

Invited Mini Review

Enzymatic DNA oxidation: mechanisms and biological significance

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DNA methylation at cytosines (5mC) is a major epigenetic modification involved in the regulation of multiple biological processes in mammals. How methylation is reversed was until recently poorly understood. The family of dioxygenases commonly known as Ten-eleven translocation (Tet) proteins are responsible for the oxidation of 5mC into three new forms, 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Current models link Tet-mediated 5mC oxidation with active DNA demethylation. The higher oxidation products (5fC and 5caC) are recognized and excised by the DNA glycosylase TDG via the base excision repair pathway. Like DNA methyltransferases, Tet enzymes are important for embryonic development. We will examine the mechanism and biological significance of Tet-mediated 5mC oxidation in the context of pronuclear DNA demethylation in mouse early embryos. In contrast to its role in active demethylation in the germ cells and early embryo, a number of lines of evidence suggest that the intragenic 5hmC present in brain may act as a stable mark instead. This short review explores mechanistic aspects of TET oxidation activity, the impact Tet enzymes have on epigenome organization and their contribution to the regulation of early embryonic and neuronal development. [BMB Reports 2014; 47(11): 609-618]

INTRODUCTION

Modification of DNA was discovered as early as the identification of DNA as genetic material. The best-known modification is base methylation, which is added to DNA after replication. In prokaryotes, base modification occurs on both

adenines and cytosines while in eukaryotes, only cytosine methylation has been found. Base modification modulates the DNA structure and function. Prokaryotic DNA modifications play a major role in marking self-DNA thus allowing specific destruction of invading viral DNA by host restriction enzymes. In higher organisms, the primary host defense mechanism of DNA methylation has evolved to repress 'genomic parasites' such as transposons and retroviral elements (1). In the meantime, it has assumed a new profoundly important role in gene regulation (2, 3).

The study of eukaryotic DNA methylation has been focused on the mammalian system over the past decades. One reason for this is the lack of DNA methylation in commonly used model organisms including yeast, *Drosophila* and *C. elegans*. Another reason is the realization that DNA methylation provides a molecular means for epigenetic inheritance and reprogramming which is a foundation of mammalian development. 5% of cytosines in the mammalian genome are methylated. Most of the methylated cytosines (5mCs) reside in CpG dinucleotides. In early development, cell-type specific genomic methylation patterns are established by *de novo* methyltransferases DNMT3A and DNMT3B. The established patterns are propagated through somatic cell divisions by the maintenance methyltransferase DNMT1 (4-6). Genomic methylation regulates a variety of biological processes related to genome function and gene expression. Its importance has been highlighted in the demonstration that each methyltransferase is essential for mouse development (6). Abnormal methylation caused by DNMT3B mutations is linked with a congenital disorder which is characterized by immunodeficiency, centromeric chromosomal instability and facial anomaly (7).

In contrast to our rich knowledge of DNA methylation, the concept of demethylation has been debated and the mechanism has remained elusive. Demethylation can take place by the removal of the methyl group from 5mC or replacement of 5mC with unmethylated C. The necessity for active demethylation was questioned, based on the fact that demethylation can result passively from inhibition of maintenance methylation during DNA replication. Since passive dilution, in conjunction with DNA replication can bring about demethylation in dividing cells, there is no absolute requirement for active removal (8). In addition to the mode of passive dilution, DNA repair in-

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cluding base excision repair (BER) and nucleotide excision repair (NER) targeted to 5mC can also contribute to the replacement of 5mC by C (9). However, repair-mediated demethylation involves localized re-synthesis of a new strand resorting to the use of DNA polymerase and a template strand. Targeted repair is therefore not a bona fide mode of active demethylation.

Are Tet enzymes demethylases?

The search for DNA demethylases has been a major endeavor in epigenetics research. Despite numerous candidate proteins including MBD2, Gadd45 and Elp3 being proposed to have demethylase function (10-12), none could be consistently demonstrated to possess this enzymatic capacity. The mere existence of active 5mC demethylation in mammalian cells has therefore been a controversial topic, not to mention the biochemical identification of an authentic demethylase.

