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Review

DNA demethylation, Tet proteins and 5-hydroxymethylcytosine in epigenetic reprogramming: An emerging complex story

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ABSTRACT

Epigenetic reprogramming involves processes that lead to the erasure of epigenetic information, reverting the chromatin template to a less differentiated state. Extensive epigenetic reprogramming occurs both naturally during mammalian development in the early embryo and the developing germ line, and artificially in various *in vitro* reprogramming systems. Global DNA demethylation appears to be a shared attribute of reprogramming events, and understanding DNA methylation dynamics is thus of considerable interest. Recently, the Tet enzymes, which catalyse the iterative oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine, have emerged as potential drivers of epigenetic reprogramming. Although some of the recent studies point towards the direct role of Tet proteins in the removal of DNA methylation, the accumulating evidence suggests that the processes underlying DNA methylation dynamics might be more complex. Here, we review the current evidence, highlighting the agreements and the discrepancies between the suggested models and the experimental evidence.

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1. Introduction

Development of an organism starts with a totipotent zygote. Through series of cell divisions and the differentiation processes, this cell will eventually give rise to the whole organism containing hundreds of specialised cell types carrying out diverse physiological functions. While the cells at the onset of development have the capacity to generate all cell types (*i.e.* are toti- or pluripotent), this developmental capacity is progressively lost as cells undertake cell fate decisions [1]. At the molecular level, the memory of these differentiation events is laid down in a complex layer of epigenetic modifications at both the DNA and the chromatin level. In accordance with the unidirectional character of the developmental progress, the key acquired epigenetic modifications are stable and inherited through subsequent cell divisions. This paradigm is, however, challenged during cellular reprogramming that requires de-differentiation (reprogramming of somatic nucleus through nuclear transfer to the oocyte — SCNT,

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generation of induced pluripotent stem cells — iPS) or a change in cell fate (transdifferentiation).

The desire to reverse cell fate and thus to challenge the directionality of development has inspired generations of cell biologists; however despite intense efforts of numerous research teams, the molecular processes underlying cellular reprogramming remain mostly unknown. At the molecular level, cellular reprogramming must involve erasure of epigenetic information, resulting in reversion of the chromatin template to a less differentiated state characterised by low DNA methylation levels [2] and high chromatin plasticity [3]. Interestingly, both repressive histone methylation [4] and DNA methylation [5] have been identified as molecular barriers to successful reprogramming process. While histone modifications have been known to be highly dynamic for quite some time, and the mechanism of removal of histone methylation through jumanji domain containing histone demethylases has been described on the molecular level [6], molecular mechanisms underlying removal of DNA methylation have only now begun to be unravelled [7]. Recent discovery of the Tet family of oxygenases, which catalyse the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine and higher oxidative derivatives [8–10] (Fig. 1), has opened up a long sought after mechanistic route for DNA demethylation. Since the discovery of their enzymatic activity in 2009 [10], Tet enzymes have been implicated in reprogramming processes *in vivo* and *in vitro*, and linked with both active and passive mechanisms of DNA demethylation. The accumulating evidence, however, suggests that the exact molecular role of Tet proteins might be more complex than originally anticipated, and that additional mechanisms of DNA demethylation are potentially at play, at least during the major changes in DNA methylation associated with *in vivo* epigenetic reprogramming. This review summarises our current understanding of DNA (de)methylation dynamics during the processes of experimental epigenetic reprogramming *in vitro*, as well as during the developmental epigenetic reprogramming *in vivo*, with particular focus on the role of Tet driven 5mC oxidation.

2. DNA demethylation, Tet proteins and 5mC oxidative derivatives

Conceptually, the mechanisms of DNA demethylation can be distinguished by their dependence on DNA replication (Fig. 2): DNA methylation patterns are typically maintained in a faithful manner due to the activity of Dnmt1 DNA methyltransferase, which associates with the replication fork through its binding to PCNA and Uhrf1 and provides specific activity on the hemi-methylated newly replicated DNA [11–13]. Loss of this maintenance methylation activity results in **passive DNA demethylation** (Figs. 2A, B), a gradual loss of DNA methylation demonstrated in systems lacking Dnmt1 or Uhrf1 tethering Dnmt1 to the replication fork and the hemi-methylated DNA [12,13].

As an alternative mechanism, **active DNA demethylation** would lead to the removal of 5mC in a replication independent manner (Figs. 2C, D). Several molecular mechanisms of active DNA demethylation have been proposed; these include "reverse" enzymatic reaction driven by DNA methyltransferases in the absence of S-adenosylmethionine (SAM, a donor of methyl group) [14–16], or an involvement of MBD binding proteins [17,18]. It should be, however, noted that these mechanisms still await *in vivo* validation.

In a manner similar to flowering plants that utilise 5mC specific glycosylases Dme and Ros1 [19], DNA repair has also been implicated in the active DNA demethylation processes in higher vertebrates, including in *Xenopus*[20], and in both mouse zygotes and developing germ cells (see below) [21]. As the protein family of 5mC specific DNA glycosylases seems to have evolved independently in flowering plants with no direct sequence homology in higher vertebrates, the described role of DNA repair in DNA demethylation in these organisms might require the existence of an additional 5mC modification that would trigger the observed DNA repair response. In alignment with this idea, 5mC has been suggested to be modified to thymine by the enzymatic activity of DNA deaminases [22] and consequently activation induced DNA deaminase (Aid, also known as Aicda) has been implicated in DNA demethylation processes in both zebrafish and mouse development [23, 24], and in *in vitro* reprogramming systems [25]. It should, however, be noted that DNA deaminases (including Aid) generally prefer unmodified cytosines in a single stranded context as their substrate, and hence their potential activity on 5mC is very limited, at least *in vitro*[26]. Further experimental evidence is thus required to clarify the extent of the contribution of this molecular pathway to observed instances of DNA demethylation.

