be excised by a putative 5hmC-specific glycosylase; (**IV**) 5hmC might be deaminated into 5hmU by AID/APOBEC demaminases; (**V**) 5hmU might be excised by SUMG and TDG; (**VI**) TETs might catalyse oxidation of 5hmC into 5fC; (**VII**) 5fC might be excised by TDG; (**VIII**) TETs might catalyse oxidation of 5fC into 5caC; (**IX**) 5caC might be excised by TDG. (**X**) Generation of AP site is followed by BER with APE1, PARP1, and XRCC1. (**XI**) 5caC converted from 5hmC by consecutive oxidation reactions could be decarboxylated by an unknown carboxylase. BE, base excision.

extracts, although 5hmC in the gene body had negligible effects on transcription; together, the data suggest that 5hmC, like other modifications, contributes to the recruitment or exclusion of factors that influence transcription (*159*). As examples, transcriptional repressors MeCP2, MBD1, MBD2 and MBD4 bind to methylated DNA through their MBD domain, but do not bind to 5hmC (*160*, *161*). In contrast, an essential factor in DNA maintenance methylation, UHRF1, can bind to either 5mC- or 5hmC-containing DNA through its SET and RING-associated (SRA) domain (*162*). Proteins that specifically associate with 5hmC have not yet been identified.

The presence of 5hmC in mammalian DNA raises a question about how this modified base is generated. Although 5hmC could be produced by oxidative DNA damage (*163*, *164*), Tahiliani *et al*. demonstrated the generation of 5hmC by enzyme-catalysed hydroxylation of 5mC (*52*). The trypanosome genome contains base J (β-D-glucosyl-hydroxymethyluracil), which is related to gene silencing in manner analogous to 5mC in mammals. Base J is a modified thymine generated by sequential hydroxylation and glucosylation of the methyl group of thymine. The first step of base J synthesis is catalysed by thymine hydroxylase, J-binding protein (JBP) 1 and JBP2. Tahiliani *et al*. envisioned an enzyme that would modify 5mC; in 2009, using a bioinformatics approach to search for mammalian homologues of JBP1 and JBP2, they identified human Ten-Eleven-Translocation 1 (TET1) as 5mC hydroxylase (*52*). The human *TET1*, previously called *LCX* (leukaemia-associated protein with CXXC domain), was originally identified as a fusion partner of the *MLL* gene in an acute myeloid leukaemia (AML) patient; other members of the TET family include the paralogous human proteins TET2 and TET3 (*165*, *166*). As with JHDMs, the TET family members are Fe(II) and α-KG-dependent hydroxylases/dioxygenases, and catalyse the same chemical reaction (hydroxylation) to produce 5hmC (Fig. 2(I)) (*52*, *167*). Even though TETs catalyse hydroxylation of the methyl group, they do not produce unmethylated C by themselves, unlike JHDMs, which can remove the methyl group as a consequence of a reaction they catalyse. In contrast to hydroxylation of the methyl group in lysine, which generates an unstable carbinolamine intermediate from which methyl group is removed as spontaneous release of formaldehyde, hydroxylation of 5mC does not produce such an unstable intermediate, and the hydroxymethyl group stays in DNA as 5hmC (Fig. 2(I)). The different outcome of these two reactions is probably due to the distinct chemical bond used in histones (nitrogen–carbon bond) *vs*. that in DNA (carbon–carbon bond) to link the methyl group. Recent studies have revealed additional activities of TETs (*168*, *169*), although earlier studies failed to detect such activity. Thymine hydroxylases isolated from fungi, such as *Rhodotorula glutinis*, *Neurospora crassa* and *Aspergillus nidulans*, catalyse sequential conversion of thymine to 5-hydroxymethyluracil, 5-formyluracil and 5-carboxyuracil (*170*, *171*). This observation prompted researchers to examine the possibility that TETs might be able to convert 5mC not only to 5hmC, but also to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Two groups demonstrated that TETs indeed possess the ability to sequentially convert 5mC into 5caC both *in vitro* and *in vivo* (Fig. 2(VI, VIII)), and that 5fC and 5caC (as well as 5hmC) are present in genomic DNA under physiological conditions (*168*, *169*, *172*). However, given that 5fC exists in genomes along with the stable modification 5hmC, the *in vivo* regulatory mechanisms of each of these three sequential reactions still need to be elucidated (*150*, *168*, *169*). It is noteworthy that 5caC cannot be distinguished from C using bisulphite sequence analysis, since 5caC behaves as C in bisulphite conversion (*169*). Therefore, methods to distinguish between 5caC and C need to be developed for future studies.