Tet proteins came into the limelight for their ability to catalyze the hydroxylation of the methyl group of 5mC (13). Oxidation is a canonical reaction to induce departure of a methyl group from organic compounds in the form of formaldehyde, formic acid, or CO₂. For instance, histone lysine demethylases found in many organisms catalyze hydroxylation of the methyl group attached

to the nitrogen of an amino group, followed by spontaneous release of the hydroxymethyl group as formaldehyde. However, single-step hydroxylation does not cause departure of the methyl group if it is linked to a carbon atom. It takes instead further reactions as shown in the thymidine salvage pathway of *Neurospora crassa* and several other fungi. This pathway demethylates thymidine to uridine through three consecutive steps of hydroxylation catalyzed by thymine-7-hydroxylase, followed by decarboxylation of the final oxidation product iso-orotate by a decarboxylase, releasing CO₂ (14). If DNA demethylation adopts an analogous mechanism, 5mC would need to be oxidized into 5caC which would in turn undergo enzymatic decarboxylation (Fig. 1). Indeed, all three Tet enzymes have been shown to be able to oxidize 5mC into 5caC efficiently *in vitro* and *in vivo* (15) and 5caC decarboxylation has been suggested to occur in mouse ES cells (16). However, a 5caC decarboxylase activity in cooperation with Tet enzymes still needs to be identified. Since the large multi-domain Tet proteins themselves do not contain decarboxylase activity (unpublished, He *et al.*), the activity might be separately provided by a Tet- or 5caC- interacting protein. The next breakthrough in the search for mammalian DNA decarboxylases might be facilitated by the structural insights recently obtained from the analysis of the fungal iso-orotate decarboxylase (17). Taken together, Tet enzymes appear to be the closest to the proposed 'demethylase' hitherto, even though they alone are not able to restore unmodified cytosine from 5mC. Identification of a 5caC decarboxylase would finally resolve whether Tet enzymes can initiate a bona fide demethylation pathway leading to the cleavage of the C-C bond linking the methyl group with the pyrimidine ring of modified cytosines.

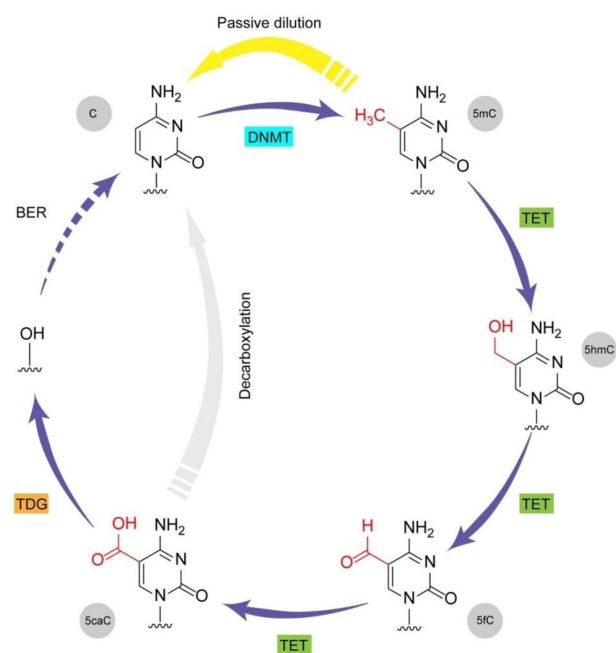


Fig. 1. Cycle of DNA methylation and demethylation. Active demethylation is achieved by iterative oxidation of the methyl group of 5mC by Tet dioxygenases and restoration of unmodified cytosines (C). The latter is thought to occur by either replication-dependent dilution (not shown) or TDG glycosylase-initiated base excision repair. Of note, TDG can recognize and excise both 5fC and 5caC. An alternative direct mechanism is feasible (grey arrow), but an enzyme responsible for 5caC decarboxylation remains to be identified.