The discovery of the Tet family of enzymes converting 5mC to 5-hydroxymethyl cytosine (5hmC), and its higher oxidative products 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), and the evidence for the presence of 5hmC in mammalian DNA, opened up a new possible mechanistic route for 5mC removal [8–10,27]. Tet (1–3) enzymes can oxidise 5mC to generate 5hmC that can be diluted through subsequent rounds of replication due to the low enzymatic activity of Dnmt1 on the hemi-hydroxymethylated DNA (Fig. 2B) [28]. In an alternative scenario, 5hmC can be further oxidised to 5fC and 5caC; both of these modified bases have been shown to be targeted by TDG DNA glycosylase and the lesion subsequently processed through the BER DNA repair pathway [8] (Fig. 2C). Tet driven 5mC oxidation thus provides a direct mechanistic route for both passive and active DNA demethylation. However, in view of current findings discussed below, it is likely that additional, as yet unidentified, molecular pathway(s) of DNA demethylation may exist. Global changes in DNA methylation observed in various reprogramming systems are thus likely to require a concerted action of several DNA demethylation mechanisms.

3. Epigenetic reprogramming and waves of global DNA demethylation *in vivo*

3.1. Zygotic DNA demethylation

Extensive epigenetic reprogramming occurs at two stages during normal development: in the early zygote, immediately following fertilisation; and in the primordial germ cells of the developing embryo (Fig. 3). In mammals, following fertilisation, the highly condensed and methylated paternal genome is decondensed through protamine removal and replacement with the histone variant H3.3 [29]. This is immediately followed by extensive and rapid DNA demethylation [30, 31], while the maternal methylome remains mostly unchanged [32, 33]. From the two cell stage onwards, both the paternal and maternal genomes undergo progressive loss of methylation until the blastocyst

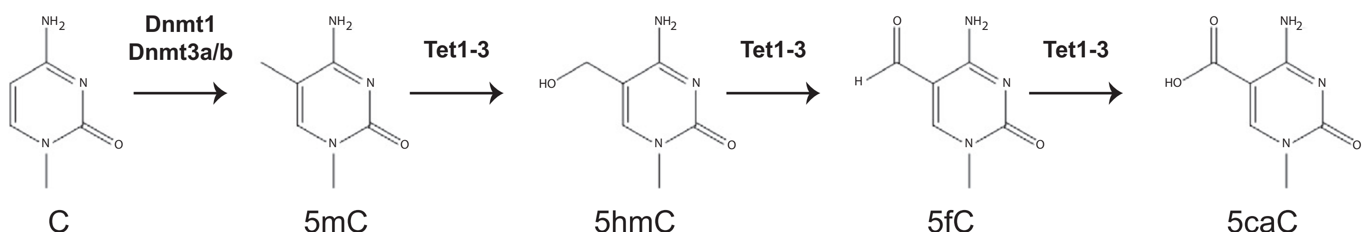


Fig. 1. Overview of enzymatic cytosine modifications observed in mammalian DNA.

