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DNA demethylases: a new epigenetic frontier in drug discovery

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DNA methylation is one of the most extensively studied, and one of the most stable, of all epigenetic modifications. Two drugs that target DNA methyltransferase enzymes are licensed for clinical use in oncology but relatively little attention has focused on the enzymatic pathways by which DNA methylation can be reversed. Recent breakthroughs have identified at least two classes of enzymes that can achieve functional reversal. This review discusses the significance of DNA demethylation in a range of human diseases, the candidate proteins that mediate the demethylation and the opportunities and challenges in targeting these candidates to develop new therapeutics.

Background to DNA methylation

Methylation of DNA is a covalent modification that occurs mainly on the 5C position of cytosines located in CpG dinucleotide motifs, and it was the first epigenetic modification to be characterized at the molecular level [1]. Whereas epigenetics is a term that has been defined several of ways, DNA methylation matches most of the criteria employed by various workers in this field (i.e. a modification that influences gene expression and other aspects of genome function, and can be inherited through cell divisions but does not alter the underlying base sequence). Although recent evidence shows that non-CpG methylation also exists, particularly at CpA and CpT sites, and is abundant in embryonic stem cells [2,3], this review focuses on the canonical CpG methylation.

In general, DNA methylation is associated with a repressive chromatin state, mediated via the binding of specific proteins (e.g. methyl-CpG binding proteins) [4] and by affecting the accessibility of transcription factors to promoters and other regulatory regions of genes [5]. DNA methylation is frequently associated with repressive modifications of histone tails such as trimethylation of the lysine 9 residue of H3 (H3K9me3), and a predominantly hypoacetylated histone state [6]. DNA methylation plays a crucial part in long-term silencing mechanisms such as genomic imprinting [7], X-chromosome inactivation [8] and silencing of retrotransposons [9]. In addition, this epigenetic mark is also important for cellular differentiation and identity [10].

Abnormal methylation is associated with several types of disease. A significant subset of human cancers show characteristic changes in DNA methylation patterns, with hypermethylation of a relatively small number of genes, including some tumour suppressors, against a background of general hypomethylation [11]. Several imprinting disorders, such as Prader–Willi/Angelman syndrome (PWS/AS) or Beckwith–Wiedemann syndrome (BWS) are caused by incorrect establishment or maintenance of parental-specific DNA methylation imprinting marks [12,13].

Establishing and maintaining DNA methylation

The establishment and maintenance of DNA methylation are relatively well-understood at a biochemical level and are performed in mammalian cells by the DNA methyltransferases (DNMTs). These catalyse the addition of a methyl group to cytosine in DNA using *S*-adenosyl-L-methionine as the methyl donor. *De novo* DNA methylation is performed by DNMT3A and DNMT3B [14], which are targeted to specific genomic regions, at least in part by the presence of repressive histone modifications and the absence of activating histone modifications, respectively [6]. This is consistent with a model whereby gene transcription is initially repressed via relatively

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transient histone modifications, and then silenced via longer-lasting DNA methylation. However, there are instances where genes are silenced directly by DNA methylation, without any apparent role of histone modifications [15]. DNMT3B is specifically required to establish methylation in pericentromeric repetitive regions [16]. Mutations in this gene in humans can cause immunodeficiency centromeric instability facial anomalies (ICF) syndrome [17,18]. DNMT3A is essential for the establishment of maternal and paternal imprints and for normal spermatogenesis, at least in part through suppression of retrotransposon expression [19]. Another factor that cooperates with DNMT3A and 3B in the establishment of methylation is DNMT3L, which lacks enzymatic activity owing to the absence of the conserved catalytic motifs [20].

DNMT1 is the maintenance DNA methyltransferase. It recognizes the hemimethylated state that is generated at a methylated CpG motif following DNA replication, and regenerates a fully methylated locus [21]. Np95 is an important partner for DNMT1 that recognizes hemimethylated DNA and is required for targeting DNMT1 to the replication fork [22].

DNA demethylation – passive and active mechanisms

DNA demethylation can occur passively (i.e. through a failure of the DNMT1 mechanism to re-establish the fully methylated state following DNA replication and cell division). This passive model does not explain the loss of DNA methylation in non-replicating cells, which has been reliably established. Active demethylation can occur on a large (i.e. potentially genome-wide) scale in primordial germ cells and in early embryos (reviewed in Ref. [23]), whereas in somatic cells demethylation occurs in a more locus-specific fashion as outlined below.