Regulation of Tet activities

DNA 5mC oxidation can pause at three intermediates 5hmC, 5fC and 5caC. What regulates progression of the oxidation reaction catalyzed by Tet? Several Tet-interacting proteins including chromatin components have been identified which might regulate the oxidation activity as well as Tet protein stability, subcellular localization and chromatin binding (reviewed by Delatte and Fuks, 2013 (18)). In addition to these proteins as a determinant for the selection of genomic loci and the extent of 5mC oxidation, several small molecules also show effects on Tet activity.

Vitamin C (Vc) and ATP, two molecules not directly involved in the hydroxylation reaction, were found to stimulate Tet enzymatic activity *in vitro* (15) and in cultured cells (19, 20). Mouse ESCs had much reduced levels of 5mC when cultured in the presence of Vc but knockout of Tet genes from ESCs blocks the effect of Vc. This result indicates that the impact of Vc on DNA methylation results from its regulatory function for Tet enzymes, rather than indirectly from its regulation of histone demethylase activities as shown for histone demethylases Jhdm1a/1b (21). In support of a role of Vc on DNA oxidation, mice deficient in Vc synthesis and grown without dietary Vc intake show reduced 5hmC and increased 5mC in tissues

(19). The stimulating effect of ATP on Tet activity was demonstrated instead only *in vitro* (15). Addition of ATP in the reaction increased the generation of the higher oxidation product 5caC from the 5mC substrate. The physiological relevance of ATP in the regulation of genomic 5hmC, 5fC and 5caC formation remains an open question. Both Vc and ATP interact with the C-terminal catalytic domain of the Tet enzymes but the exact molecular mechanism for stimulation is unclear. Measurement of intracellular availability of these small molecules and their interaction with Tet enzymes will be key to determining their regulatory roles in physiological settings.

Two distinct demethylation functions of Tet-mediated 5mC oxidation

While an immediate purpose of Tet enzymes is to remove 5mC, either by means of triggering replicative dilution or the enzymatic excision of the oxidation products, a fine distinction can be made with regard to the functional needs they meet (Fig. 2). In pronuclear-stage zygotes, Tet3 as a maternal factor eliminates methylation deposits differentially placed on paternal alleles such as the *Nanog* promoter during gametogenesis (22). Failure to remove methylation from the paternally methylated genes is expected to cause haploinsufficiency in early embryonic gene expression. This mode of erasure of pre-existing methylation also contributes to demethylation at a subset of imprinted alleles in primordial germ cells (23, 24). In contrast to the association of 5mC oxidation with regional restoration of an unmethylated state during reprogramming, in somatic cells such as stem cells and neurons, 5mC oxidation more often appears to contribute to the maintenance of methylation fidelity by counteracting aberrant *de novo* methylation. Williams *et al.* (2011) have reported that in ES cells, Tet1

keeps CpG-rich promoters hypomethylated by removing erroneously placed methylation (25). This methylation-antagonizing role of Tet1 is also seen in neural precursor cells in the brain and in postnatal germ cells. Deficiency of Tet1 in the adult brain has been shown to cause 5mC accumulation at the promoters of neurogenesis-associated genes, potentially causing impairment of cell proliferation (26). Yamaguchi *et al.* (2012) have reported that oocytes from Tet1-deficient mice suffered from abnormal methylation and decreased expression of a few meiotic genes, potentially causing reduced fertility (27). Although the contribution of Tet in some cell types to preventing accretion of additional methylation seems clear, details of this surveillance mechanism to antagonize aberrant methylation at individual CpG sites remain to be explored.

Do Tet enzymes and 5hmC have important functions in ES cells?

Mouse ES cells (ESCs) have been used as a model to study DNA demethylation for two particular reasons. First, all DNA methyltransferases and Tet1/2 are highly expressed. Second, all three oxidative derivatives 5hmC, 5fC and 5caC are abundant relative to most other cell lines. These features promise to facilitate analyses of epigenetic roles of DNA modification in pluripotency maintenance and lineage commitment. However, their disadvantage is the dynamic cell equilibrium and heterogeneity with regard to the co-existence of distinct functional and epigenetic states (28). ESC populations are thus highly susceptible to environmental variations and culture conditions. The presence of a small fraction of differentiating or differentiated cells is also a major concern. Prolonged culture required for the establishment of stable cell lines carrying gene manipulations would further disturb the cell balance, resulting in uncontrolled property alterations, complicating interpretation of phenotypic observations.