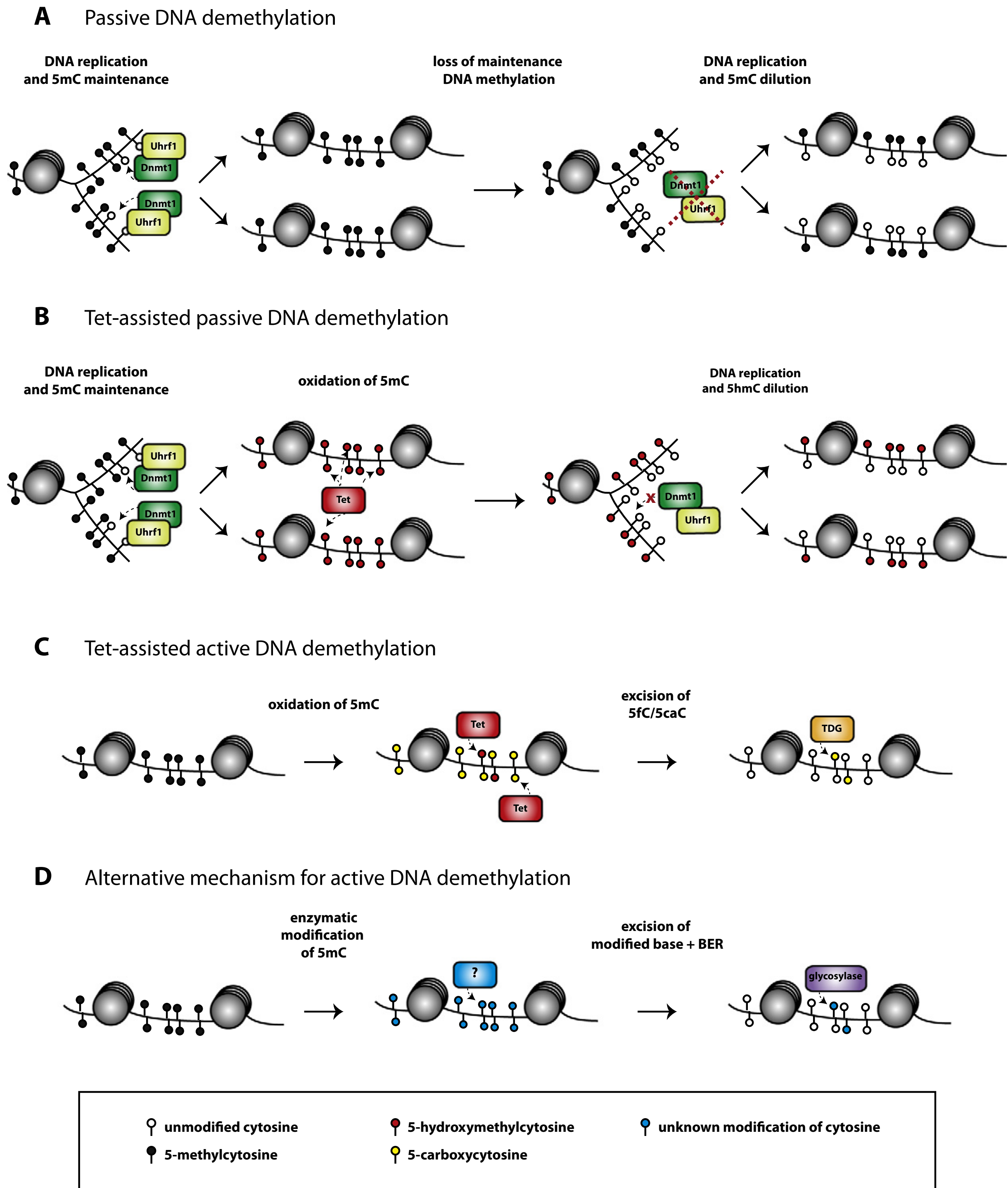


Fig. 2. Proposed mechanisms of DNA demethylation. (A) Lack of methylation maintenance during DNA replication leads to 5mC dilution, eventually leading to fully unmethylated DNA. (B) Conversion of 5mC to 5hmC impacts on the maintenance activity of Dnmt1 (*in vitro*) resulting in dilution of 5hmC through DNA replication, eventually leading to fully unmethylated DNA. (C) 5hmC can be further converted by Tet enzymes to 5fC and 5caC that are targeted for excision by TDG glycosylase followed by BER repair. (D) Alternative (as yet uncharacterised) pathway for DNA demethylation involving BER repair.

stage [32,33] when the embryonic genome contains very low levels of DNA methylation just prior to implantation [2]. High levels of DNA methylation are subsequently re-established in the epiblast [34] immediately following the implantation due to a specific expression of Dnmt3b *de novo* DNA methyltransferase [35].

Numerous reports have demonstrated that the loss of paternal DNA methylation occurs a few hours following fertilisation *via* a rapid replication-independent mechanism [21,30,36,37]. In agreement with the possible involvement of 5hmC in DNA demethylation, Tet3 oxygenase has been shown to be highly expressed in mouse oocytes [38,39]. Additionally, immunofluorescence staining using 5hmC specific antibodies identified this modification enriched in the paternal pronucleus following DNA demethylation [38,39]. Subsequently, in the 2 cell stage embryos, clear 5hmC signal is still detectable in half of the nucleus

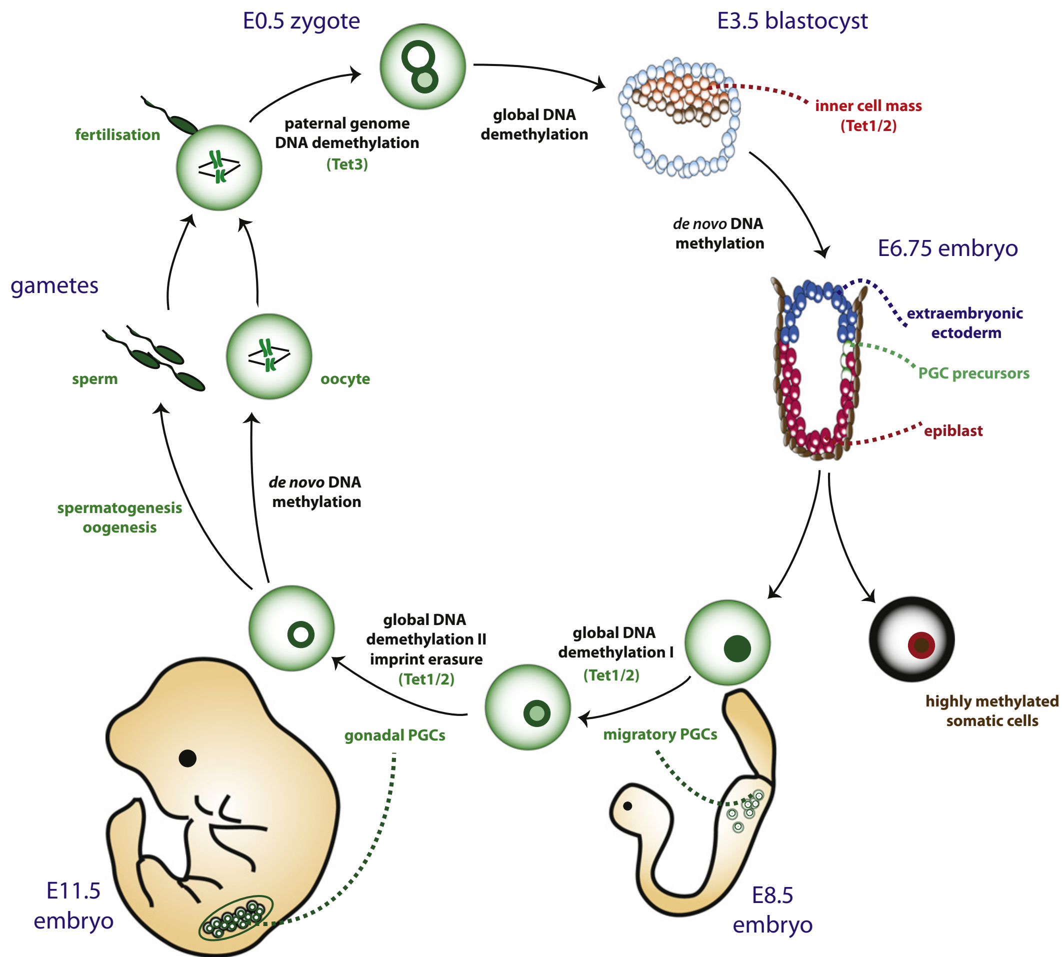


Fig. 3. Overview of key developmental stages with genome-wide DNA demethylation during mouse development indicating the expression of relevant Tet proteins.