Reprogramming of methylation patterns in the early embryo is needed for the acquisition of pluripotency and for subsequent lineage commitment. The developmental waves of active DNA demethylation during development are shown in Fig. 1. In the zygote there is rapid DNA demethylation of the paternal pronucleus post-fertilization but before cell division, and at least partly also before DNA replication. This is in contrast to the gradual loss of methylation from the maternal genome through the first few cleavage divisions in the embryo. The latter is probably a case of passive DNA demethylation but the former is likely to occur through an active mechanism.

The second example of genome-wide erasure of DNA methylation occurs in primordial germ cells (PGCs) following their migration into the gonadal anlagen. Genome-wide DNA demethylation occurs around days 11.5–13.5 post-conception in the mouse. Although this occurs in the presence of cell division, it occurs

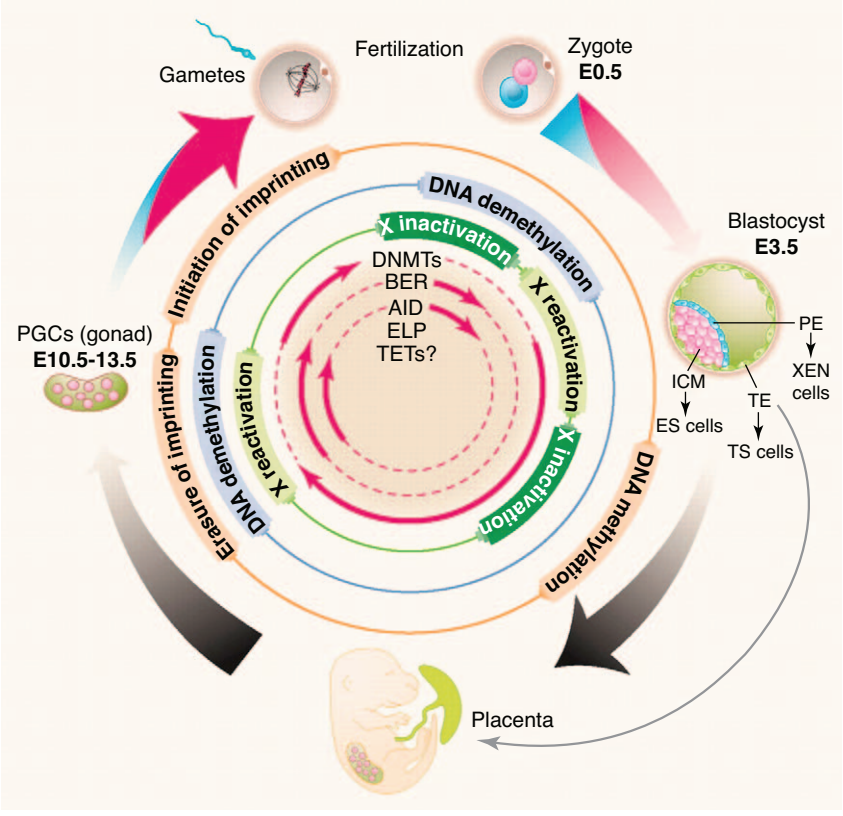

FIGURE 1

DNA demethylation during development. The diagram shows the two major phases of genome-wide erasure of DNA methylation in the early embryo and in primordial germ cells (PGCs) of the mouse. The levels of DNA methylation are indicated by the thickness of the outer arrows. The maternal genome is indicated in red and the paternal in blue. The rapid post-fertilization of the paternal genome is indicated. The inner circle shows candidate factors implicated in *de novo* methylation, maintenance of methylation and demethylation, respectively. The developmental time-points during which these factors are active are indicated by the solid arrows. *Abbreviations*: ES, embryonic stem; TS, trophectoderm stem; XEN, extra-embryonic endoderm; ICM, inner cell mass; TE, trophectoderm; PE, primitive endoderm; ELP, elongation protein. Reproduced from [23].

surprisingly rapidly and is also accompanied by the engagement of base excision repair, so could at least in part be an active process [23]. The outcomes of reprogramming of DNA methylation in PGCs are the erasure of somatic imprinting marks, enabling later-stage germ cells to establish parental-specific patterns according to the sex of the germ line, and the erasure of acquired epimutations so that their transgenerational inheritance is prevented [23].