Early experiments by Ito *et al.* (2010) based on knockdown of Tet1 in ES cells led to the claim that Tet1 has an important role in ESC self-renewal and inner cell mass specification (29). This claim has not been supported by subsequent studies based on mouse gene targeting. ES cells of Tet1-single or Tet1/Tet2-double knockout appear to be unaffected in self-renewal and pluripotency, and Tet-deficient mice can be born without overt developmental defects (26, 30, 31). In fact, all three Tet genes can be deleted from ESCs without significantly compromising their ability for self-renewal and pluripotency (32). The disagreement between Ito *et al.* and subsequent work might be explained by the aforementioned limitations of ESCs. Therefore, while the ESC system is a valuable tool for the dissection of molecular pathways surrounding DNA demethylation, caution has to be exercised to avoid drawing a wrong conclusion regarding developmental relevance.

Tet-mediated 5mC oxidation of parental genomic DNA in zygotes

The decision to either maintain or erase 5mC deposits at me-

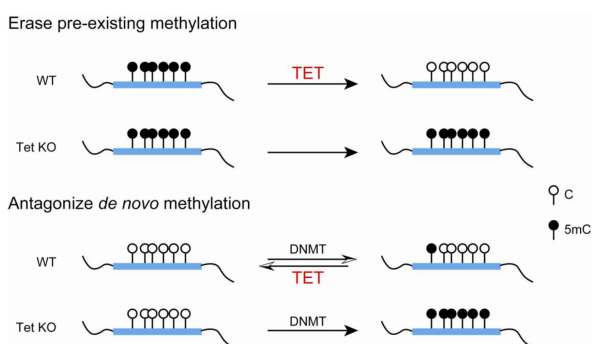


Fig. 2. Two scenarios of DNA demethylation via Tet-mediated 5mC oxidation. The first concerns erasure of regional methylation in association with timely reactivation of methylated genes as demonstrated for pluripotency loci in the paternal genome in mouse post-fertilization early embryos (see main text). The second deals with the removal of excessive methylation by pruning methyl groups stochastically placed due to an inaccurate methylation machinery. This might be a mechanism to keep CpG islands free of 5mC in the mammalian genome.

thylated CpGs in parental genomic DNA has to be made in the single embryo shortly after fertilization. Early work to explore 5mC dynamics relied on the anti-5mC antibody which can distinguish 5mC from unmodified cytosine. By using immunofluorescence assays, a substantial 5mC loss was observed in the male pronucleus in mouse zygotes (33). Relative to the drastic loss of 5mC in the bulk paternal genome, specific genomic loci seemed to undergo limited degrees of demethylation as revealed by bisulfite sequencing (34). The 5mC signal loss was interpreted as DNA demethylation. As DNA replicates only once, the abrupt loss of 5mC was considered to involve an active process. A more important reason is that inhibition of DNA replication had no effect on 5mC loss in the paternal DNA. Active demethylation in the zygote has since become a paradigm for epigenetics research which is also anticipated to shed light on epigenetic inheritance across generations. However, the biochemical mechanism has long remained unsettled.

The first mechanistic clue about active demethylation came from the inspection of JBP protein, which catalyzes the hydroxylation of the 5' methyl group of thymine in trypanosomes. As mammalian homologues, the Tet family proteins were confirmed to be capable of catalyzing hydroxylation of 5mC into 5hmC (13). The reaction was immediately proposed as a step in the route toward DNA demethylation. By using antibodies specific for 5hmC, several labs observed DNA oxidation in the male pronucleus starting from the PN3 stage (22, 35, 36). Mouse genetic studies established that Tet3, the only one of the three 5mC dioxygenases present in oocytes, was responsible for oxidative demethylation of the paternal genome (22, 36). In addition to its contribution to global loss of 5mC, Tet3 has been shown to be required for demethylation at a range of specific genomic loci.