corresponding to paternal chromatin [40]; this signal gradually diminishes during further preimplantation development along with the decreased expression of *Tet3* [38,40]. In this context, it has been previously shown that hemi-hydroxylated DNA is not efficiently recognised by Dnmt1 as a substrate [28]. Consequently, and in combination with the findings above, it has been suggested that the zygotic DNA demethylation proceeds through oxidation of 5mC to 5hmC that is followed by a passive loss of 5hmC through DNA replication [39,40]. In addition to the correlation between 5hmC accumulation and 5mC disappearance, Tet3-deficient oocytes unable to catalyse the formation of 5hmC, showed global increase of 5mC on the paternal pronucleus and at specific loci, including *Oct4* (also known as *Pou5f1*) [39].

More recent results, however, have shed new light on the role of 5hmC during early embryo reprogramming. A detailed analysis of the kinetics of 5mC and 5hmC on the paternal pronucleus revealed that 5mC disappears from the paternal genome by early PN3 stage, while 5hmC starts to accumulate only after the major drop in 5mC has occurred, and increases considerably from PN4 onwards ([41] and R. A., P.H. unpublished data). This observation is not consistent with the idea of direct conversion of 5mC to 5hmC. It should be also noted that, while targeting of 5hmC to the demethylating genome appears to be conserved in mammals [38], 5hmC is not enriched on the demethylated genome in other vertebrates during early embryo reprogramming [42,43].

The suggested model of zygotic demethylation through 5hmC formation also implicates the presence of high levels of 5hmC in the paternal genome (*i.e.* all 5mC being converted to 5hmC). To the contrary, the actual 5hmC levels in the zygote are very low (R.A., P.H. unpublished). Consistent with this, Tet-assisted bisulphite sequencing (TAB-Seq), which allows for locus-specific quantification of 5hmC at CpG dinucleotides, revealed that average hydroxymethylation of the paternal genome is only 5% at the 2-cell stage, compared with the average methylation level in sperm, which is 80% [32]. In addition, only 10% of CpGs demethylated between sperm and 2-cell embryos are enriched for 5hmCpG at the 2-cell stage [32]. These results thus argue against a model whereby 5mC from sperm is converted to 5hmC and passively diluted during preimplantation development. Last, but not least, although Dnmt1 seems to have lower activity on hemi-hydroxylated DNA *in vitro*, Uhrf1, responsible for Dnmt1 recruitment to the replication fork, has been shown to bind to both 5mC and 5hmC [44,45], further questioning a simple model of 5hmC driven passive DNA demethylation.

In an alternative explanation, it is possible that the zygotic 5hmC undergoes fast turnover through higher 5mC oxidative derivatives (5fC and 5caC) followed by excision and BER driven repair (Fig. 2C). Both 5fC and 5caC have been detected in the paternal pronucleus of late zygotic stages [46]. Additionally, BER and DNA breaks have been previously linked to the DNA demethylation process in the zygote [21,37]. However, there is currently no evidence linking activation of BER in the early zygote and Tet driven 5hmC formation, and given the detectable presence of 5hmC only in late stage zygotes (a few hours following DNA demethylation), it is conceivable that an alternative molecular mechanism is implicated in the initial rapid loss of 5mC in the early zygote, with 5hmC possibly playing a separate role during the late zygotic stages (Fig. 5A). These results thus advocate a necessity for further investigation into the extent of 5hmC contribution and the exact molecular mechanism of zygotic DNA demethylation.

3.2. Germline DNA demethylation

3.2.1. Loss of DNA methylation in nascent and migratory PGCs

DNA methylation that has accumulated in the embryo following implantation needs to be reset during the development of the early germ line in primordial germ cells (PGCs), the embryonic precursors of gametes. In mice, PGCs are first specified from the pluripotent epiblast cells [47], followed by extensive global changes to histone modifications [48,49]. Following specification, nascent germ cells actively migrate from their location in the underlying endoderm of the invaginating hindgut into the genital ridge [50,51], which contains the embryonic gonads. In comparison to the epiblast cells from which PGCs are derived, migratory germ cells show low level of global DNA methylation [34,52]. Several observations have given insight into the possible molecular mechanism by which global DNA methylation is reduced in migratory PGCs. Following specification, nascent PGCs downregulate Glp, a cofactor of G9a H3K9 histone methyltransferase, leading to a genome wide loss of H3K9me2 specifically in early germ cells [48,49]. The combination of a lack of the usual cross-talk between H3K9me2 and DNA methylation [53], and the additional observed downregulation of *de novo* DNA methyltransferases Dnmt3a and Dnmt3b in nascent germ cells [54], could (at least partially) explain the erosion of DNA methylation following PGC specification. Moreover, slight downregulation of *Dnmt1* and *Uhrf1* is detectable on the mRNA level following PGC specification [54], although this could be linked to the change in the replication time observed in nascent PGCs (both aforementioned factors are cell cycle regulated). Considering these observations, a model of passive DNA demethylation has been put forward in migratory PGCs [34]. However, the complete lack of both maintenance and *de novo* DNA methylation would lead to much faster and extensive DNA demethylation than is observed in migratory PGCs [34,55,56]. Additionally, some of the genomic regions, such as genomic imprints, seem to maintain DNA methylation levels throughout this period of PGC development [21,34,48,52,57]. This suggests that the mechanism implicated in the loss of 5mC in pre-gonadal PGCs is likely to be more complex.