More recently, active DNA demethylation has been proposed as a gene regulation mechanism in selected adult tissues. In proliferating cells it is difficult experimentally to distinguish between passive and active demethylation. It is easier to identify active DNA demethylation unequivocally in non-dividing cells, so it is perhaps unsurprising that the majority of data for this process in adult tissues have come from that archetypal post-mitotic cell type – the neuron.

Some of the most striking data have been generated in animal models of emotional stress, focusing on genes that play key parts in the limbic–hypothalamic–pituitary axis (LHPA) and mediate long-term responses to stress via glucocorticoid secretion. Arginine vasopressin (AVP) is a key stimulator of adrenocorticotrophin (ACTH) release from the pituitary. In mice subjected to early life stress (maternal–infant separation) a specific AVP enhancer was hypomethylated in the paraventricular nucleus resulting in persistent overexpression of the gene [24]. Like AVP, corticotrophin-releasing factor (CRF) is secreted by the cells of the paraventricular nucleus and activates the LHPA system. In an alternative mouse model of emotional stress (i.e. social defeat) the CRF promoter was hypomethylated in those mice susceptible to stress, and this was associated with increased expression of the gene. If the neurons in this brain region are genuinely post-mitotic, this argues for the presence of an active DNA demethylation process [25]. Active DNA demethylation does not appear to be restricted to the central nervous system, having also been reported in renal cells [26] and in post-replicative human peripheral blood mononuclear cells [27].

Mechanisms of active DNA demethylation

The biochemical process behind active DNA demethylation has been an area of significant controversy, with thermodynamic predictions suggesting that the 5C-methyl modification would be highly stable. Hence, it is still unclear if chemistry exists around which the methyl group can be directly removed from 5-methylcytosine by breaking the carbon–carbon bond. Candidate proteins involved in active DNA demethylation are listed in Table 1. Broadly, these either reverse the methylation in a multistep process coupled to DNA repair mechanisms or further modify the 5-methylcytosine into a different compound.

In *Arabidopsis*, a family of DNA glycosylases [e.g. DEMETER (DME), DEMETER-LIKE 2 and 3 (DML2, DML3) and REPRESSOR OF SILENCING 1 (ROS1)] has been identified that is involved in the regulation of imprinting and other epigenetic gene silencing processes [28–31]. The members of this family are large bifunctional DNA glycosylases that recognize and excise 5-methylcytosine in addition to having lyase activity. Subsequent base excision repair (BER) steps then insert an unmethylated cytosine in the place of the methylated one that was removed. These large plant glycosylases do not appear to have mammalian counterparts. Although it has been claimed that the mammalian glycosylases Thymine-DNA glycosylase (TDG) [32,33] and Methyl-CpG binding domain 4 (MBD4) might have activity against 5-methylcytosine, these claims need further clarification, especially through *in vivo* studies.

Because this process of demethylation is a multistep one, it can be difficult to recreate in its entirety by *in vitro* biochemical assays. In addition, some of the proteins that have been put forward as candidates for active DNA demethylation could facilitate or increase the efficiency of demethylation, without being key catalytic components themselves. Growth arrest and DNA-damage-inducible protein 45 (GADD45) is an example of such a protein [34].

Of the candidates shown in Table 1 the greatest interest is currently focused on the cytidine deaminases: activated induced cytidine deaminase (AID) and Apolipoprotein B mRNA editing enzyme 1 (APOBEC1), and on the TET family. Figure 2 is a schematic of the biochemical modifications performed by these candidate proteins.

AID was originally identified as an enzyme that is essential for the maturation of antibody responses, because it is required for somatic hypermutation of immunoglobulin genes and immunoglobulin class switching [35]. AID and APOBEC1 can deaminate cytosine and 5-methylcytosine *in vitro* [36,37]. AID has now been implicated in active DNA demethylation, potentially working in concert with the BER pathway [38]. The AID-mediated deamination of 5-methylcytosine leads to a T:G mismatch that can be resolved by DNA glycosylases such as TDG working as part of the BER pathway. This has clear parallels with the role of the DNA glycosylases in plants. In zebrafish AID has worked cooperatively with one of the MBD family members, MBD4, to promote DNA demethylation [39]. One of the other proteins listed in Table 1, GADD45, might also cooperate in this process.