Tet and TDG-dependent active demethylation represents an attractive potential mechanism to remove oxidized 5mC following further oxidations of 5hmC by Tet enzymes to create 5fC and 5caC, which are recognizable by TDG (Fig. 1). While TDG function is believed to be responsible for the restoration to unmodified cytosine from 5mC in ESCs (15, 37), recent work by Guo *et al.* (2014) showed that the zygotic demethylation process was unaffected by deletion of TDG from the zygote (38). This result suggests the existence of as-yet-unknown demethylation mechanisms downstream of Tet3-mediated oxidation.

Both parental genomes undergo active demethylation at the pronuclear stage

Preimplantation DNA demethylation of the two parental genomes differs both in timing and mechanism. The paternal genome undergoes active demethylation in the one-cell embryo, a few hours after fertilization. The maternal genome loses methylation by passive dilution thanks to DNA replication later during blastomere cleavage. The overall methylation level of a blastocyst reaches its lowest point at embryonic day E3.5.

The apparent asymmetry in reprogramming was initially based on the comparison of signal intensity in 5mC staining between the two pronuclei (33) and bisulfite methylation profiles for a limited number of specific sequences in the pronuclear DNA (34). These experiments could not exclude the possibility that the maternal genome is also subjected to active demethylation. The preferential demethylation observed in the male pronucleus could be just a reflection of the need to erase the hypermethylation found in sperm DNA. Recent genome-scale 5mC mapping using isolated pronuclear DNA has indicated that indeed the maternal genome also undergoes Tet3-mediated active demethylation in fertilized eggs, although the number of CpGs losing 5mC is much smaller than in the paternal genome (38, 39). The demethylation process in both pronuclei is independent of DNA replication as treatment of zygotes with replication inhibitor did not have any effect.

Mechanism for selective demethylation in pronuclear DNA

Based on the initial observation of 5mC loss predominantly in the male pronucleus, the chromatin organization of the sperm genome was hypothesized to attract a putative demethylase (34). However, the existence of male genome-specific features as a determinant of active demethylation is incompatible with the recent discovery of maternal DNA demethylation in female pronuclei (38, 39). A more plausible alternative deals with a protective mechanism provided by PGC7 (also referred as Dppa3 or Stella) which is known to be a negative regulator of DNA methylation in the early embryo (40). The maternal genome and stably methylated DMRs that control genomic imprinting, but not the bulk paternal genome, were found to be enriched in histone H3 lysine 9 dimethylation (H3K9me2), one of the prominent marks of transcriptional repression (41). H3K9me2 was further demonstrated to serve as a signal for PGC7 binding. Chromatin-bound PGC7 was suggested to prevent conversion of 5mC to 5hmC, at least in part, by disengaging Tet3 from chromatin interaction and/or directly suppressing its enzymatic activity (41, 42). Consistent with this, in PGC7 knockout zygotes, 5mC in the female pronucleus became accessible to oxidation into 5hmC (36). This model thus suggests a repressive histone mark is shielding methylated regions from oxidative demethylation rather than using a positive signal to guide the demethylation. However, the ultimate confirmation for an anti-correlation between H3K9me2 and 5mC oxidation awaits genome-wide mapping of H3K9me2, 5mC/5hmC and PGC7 binding sites, which is still technically challenging in pronuclear samples due to limiting material. Obviously, PGC7-based masking is not the only mechanism responsible for selective demethylation as loss of methylation was not induced at a subset of imprinted loci in PGC7 knockout embryos (40). Additional factors regulating Tet3 function are expected to be discovered in the coming years.