Expression of both Tet1 and Tet2 oxygenases and clear immunofluorescence signal for 5hmC have been detected in migrating PGCs, alluding to a possible role for 5hmC in the observed loss of DNA methylation during this phase [58,59]. However, *in vitro* studies using *in vitro* derived PGC-like cells (PGCLCs) or induced PGCs (iPGCs), which mimic migratory PGCs [60,61], have come to conflicting conclusions regarding the requirements of Tet1 and Tet2 during pre-gonadal germline development [58,59]. It is also interesting to note that the epigenetic regulation in migrating PGCs shares many similarities with that observed in naïve mouse pluripotent stem cells cultured in “2i” conditions [2,62]. Both systems are characterised by the downregulation of *de novo* DNA methyltransferases [2,54] and by the lack of MAPK signalling [62,63]. To this point, and consistent with the studies in PGCLCs, low levels of global 5mC have been recapitulated in Tet1/2 DKO mouse ESCs cultured in the presence of 2i, suggesting that Tet1/2 oxygenases play potentially only a minor role in the 5mC reduction observed in early mouse germ cells [64].

3.2.2. Epigenetic reprogramming in gonadal PGCs

The last major event that characterises early germline development is the rapid and global epigenetic reprogramming that occurs in PGCs upon colonisation of the gonads, where the germ cells will reside throughout the rest of embryonic and adult life (Fig. 3). This process includes extensive DNA demethylation during which most of genomic DNA methylation, including genomic imprints, is erased, and methylation on most repetitive elements severely reduced [21,34,52,57]. This reprogramming process additionally entails a transient loss of heterochromatin-associated modifications [48], and the reactivation of the inactive X-chromosome in female germ cells [65]. Following gonadal epigenetic reprogramming, the genome of PGCs shows the lowest level of genome-wide DNA methylation seen at any point of development. This global hypomethylated state is long lived and lasts until postnatal development in female germ cells that start to reacquire DNA methylation only during the oocyte growth in postnatal ovaries [66]; to the contrary, male germ cells start to accumulate DNA methylation relatively quickly only a few days following the global reprogramming [66].

The molecular mechanism underlying the rapid genome-wide gonadal removal of DNA methylation, including the erasure of genomic imprints, has been a subject of intense scientific debate, with both passive [55,56,58] and active [21,23] models of DNA demethylation proposed. Most recently, the observation that PGCs upregulate the expression of Tet1 oxygenase [21,58] has raised the possibility of a role for 5hmC in this process. Moreover, a 5hmeDIP approach revealed the presence of 5hmC at genomic imprints in PGCs undergoing DNA demethylation, implicating Tet1 driven 5hmC formation in germline imprint erasure [58]. Based on this study, the proposed model of germline DNA demethylation involves oxidation of 5mC to 5hmC followed by a passive loss of 5hmC through DNA replication (Fig. 2B). This model is thus reminiscent of the molecular mechanism proposed to explain zygotic DNA demethylation. Again, however, there are numerous inconsistencies between the predicted scenario and the experimental observations. For example, as bisulphite sequencing does not distinguish between 5mC and 5hmC [67], conversion of 5mC to 5hmC followed by gradual 5hmC dilution should be observable by gradual signal dilution in the Bis-Seq datasets derived from gonadal PGCs. This is, however, in stark contrast with the observed rapid signal loss ([34] and P.W.S.H., P.H. unpublished). Additionally, similar to the situation in the zygote, the absolute level of 5hmC in PGCs is very low, further arguing against 5mC to 5hmC conversion followed by replication driven dilution (P.W.S.H., R.A., P.H. unpublished). Again, both discrepancies could be potentially explained by rapid 5hmC turnover caused by 5hmC progression to 5fC and 5caC intermediates followed by the excision and BER driven DNA repair (Fig. 2C), which would be consistent with the observed activation of the BER machinery at this time[21]. It should, however, be pointed out that it is currently not known, whether the observed activation of BER in PGCs is mechanistically dependent on Tet1/2 activity.

Further information regarding potential roles of 5hmC and Tet oxygenases in germline DNA demethylation has come from studies using Tet1 single knockout (Tet1 KO) and Tet1/2 double knockout (Tet1/2 DKO) mice [68–70]. Studies on Tet1 KO PGCs revealed increased DNA methylation on some of the imprint control regions and meiotic gene promoters [69,70]. Moreover, Tet1 KO and Tet1/2 DKO mice have a fertility phenotype consistent with defective imprinting [68,69], suggesting that genomic imprints have not been properly reprogrammed. Interestingly, this does not seem to affect all the embryos, as a proportion of embryos generated from mating Tet1/2 DKO males or females with Tet1/2 double-heterozygous (Dhet) partners shows normal imprinting methylation pattern and survives to adulthood [68]. Additionally, immunostaining revealed that the genome-wide levels of DNA methylation in Tet1/2 DKO and wt PGCs are comparable [68], suggesting that the germline DNA demethylation is to a large extent Tet independent, and that only a subset of loci may be affected in the absence of 5hmC formation ([55,58,68,69] and P.W.S.H., P.H. unpublished). In light of these observations, further investigation into the extent and mechanism by which 5hmC regulates DNA methylation during PGC reprogramming is required.