In PGCs, described earlier as one of the key tissues in which large-scale DNA demethylation occurs, knocking out AID results in a significantly altered methylation profile [38]. AID deficiency therefore interferes with global DNA demethylation. In fusions of embryonic stem cells and somatic cells, Aid appears to be involved in aspects of reprogramming and demethylation of pluripotency genes such as Octamer-binding protein 4 (*OCT4*) or *NANOG* [40].

TET1, TET2 and TET3 were initially identified as candidate DNA demethylases via their homology to trypanosomal proteins that hydroxylate the methyl group of thymine as part of a synthetic pathway that creates a non-canonical nucleoside, base J [41]. Human TET1 protein has catalysed the conversion of 5-methylcytosine to 5-hydroxymethylcytosine in cultured cells and in cell-free biochemical assays. Further studies in mice have demonstrated that all three TET family members can convert 5-methylcytosine to 5-hydroxymethylcytosine, and the generation of Tet1 knockdowns has indicated a potential role for this protein in early embryo development and in the function of embryonic stem cells [42]. TET-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine in ES cells is preferentially located at the bodies of actively transcribed genes and in the promoter regions of Polycomb-repressed developmental regulators, and is associated with increased gene transcription [43–45]. 5-Hydroxymethylcytosine accumulates in the paternal pro-nucleus during zygotic reprogramming, during the period when 5-methylcytosine levels drop so dramatically [46].

Recent work suggests that 5-hydroxymethylcytosine can be further modified enzymatically in a cell in a reaction that might be carried out by the AID or APOBEC deaminases [47].

TABLE 1

Biochemical pathways of DNA demethylation

Gene families and pathways	Gene	Proposed role in demethylation of 5-methylcytosine	References
Cytidine deaminases			
AID	Activation-induced cytidine deaminase	Deamination of cytosine and 5-methylcytosine	[38,40]
APOBEC1	Apolipoprotein B mRNA editing enzyme 1		
TET proteins			
TET1	Tet oncogene 1	Conversion of 5-methylcytosine to 5-hydroxymethylcytosine	[41,42]
TET2	Tet oncogene 2		
TET3	Tet oncogene 3		
Elongator complex			
ELP1	Elongation protein 1 homolog	Identified via genetic screens for factors that affect paternal genome demethylation	[68]
ELP2	Elongation protein 2 homolog	Biochemical mechanism of action unknown	
ELP3 (KAT9)	Elongation protein 3 homolog		
Base excision repair			
RNF4	RING finger protein 4	Candidate members of base excision repair pathway that act to promote active demethylation following deamination of 5-methylcytosine	[69,39,70–73]
TDG	Thymine-DNA glycosylase		
APE1	APEX nuclease		
MBD2	Methyl-CpG binding domain protein 2		
MBD3			
MBD4	Methyl-CpG binding domain protein 3		
XRCC-1	Methyl-CpG binding domain protein 4		
PARP-1	X-ray repair complementing defective repair in Chinese hamster cells 1		
Nucleotide excision			
GADD45A	Growth arrest and DNA-damage-inducible protein 45alpha and 45beta	Promote demethylation, possibly as part of this pathway. Non-enzymatic proteins	[73,34]
GADD45B			
DNA methyltransferases			
DNMT3A	DNA methyltransferase 3A	Mechanism unknown, possibly deamination	[74]
DNMT3B	DNA methyltransferase 3B		
DNA glycosylases			
DME	Demeter	Excision of 5-methylcytosine which is followed by base excision repair. Only reported in plants	[28–31]
DML2	Demeter-like 2		
DML3	Demeter-like 3		
ROS1	Repressor of silencing 1		

The table summarizes the biochemical pathways that have been proposed to function in active DNA demethylation. It attempts to capture the overall landscape of publications on active DNA demethylation but it should be noted that this field is complex and many reports are considered contentious. For a useful summary of the issues and controversies, see Ref. [75].