Biological significance of mouse zygotic demethylation

Pronuclear DNA demethylation is a conspicuous post-fertiliza-

tion event. There are at least three potential roles this event might play. First, abnormal methylation might be imposed during gametogenesis and would thus need to be 'pruned' in the early embryos. Second, zygotic demethylation might serve to remove sperm methylation to selectively retain maternal methylation. This hypothesis provides an explanation for the scarcity of paternal methylation imprints (limited to three imprinting control regions in mouse) identified so far. Third, certain genes undergoing down-regulation and methylation-based silencing in germ cells need to be reactivated in the embryo. Compelling evidence for the third hypothesis has been obtained over the last few years. Several labs have noticed that the promoter and enhancer regions of critical pluripotency genes were methylated in sperm but unmethylated in oocytes, early embryos and ES cells (43-45), and therefore there exists a need for prompt demethylation in zygotes. Gu *et al.* (2011) have provided a direct demonstration for the importance of zygotic demethylation of pluripotency loci in the establishment of full developmental potential in early embryos (22). Loss of zygotic 5mC is caused by its oxidation catalyzed by Tet3. Early embryos derived from Tet3-deficient oocytes fertilized with wild-type sperm have reduced expression of the paternal *Oct4* gene. Moreover, over half of these embryos show serious abnormalities by embryonic day E11.5 and degenerate by E14.5. Thus, zygotic demethylation, though not absolutely essential for embryo survival, clearly maintains developmental potential by contributing to the reactivation of embryonic genes silenced during germ cell development. The basis of the individual variation in lethality caused by Tet3 deletion is not clear.

Embryonic methylation reprogramming in different animals

Methylation disparity is recognized to exist between the dimorphic male and female gametes of mammals. The first class of genomic sequences found to be subjected to differential methylation was the imprinted control regions. These are either methylated in sperm or oocytes and the resultant allele-specific differential methylation persists in order to regulate monoallelic gene expression in diploid somatic cells in development. The demethylation that occurs in embryonic germ cells serves to erase the pre-existing pattern inherited from somatic cells to generate haploid gametes with a homogenous epigenome. It has become clear that methylation reprogramming facilitates the establishment of biparental genetic totipotency after fertilization. By contrast, lower vertebrates with external fertilization and embryonic development lack genomic imprinting. However, recent studies have revealed extensive methylome differences between sperm and eggs in zebrafish which are eliminated by embryonic reprogramming in embryonic development (46, 47). The zebrafish maternal genome emulates the relatively hypermethylated paternal genome through *de novo* methylation to create a homogenous epigenome without allelic difference by the 1000-cell embryo stage. However, the biological significance of this is ques-

tioned by the fact that haploid zebrafish embryos can be produced by *in vitro* fertilization and survive in a haploid state for several days (48). In vertebrates in which haploidization and parthenogenesis (without contribution of a sperm) can occur artificially or naturally, methylation reprogramming of parental DNA is likely to be inessential for embryo viability and development. Given the known diversity of the animal kingdom, the ways reprogramming is achieved are expected to be intriguingly complicated.

Methylation changes during brain development

It has been known for many years that as the inner cell mass differentiates into different tissues post-implantation, there is a major remodeling of 5mC patterns, with widespread *de novo* methylation driven by DNMT3A and DNMT3B. It now appears that 5hmC patterns are also reworked during this period, with a decrease from the levels seen in ESC such that most adult tissue has considerably less 5hmC than seen in the stem cells (49, 50). The major exception here is of course the neural tissues, where 5hmC levels can reach up to 1% of total cytosine (13, 51, 52). It seems that rather than retaining 5hmC, early neural tissues have lost the 5hmC present in ESC and instead it is in late neural development that 5hmC levels start to rise, and they continue to do so with age (53). 5hmC enrichment can be found at transcriptional start sites (TSS) and gene bodies in neural tissues (52-55) and also appears to be enriched at enhancers as for ESC, although there are conflicting reports here (54, 56, 57). Both the timing of 5hmC increase and the areas of relative enrichment appear to be conserved between mouse and human, suggesting an important function (53, 58).

This is strengthened by the phenotypes seen on dysregulation of TET levels. Tet1 knockout resulted in decreased proliferation of neural progenitor cells in the adult mouse brain and a reduction in the progenitor pool (26). This was accompanied by a gain in 5mC at promoters of target genes and decreased transcription of adult neurogenesis-related genes. Overexpression of TET1 specifically in the brain on the other hand gave a decrease in 5mC and resulted in up-regulation of some genes (59-61). Interestingly, for the overexpression studies transcriptional effects were quite small, with two groups reporting the effects to be independent of TET1 catalytic activity. There was also an increase in 5mC levels at developmentally-important enhancers seen in TET2-deficient neural progenitor cells (NPC), suggesting that TET2 drives the timing of demethylation specifically at enhancers, thus regulating their function (54). Two independent studies using TET3 overexpression in the brain support a role for 5hmC in demethylation of 5mC at promoters (62, 63). Interestingly however, alterations in *intra-genic* 5hmC were more consistent with a role for gene body hydroxymethylation in *promoting* transcription directly, rather than as an intermediate in the conversion of 5mC to C, since for most loci examined levels of intragenic 5mC did not appreciably alter. In fact a number of lines of evidence support a role