Possible involvement of hydroxylation of 5mC in DNA demethylation and functions of TET hydroxylases

Accumulating evidence indicates that TET-catalysed hydroxylation of 5mC is involved in the process of DNA demethylation; this implication has led to the study of the physiological relevance of these proteins. Widespread epigenetic reprogramming of PGCs, including genome-wide DNA demethylation, occurs at E11.5. Therefore, significant expression of *Tet1* coincident with the transcription of BER components in E11.5 PGCs implied that hydroxylation of 5mC by TET1 might participate in active DNA demethylation as well as the BER pathway (*139*). *Tet1* is highly expressed in mouse ESCs, and is rapidly down-regulated during their differentiation (*52*, *149*). Chromatin immunoprecipitation (ChIP)-seq analyses of TET1 have revealed a significant preference of TET1 for CGI-containing gene promoters (*153*, *155*, *173*). Unexpectedly, microarray and RNA-seq analyses in *Tet1*-depleted mouse ESCs revealed that TET1 has predominantly repressive, rather than activating, effects on its direct target genes (*153*, *155*, *156*, *173*). Currently, however, TET1 binding alone is unable to predict whether a gene is active or silenced. TET1 and TET2 are responsible for 5hmC production in mouse ESCs (*167*, *174*, *175*), but the role of TET1 in ESC self-renewal and maintenance of pluripotency is controversial. While one study reported that knockdown of *Tet1* in mouse ESCs resulted in down-regulation of *Nanog* expression, impairment of ESC self-renewal, and maintenance of pluripotency (*167*), other studies showed that knockdown of neither *Tet1* nor *Tet2* affects *Nanog* expression, ESC self-renewal, or pluripotency (*153*, *175*). These differences might be attributable to the differences in the ESC backgrounds and/or off-target effects of shRNAs (*153*). In addition, depletion of *Tet1* results in differentiation patterns skewed toward trophoectoderm, primitive endoderm, and endoderm lineages, both *in vitro* and in a teratoma formation assay (*167*, *175*). Although the studies utilizing knockdown approaches to reduce the expression of *Tet1* collectively suggest a potential requirement for TET1 in maintenance of pluripotency and normal development (*167*, *175*), the study of *Tet1*-null ESCs and mice

has indicated that TET1 is dispensable for ESC maintenance, and that its loss is compatible with embryonic development and post-natal survival. The generation of viable *Tet1* knockout mice provides a possible explanation for the observed defect in lineage specification due to loss of TET1. *Tet1*-depleted ESCs exhibit skewed differentiation under non-physiological conditions, e.g. in teratoma or *in vitro* embryoid body assays, but such defects in lineage specification are less pronounced in the context of an embryo and are compatible with embryogenesis. Although the generation of viable and fertile *Tet1*-null mice unequivocally indicated that TET1 deficiency does not prevent embryonic or post-natal development, the average litter size of homozygous parents seems to be smaller than wild type, and homozygous mutant pups have a slight reduction in body size and weight. Therefore, further study is required in order to determine the role of TET1 in active DNA demethylation in PGCs, gametogenesis, fertility and embryonic development. It has also been suggested that TET1 participates in neuronal activity-induced, locus-specific DNA demethylation in the dentate gyrus of the adult mouse brain *in vivo* (*176*).