4. Epigenetic reprogramming *in vitro*

4.1. The role of 5hmC and Tet proteins in iPS cell reprogramming

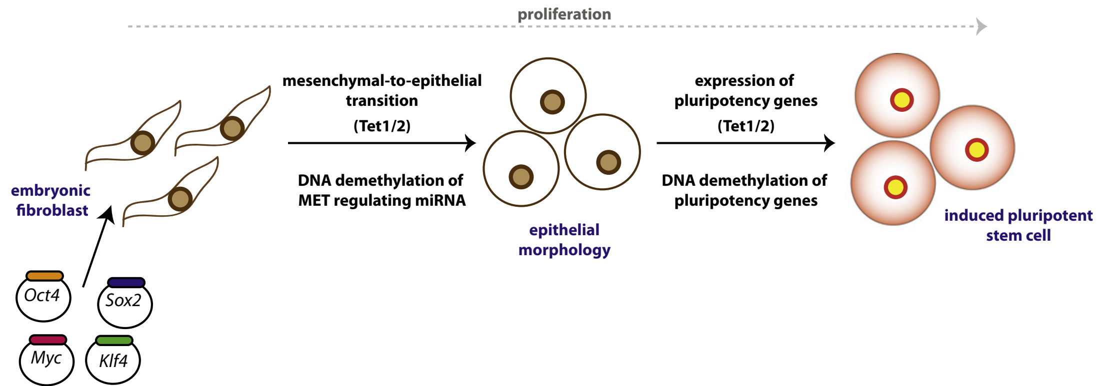
While originally considered to be irreversibly committed to their fate, it has now been shown that terminally differentiated nuclei retain considerable plasticity and can be reprogrammed to a less differentiated state *in vitro*. The best of the *in vitro* reprogramming methods studied is

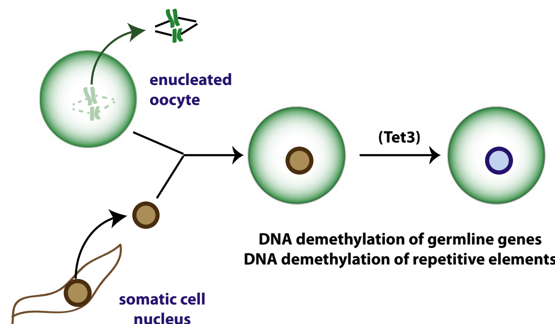
the transcription-factor based approach developed by Takahashi and Yamanaka, whereby ectopic expression of the transcription factors Oct4, Sox2, Klf4 and Myc (OSKM) results in the generation of induced pluripotent stem (iPS) cells [71] (Fig. 4A). Inducing pluripotency through the ectopic expression of transcription factors requires extensive chromatin remodelling, including DNA demethylation which has been shown to be rate limiting to the reprogramming process [5,72,73].

Similar to *in vivo* reprogramming, 5hmC levels are increased during iPSC generation, most likely as a direct result of increased Tet1 and Tet2 expression [74–79]. Depletion of Tet1 or Tet2 significantly reduces iPSC reprogramming efficiency in conventional reprogramming medium [75–79], and triple knockout Tet1/2/3 (Tet-TKO) mouse embryonic fibroblasts completely fail to generate iPSC colonies following OKSM overexpression [79]. Although these results clearly implicate a role for Tet proteins during iPSC generation, there is considerable debate regarding which loci are targeted by Tet proteins and 5hmC [76–79], and whether these are in fact necessary only in certain contexts, and not for the general reacquisition of pluripotency.

Whether Tet1 is implicated in promoter and enhancer demethylation and gene activation during iPSC reprogramming has been partially addressed in a study reporting that Tet1 is necessary for the demethylation of the *Oct4* enhancer and promoter regions followed by the transcriptional reactivation of this gene [78]. The authors studied the kinetics of 5mC and 5hmC by MeDIP and hMeDIP respectively, and observed an unexpected accumulation of 5mC at the regulatory elements of *Oct4* and other genes on day 1 post reprogramming [78]. This DNA methylation increase is followed by 5hmC accumulation, eventually resulting in DNA demethylation of the target sequences. The observed 5mC accumulation was greatly increased upon depletion of Tet1, ultimately leading to the incomplete reactivation of *Oct4*, while overexpression of a catalytically active, and to a lesser extent catalytically inactive, Tet1 prevented this increase [78]. The authors also show that a catalytically active, but not mutant, Tet1, can replace Oct4 in the OSKM transcription factor cocktail, suggesting a functional role of Tet1 driven 5-hydroxymethylcytosine in Oct4 reactivation and iPSC reprogramming [78].

Recent studies have provided further details regarding a functional role for Tet1 and Tet2 during iPSC reprogramming [76,79]. Interestingly, these studies show that Tet1 driven 5hmC is not responsible for DNA demethylation of *Oct4* regulatory elements [76]. Rather, the Tet family of proteins is only necessary for cells to undergo mesenchymal-to-epithelialtransition (MET) [76,79]. Supporting this claim, Tet-TKO mouse embryonic fibroblasts (MEFs) showed no indication of epithelium-like morphology upon expression of OSKM reprogramming factors, a phenotype that could only be rescued by the overexpression of a enzymatically-active Tet2 catalytic domain [79]. Moreover, epithelium-like somatic cells lacking all three Tet proteins, as well as MEFs acutely depleted of Tet proteins immediately following MET, can reprogram to pluripotency efficiently in the presence of vitamin C [79]. The authors further argue that Tet proteins catalyse the oxidation of 5mC at the *mir200* cluster, which has been previously shown to regulate epithelial markers [79]. Oxidized 5mC is subsequently excised by TDG, resulting in DNA demethylation and thus *mir200* family expression enabling MET. This model is supported by the observation that 5hmC accumulation, DNA demethylation and the expression of the *mir200* family do not occur in Tet-TKO MEFs (with Tet2 single knockout





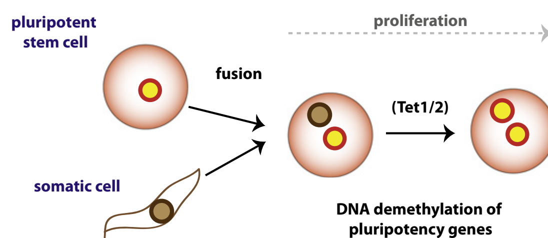


Fig. 4. Overview of *in vitro* reprogramming systems with a described role for Tet proteins.

having the most pronounced effect) [79]. Moreover, TDG-deficient MEFs show an iPSC conversion defect similar to Tet-TKO MEFs, supporting the model of DNA demethylation through TDG driven excision of higher 5mC oxidative derivatives [79].