for intragenic 5hmC as a stable epigenetic mark in its own right as recently proposed (64, 65), and bear looking at in more detail at this point.

Does intragenic 5hmC have a separate role?

While the generation of 5hmC from 5mC during PGC and zygotic development has been clearly linked to the processes of demethylation and remodeling occurring at these phases, as indicated in earlier sections of this review, an accumulation of circumstantial evidence suggests that at least some of the 5hmC seen in neural tissues may be acting as an epigenetic mark in its own right, and not just as a mechanism by which to regenerate cytosine from 5mC. Firstly, the levels of 5hmC in brain are far higher than in any other tissue and represent a substantial fraction of total methylated cytosine, suggesting turnover is extremely slow, if occurring at all. Levels of 5fC and 5caC, the next steps in the proposed demethylation pathway, are 1-2 orders of magnitude lower on the other hand, despite all three bases being generated by the same enzyme (15, 66). There is in fact some indication that there appears to be a regulatory step blocking further oxidation of 5hmC. 5caC increases without any change in 5hmC in TDG^{-/-} ESC, suggesting that TDG normally removes 5caC specifically (15). Likewise Tet3 overexpression gave a decrease in 5hmC in gene bodies without an increase in 5fC (62). 5hmC levels also increase during normal brain development without a decrease in 5mC (53), which would be expected if its sole purpose is to serve as an intermediary in the pathway. Tellingly, 5hmC is only found at CpG, whereas 5mC is also found at CpH (53, 56): this suggests that, at least at some sites, removal of 5mC either does not occur during the lifetime of the tissue, or does so by another pathway. In germ cells and particularly in zygotes, 5mC is converted to C with few or no cell divisions, suggesting that the build-up in brain is not just due to low rates of cell division. One hallmark of active demethylation would be the colocalisation of 5hmC and further oxidation products such as 5fC and 5caC. Only a fraction of sites show such colocalisation, further supporting a separate role for 5hmC (37, 67). Recent studies show that during neural development, 5fC and 5caC transiently accumulate at enhancer regions prior to and during the period when these elements lose 5mC and become activated (37, 68), suggesting that it is these elements rather than the gene bodies which are undergoing active demethylation.

If indeed 5hmC is a separate epigenetic mark, it would need to be specifically interpreted by elements of the transcriptional machinery. Consistent with this hypothesis, two groups have identified proteins which can bind to 5hmC using proteomics approaches (69, 70). As well as proteins which were already known to bind 5mC and which also appear to be capable of binding 5hmC, such as MeCP2 (71) and UHRF1, both groups identified proteins which appear to bind solely to 5hmC, although the same set of proteins was not identified in both studies, perhaps due to differences in tissues chosen or technical approaches. Furthermore, both groups identified a relatively

larger number of proteins which specifically bind to 5fC or 5caC, further supporting a separation of functions for 5hmC and further oxidation products.