Although *Tet2* is expressed, and together with *Tet1* is responsible for 5hmC production in mouse ESCs, TET2 does not seems to play a significant biological role in ESCs (*167*, *175*). However, among all three TET family members, *TET2* is most frequently mutated in myeloid malignancies (*177*). The overall mutation rate is 19.5%, but the frequency of mutations varies between different diseases, including myelodysplastic syndromes (MDS), myeloproliferative neoplasm (MPN), chronic myelomonocytic leukaemia (CMML), AML and secondary AML (sAML) (*177*, *178*). RNAi-mediated depletion of *Tet2* in mouse hematopoietic precursors results in differentiation patterns in culture that are skewed towards monocyte/macrophage lineages, indicating that TET2 is important for normal myelopoiesis (*178*). The *TET2* mutations associated with myeloid malignancies impair enzymatic activity and are associated with global hypomethylation (rather than the expected hypermethylation) at differentially methylated CpG sites (*178*). Further study of the role of TET2 in hematopoietic cells will reveal the function of 5hmC in DNA demethylation and the relationship between DNA methylation changes and myeloid neoplasia.

It has also been suggested that hydroxylation of 5mC is involved in genome-wide DNA demethylation in the paternal genome of the zygote. Immunofluorescence analyses using antibodies against 5hmC showed that the appearance and accumulation of 5hmC in the paternal genome coincide with loss of the signal for 5mC and that 5hmC signal persists during cleavage stage of embryos, suggesting that the genome-wide loss of 5mC is attributable to the conversion of 5mC to 5hmC (*53*, *179*). Consistent with the passive DNA demethylation of maternal DNA in early embryo, 5hmC generated in paternal DNA is lost by a DNA replication-dependent passive process in the cleavage stage (*180*). The expression of the three TET hydroxylases/dioxygenases is differentially regulated during early development. While *Tet3* is expressed in the oocyte and zygote and is suddenly down-regulated at the two-cell stage (*53*, *179*), *Tet1* and *Tet2* are mainly expressed in the inner cell mass (*167*, *174*), indicating that the conversion of 5mC to 5hmC in the zygotic paternal pronucleus is catalysed by TET3. To study the physiological role of TET3, conventional and conditional knockout (CKO) mice of *Tet3* were generated (*54*). Female CKO mice, in which germline-specific deletion of *Tet3* from PGC has been achieved, are normal in growth and morphology. Mating between CKO female and wild-type male mice produces zygotes in which neither the appearance of 5hmC signal nor the loss of 5mC signal from paternal DNA is observed. In contrast, zygotes generated by the converse mating do not exhibit such a change. Thus, the genome-wide loss of 5mC in the zygotic paternal genome is attributable to the hydroxylation of 5mC into 5hmC by maternal TET3. In addition, deletion of *Tet3* from the female germ cells impairs DNA demethylation at *Line1* and octamer-binding transcription factor 4 (*Oct4*) in the paternal genome, and impedes expression of *Oct4* derived from paternal DNA in the early embryo. Female CKO mice do not show obvious defects in epigenetic reprogramming in embryonic germ cells, oocyte development, maturation and fertilization. However, their fecundity is significantly lower, both in terms of frequency of successful pregnancy per mating and litter size. *Tet3* (Mat^{-}/Pat^{+}) mutant embryos exhibit a high frequency of degeneration and morphological abnormalities, starting from midgestation. Further study will reveal the role of 5mC hydroxylation in the zygotic paternal genome. On the other hand, conventional homozygous mutation of *Tet3* results in neonatal lethality (*54*), indicating that TET3 might have roles in the embryo as well as the oocyte and zygote.

Accumulating evidence strongly supports the idea that hydroxylation of 5mC to 5hmC, catalysed by TET hydroxylases/dioxygenases, participates in both genome-wide and locus-specific DNA demethylation. However, several pathways from 5hmC to C have been proposed, as follows (Fig. 2). A few reports support a direct conversion of 5hmC into C (Fig. 2(II)). When 5hmC is exposed to UV radiation, very efficient generation of cytosine is observed, suggesting a plausible mechanism for conversion of 5hmC into C: photochemical hydration of 5hmC followed by elimination of formaldehyde (*181*). It has also been noted that 5-hydroxymethylpyrimidine derivatives prepared with radioactively labelled formaldehyde lose their label when stored in or expose to alkaline solution, indicating that the hydroxymethyl group is removed from 5-hydroxymethylpyrimidine derivatives as formaldehyde under high pH (*182*, *183*). On the other hand, DNA cytosine-5-methyltransferases (C5-MTases) could catalyse the removal of formaldehyde from 5hmC and thereby generate C (*184*). Thus, these reports suggest that 5hmC could be directly converted into C through loss of formaldehyde, either upon exposure to ultraviolet light or high pH, or by C5-MTases (Fig. 2(II)). 5hmC may be an intermediate in the conversion of 5mC to C via