It is important to note that, although the TET enzymes do not apparently convert 5-methylcytosine to the fully unmodified form of the base, the consequences can be similar to total reversion. This is because many of the cellular effects of cytosine methylation are mediated via the binding of specific proteins, such as Methyl CpG binding protein 2 (MeCP2) and members of the MBD family [4]. The methyl-binding proteins that have so far been tested are unable to bind to the unmodified base or the 5-hydroxymethylcytosine, and therefore the TET-mediated modification is functionally inactive with respect to these mediators of epigenetic gene repression [48]. If, however, proteins are identified in the future that bind specifically to 5-hydroxymethylcytosine then this modification itself might be an epigenetic mark that can signal via specific mediators.

Commercial utility of active DNA demethylation

One of the most exciting breakthroughs in biology in the first decade of the 21st century has been the creation of induced pluripotent stem (iPS) cells. The seminal 2006 publication from Kazutoshi Takahashi and Shinya Yamanaka showed that transduction with four pluripotency genes was sufficient to convert fully differentiated cells into pluripotent non-lineage committed stem

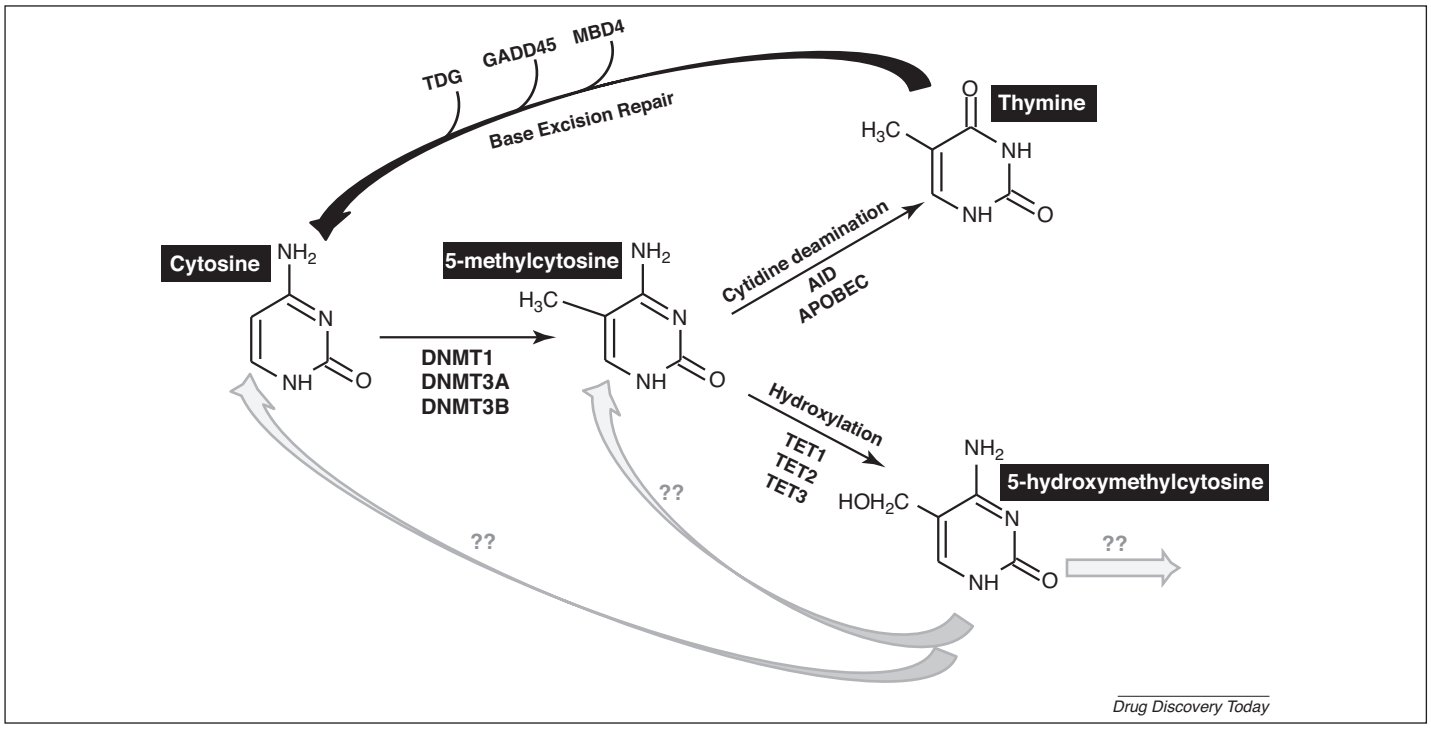

FIGURE 2

The chemistry of cytosine methylation and demethylation. A schematic representation of the major mechanisms of vertebrate cytosine methylation and active demethylation. Gene names are as stated in the manuscript text and in Table 1. BER refers to base excision repair. Gene names in grey circles refer to proteins implicated in BER but whose precise roles in active DNA demethylation have not yet been fully determined. The asterisk indicates more than one member of a protein family has been implicated in the pathway.

cells [49]. Although it is still a long way from clinical use, this technology has the potential to transform cellular therapies because it offers the promise of patient-specific stem cell implantation. In the shorter term the technology will be used by pharmaceutical companies to generate new disease screening platforms and additional toxicology assays. Significant progress has been made since the original publication in improving the applicability and success of the technique; but cellular reprogramming remains relatively inefficient, in terms of both the number of cells converted and the time that the process takes [50]. Additionally, iPS cells still do not exhibit exactly the same functional characteristics as 'normal' embryonic stem cells [51]. The inefficiency and the disparity appear to be strongly related to epigenetic status, including DNA demethylation [52]. Indeed, the rate-limiting step currently looks to be the efficiency with which the treated cells can strip out the DNA methylation marks associated with the differentiated state and return to a zygote-like demethylated genome.