Together, these results argue that, while Tet-mediated 5mC oxidation is necessary for MET, Tet proteins are dispensable for the reacquisition of pluripotency in the presence of vitamin C. Further studies will thus be necessary to clarify the role of vitamin C and the exact molecular pathway leading to Tet independent DNA demethylation of the promoters of pluripotency genes in this system.

4.2. The role of 5hmC in other in vitro reprogramming systems

Epigenetic reprogramming of somatic nuclei can also occur by a somatic cell nuclear transfer (SCNT) or through cell fusion. SCNT involves the transfer of a somatic nucleus into an enucleated oocyte [80]

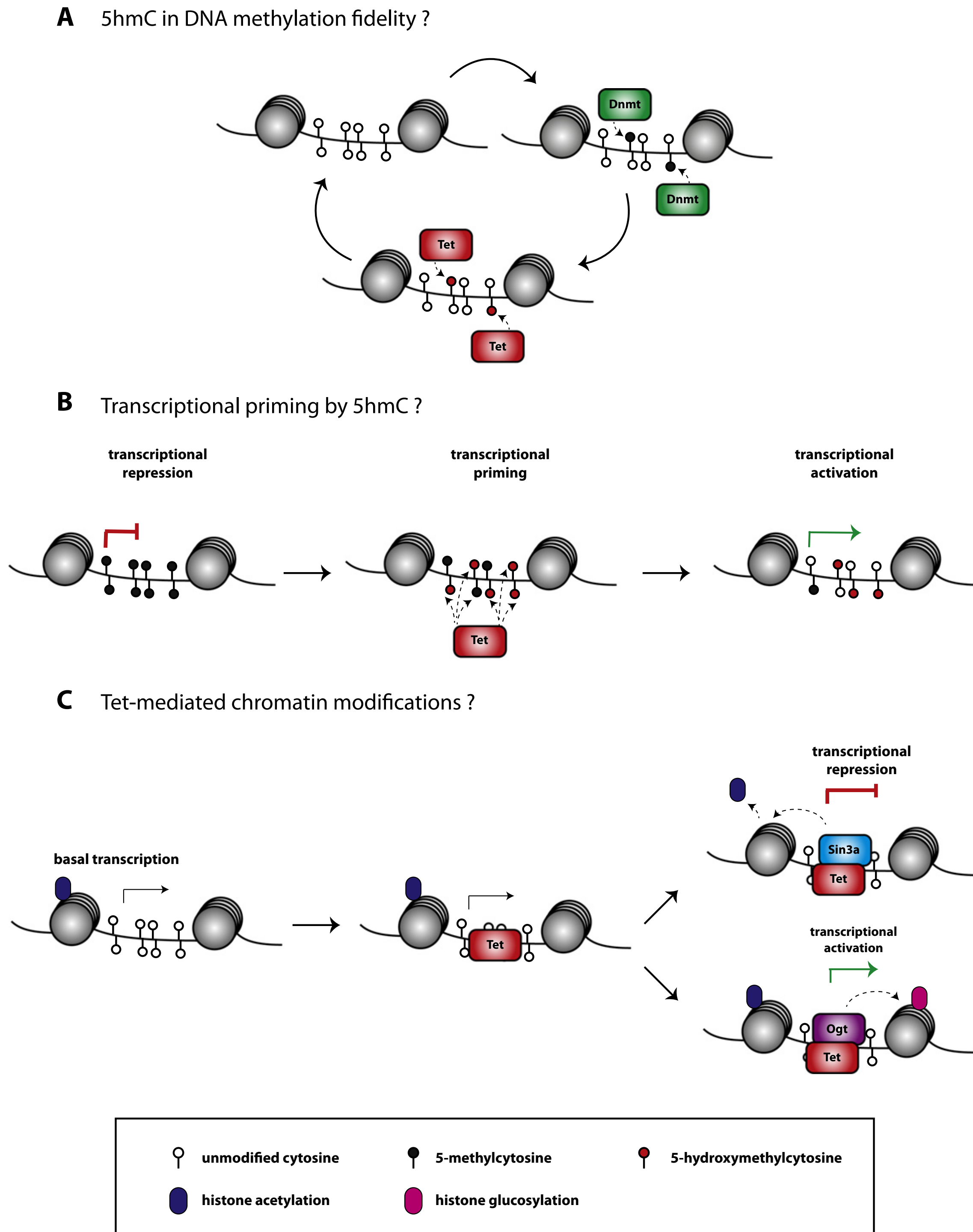


Fig. 5. Potential roles for Tet proteins and Tet driven 5hmC formation in reprogramming. (A) Tet driven 5hmC has been implicated in counteracting aberrant *de novo* DNA methylation activity. (B) 5hmC might be directly implicated in removal of repressive 5mC in a locus specific manner to allow for transcriptional induction. (C) Biochemical studies revealed that Tet proteins are found within complexes containing various chromatin modifying activities that can affect chromatin configuration and the transcriptional status of the target loci independent of 5mC oxidation.