the BER pathway (Fig. 2(III–X)), since BER components that function in the process after base excision are involved in DNA demethylation (*139*, *140*, *176*). The generation of an AP site to initiate BER could be achieved in several ways (Fig. 2(III–IX)). Given that 5hmC-specific DNA glycosylase activity exists in calf thymus extracts (*185*), it might be possible that excision of 5hmC by a 5hmC-specific DNA glycosylase initiates BER, as in plant (Fig. 2(III)). Several lines of evidence support the possibility that 5hmC may be deaminated into 5hmU by AID/APOBEC deaminases, followed by excision by 5hmU glycosylases such as single-strand uracil DNA glycosylase (SUMG1) and TDG, in order to initiate BER (Fig. 2(IV, V, and X)). First, AID/APOBEC deaminases facilitate 5hmC demethylation as well as deamination to convert 5hmC into 5hmU (*176*). Second, 5hmU can be excised by 5hmU glycosylases, TDG and SUMG1 (*129*, *176*). Finally, TDG interacts with AID and functions in a process that engages APE1 and XRCC1 (*128*, *129*). TETs catalyse a consecutive oxidation of 5hmC to 5fC and 5caC, and TDG can excise both of 5fC and 5caC (Fig. 2(VI–IX)) (*168*, *169*, *186*). Therefore, it may be possible that further oxidation of 5hmC followed by excision of oxidation products by TDG initiates BER (Fig. 2(VI–X)). The conversion of 5hmC into C could be achieved by TET-catalysed sequential oxidation of 5hmC into 5caC, followed by decarboxylation by a putative decarboxylase (Fig. 2(XI)). Although a similar process that converts thymine to uracil exists in fungi (*170*, *171*), an enzyme capable of decarboxylating 5caC in DNA has yet to be identified.

The rediscovery of 5hmC, and the identification of TETs that catalyse the hydroxylation of 5mC, provide an important advance in understanding of DNA demethylation. Although there is not yet a consensus on the mechanism of active DNA demethylation in mammals, accumulating evidence suggests that hydroxylation of 5mC and BER may be involved. However, these mechanisms generate many pyrimidine derivatives in DNA, including, 5fC, 5caC and 5hmU. Therefore, further study will require development of methods to distinguish these pyrimidine derivatives, in addition to C, 5mC and 5hmC. Besides the mechanism, the precise timing and extent of genome-wide DNA demethylation also remains unclear. Genome-wide profiling of the pyrimidine derivatives involved in the process will capture the entire picture of genome-wide DNA demethylation, and thereby contribute to our understanding of its mechanism.

Conclusion

Recent advances have revealed that demethylation of both histones and DNA in chromatin employs the same chemical reaction, hydroxylation, which is catalysed by Fe(II) and α-KG-dependent hydroxylase/dioxygenase: JHDMs and TET hydroxylases/dioxygenases. These enzymes are now emerging as important players in development, and are linked to human diseases. In addition to the direct removal of the methyl group from histones, mechanisms for the active turnover of methyl groups on histones and for antagonizing the effect of histone methylation have been proposed; these include clipping of methylated histone tails, replacement of methylated histones with unmethylated histones, a 'binary switch' model in which the effect of methylation is counteracted by another histone modification, and antagonizing methylation on arginine residues by converting monomethyl-arginine in histone to citrulline (*65*). The existence of these multiple mechanisms suggests that histone demethylation utilizes each mechanism in a context-dependent manner. In mammals, although several mechanisms have been suggested, the specific mechanisms by which DNA demethylation is accomplished remain elusive and highly debated. Given that methylation of chromatin contributes to fundamental processes such as establishment of cell identity in multicellular organisms, further study will provide us with a better understanding of epigenetic regulation, reprogramming, and disease, and therefore has implications for regenerative medicine and human health.

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Conflict of interest
None declared.

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