A large proportion of 5hmC in brain is intragenic rather than at promoters or enhancers (55, 71, 72). It is possible that there may be a separation of roles with respect to establishment of 5hmC patterns. TET1 and TET3 contain CXXC domains, while the CXXC domain of TET2 appears to have been split off to form the IDAX protein, with which TET2 associates. CXXC domains are generally found in proteins which bind to CpG islands and in keeping with this the bulk of TET1 binding in ChIP studies was found to be at promoter regions and to depend on its CXXC domain (73). Furthermore, loss of TET1 led mainly to a gain in 5mC levels at promoters and enhancers, as well as marked effects on transcription (26, 73) (Fig. 3). In contrast TET2 lacks a CXXC region and depletion of this protein in neural tissue led to decreases in intragenic 5hmC levels mainly, with more subtle effects on mRNA levels (62). Association of TET protein with actively transcribing genes also leads to H3K4 methylation through its interaction with O-linked N-acetylglucosamine transferase (Fig. 3) (74, 75). This is more consistent with 5hmC facilitating transcription rather than being a strong activator. In keeping with a role in transcriptional read-through, the presence of 5hmC did not hinder polIII progression through a transcriptional unit, while 5fC and 5caC both did (76). Recently a similar role for intragenic 5mC has also been described by a number of groups (77, 78). Loss of 5mC in gene bodies, with attendant loss of its derivative 5hmC, has been shown to cause a decrease in transcription rather than the increase normally associated with demethyla-

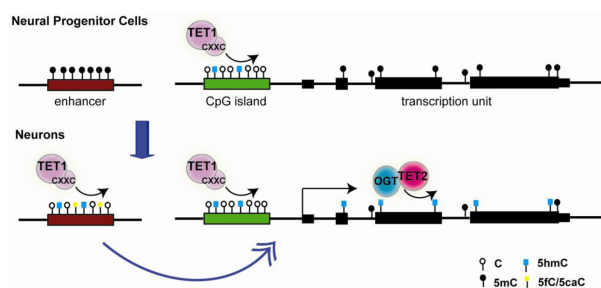


Fig. 3. Possible scenario for TET-mediated events during neural development. In progenitor cells promoter CpG islands may be maintained free of 5mC by the action of a TET protein, likely targeted to this region by its CXXC domain, while the gene body and distal enhancer elements are relatively heavily methylated. During differentiation a transient wave of TET1 activity frees enhancers of 5mC by oxidation to 5hmC then further to 5fC and 5caC, allowing interaction with the proximal promoter and driving transcription. TET2 association with the transcription unit drives the production of 5hmC and also chromatin alterations through its recruitment of O-linked N-acetylglucosamine transferase (OGT). Gene bodies are characterised by a high level of 5hmC, particularly at exons and near splice junctions, a moderate level of 5mC and the absence of substantial 5fC or 5caC.

tion of promoter regions. In brain tissue this contrast is particularly striking for testis-specific genes and neuronal genes: in the former, 5mC at the promoter represses transcription, while for the latter intragenic methylation (5mC and 5hmC) appear to facilitate transcription (72, 79). However the effect on transcription for the latter is small and other functions cannot be excluded. A role for intragenic methylation in exon choice and splicing has also been indicated in some studies, for example (56, 80). It is likely that careful study of the 5hmC-specific readers may help to identify if 5hmC has indeed a unique role as an epigenetic mark in its own right.

CONCLUDING REMARKS

The discovery of Tet-mediated 5mC oxidation has created a 'Tetonic' advance in our understanding of DNA demethylation (81). While many proposed demethylation paths have gone astray, Tet-mediated 5mC oxidation appears to hold true. Robust biochemical characterization of purified enzymes in combination with vigorous genetic analyses using gene-targeted mouse models has firmly established the central position of Tet enzymes in demethylation processes so far. However, one has to bear in mind that the above mechanism of DNA demethylation may not be exclusive. An interesting question remaining is whether there exist any enzymes or pathways other than Tet to oxidize and/or remove 5mC *in vivo*. A definitive answer to this question is however within reach now as a clear-cut result showing either the presence or absence of 5hmC signal in Tet-triple knockout mouse can soon be expected. As for biological function, Tet homologs in lower organisms seem to assume totally different roles as already seen for the JBP enzymes in trypanosomes. The driving force behind the evolutionary shift of Tet proteins toward epigenetic regulation in higher organisms deserves more attention and the study of Tet homologs in diverse organisms may hold promise for improving our comprehension of the essence of enzymatic DNA oxidation. The significance of mammalian methylome reprogramming, in particular during the zygotic stage, requires further investigation. Dissection of the regulatory networks governing 5mC oxidation to create epigenomic states compatible with lineage commitment and diversification remains another major task of future research.

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