(Fig. 4B), which initiates extensive epigenetic reprogramming, comprising DNA demethylation of numerous genomic loci including repetitive elements and germline genes [81,82]. It should be noted that, while DNA demethylation is extensive during SCNT, it pales in comparison with the zygotic DNA demethylation observed *in vivo* on the paternal pronucleus following fertilisation [82]. Cell fusion reprogramming approaches involve fusion between somatic cells and pluripotent stem cells [83,84], initiating reprogramming of the somatic epigenome, including DNA demethylation at regulatory elements of pluripotency genes [84,85] (Fig. 4C).

While the role for 5hmC has been investigated extensively during iPSC reprogramming, limited studies currently exist describing the role for 5hmC during other *in vitro* types of somatic cell reprogramming. Depletion of Tet2 during reprogramming following cell fusion of somatic cells with embryonic stem (ES) or embryonic germ (EG) cells revealed that Tet2, but not Tet1, is required for a normal reactivation of *Oct4*, *Nanog* and *Cripto* (also known as *Tdgf1*) expression, as well as for 5hmC formation on the *Oct4* gene body [86]. Investigations using Tet1/2 DKO and Tet-TKO ES cells would be informative to elucidate further details and functional requirements for Tet driven 5hmC formation during the reprogramming in this system.

During SCNT, it has been shown that the Tet3 protein originating from the oocyte cytoplasm becomes concentrated in the pseudo-pronucleus (PPN), resulting in the oxidation of 5mC specifically in the PPN [39]. Upon depletion of Tet3, 5hmC formation on the PPN does not occur, and no substantial demethylation of the *Oct4* promoter or reactivation of *Oct4* expression is observed [39].

5. Conclusions and remaining questions

To date, research into the role of Tet proteins during reprogramming processes has focussed on the role of 5hmC as an intermediate in DNA demethylation pathways. *In vitro*, studies using iPSC reprogramming models have established a definitive requirement for Tet driven 5hmC and TDG in active DNA demethylation of loci regulating MET [79]. While a requirement for 5hmC during DNA demethylation has been suggested for other loci during *in vitro* reprogramming [77,78], including a number of pluripotency genes, Tet proteins seem dispensable for the reacquisition of pluripotency [79]. Thus, further work is required to assess the exact dynamics of 5mC and 5hmC modifications and elucidate the exact function of 5mC to 5hmC conversion in these instances.

In vivo, the relationship between 5hmC and DNA demethylation is more unclear. Early models suggesting that the passive dilution of 5hmC following the conversion of 5mC to 5hmC drives global DNA demethylation were based on experiments using non-quantitative, antibody based approaches [40,46,58]. At least in the early mouse embryo, more recent quantitative data has suggested significant inconsistencies with the original model, including the observations that the absolute level of 5hmC is very low compared to 5mC levels, and that 5hmC enriched regions at the two cell stage do not overlap with the regions demethylated between sperm and the early embryo [32].

While the recent studies have mainly focused on the role of Tet proteins and 5hmC during the waves of genome-wide DNA demethylation, there is an increasing evidence that the Tet oxygenases have other regulatory functions, such as in DNA methylation fidelity and in transcriptional regulation [87] (Fig. 5). With respect to the maintenance of DNA methylation patterns, studies in ES cells have suggested that Tet1 activity counteracts aberrant *de novo* DNA methylation, allowing for the maintenance of the unmethylated state [87]. This is further supported by studies in HEK293 cells showing that the overexpression of full length Tet1 protein affects DNA methylation only at sparsely methylated genomic loci [88] (Fig. 5A). With respect to transcriptional regulation, it has been shown that 5hmC is enriched at enhancers and gene bodies of actively transcribing genes in a variety of cell types both *in vitro* and *in vivo*[89,90] (see also the reviews by Wen and Tang and by Marques and colleagues, this issue). Moreover, a role for 5hmC in transcriptional priming has been hypothesized [77] (Fig. 5B). Intriguingly, studies in ES cells have revealed that the majority of Tet1 transcriptional regulation is independent of 5mC-to-5hmC conversion [87] (Fig. 5C). Tet1 appears to bind predominantly to unmethylated, CpG rich regions [87], and can recruit chromatin modifiers either directly (Sin3a [87]) or indirectly (PRC2 [91]); similarly, Tet2 interacts with and guides Ogt to chromatin, enabling glucosylation of H2B and thus high levels of transcription [92].

In this context, there is only limited information regarding potential roles of 5hmC and Tet-mediated chromatin modifications in transcriptional regulation during epigenetic reprogramming processes both *in vivo* and *in vitro*. However, certain observations suggest that this function of Tet proteins may be of a fundamental importance. For example, during PGC reprogramming, depletion of Tet1 leads to the down-regulation of a large subset of genes necessary for a normal meiotic progression [70]; remarkably, however, there was less than a 5% overlap between differentially methylated regions and altered gene expression [70], suggesting that the observed phenotype may in fact be regulated by an indirect or a DNA demethylation-independent mechanism.

In summary, the emerging picture regarding the role of Tet proteins is complex. The combined evidence implicates a role for 5hmC and Tet oxygenases in the locus specific control of 5mC levels, while further investigations will be required to elucidate the exact role of 5hmC and Tet proteins during the waves of genome wide DNA demethylation connected with developmental reprogramming. The experimental evidence further suggests that 5hmC might be more dynamic than originally assumed, while biochemical studies of the Tet(1–3) containing complexes clearly argue for the necessity to consider biological roles of Tet proteins outside 5mC oxidation, in particular during transcriptional regulation and in the modulation of local chromatin structure.

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