AID-dependent DNA demethylation is essential for reprogramming, albeit it in a different model system (i.e. heterokaryon formation rather than genetic reprogramming with pluripotency genes) [40]. Relatively little is understood about how endogenous active DNA demethylation is targeted in cells. However, in experimental systems it could be possible to target components of the DNA demethylation pathway to specific cellular loci using technologies such as zinc finger localization. This will facilitate locus-specific DNA demethylation and reactivation of specific target genes. Although the importance of the other non-AID putative DNA demethylation pathways in cellular reprogramming remains to be established, it is possible that strategies enhancing active DNA demethylation could improve the commercial viability of iPS cell technology.

Small-molecule inhibitors of active DNA demethylation pathways

As described above, active DNA demethylation can be associated with long-term gene regulation in the central nervous system, particularly those regions associated with stress. The Arginine vasopressin (*Avp*) and Corticotrophin-releasing factor (*Crf*) genes are not unique in becoming hypomethylated in post-mitotic neurons. For example, experiments in isolated murine hippocampal neurons have demonstrated selective DNA demethylation of the promoter of the brain-derived neurotrophic factor (BDNF) gene in response to synaptic activity [53].

It has been recognized for many years that children who suffer severe early life neglect or abuse are at substantially elevated risk of several long-term neuropsychiatric conditions, including drug addiction, depression and suicide [54]. These appear to correlate with the chronic over-activation of the LHPA pathways and it is certainly possible that hypomethylation of the key activating genes is a feature in these long-term sequelae. Some of the hypomethylation changes seen in rodent models and described earlier have been reported in human patients with a history of childhood abuse [55].

This raises the possibility of creating novel therapeutics in neuropsychiatric disorders by the development of inhibitors of active DNA demethylation. There are, however, several hurdles to overcome before this can become a reality. Some of these are scientific – and the majority of the evidence in support of this

approach is largely correlative at present. As such, it is unclear which of the known DNA demethylation mechanisms is operating in these central nervous system events, and understanding this will be a prerequisite for a rational drug discovery approach. Although identification of the target enzymes will be a helpful step forward, there are few known starting points for a medicinal chemistry campaign for any of them, which could necessitate a HTS of several million compounds to identify useful hits.

Drugs to target neuropsychiatric indications will have to penetrate the blood–brain barrier. It is unclear whether it would be necessary or feasible to ensure clinical delivery into selected brain regions, nor can we predict the effects of these currently hypothetical compounds on global brain methylation levels, as opposed to just the abnormally demethylated genes of the LHPA axis.

There is also an ethical aspect that will require careful consideration. The most appropriate therapeutic intervention under this model might be the inhibition of active DNA demethylation at a crucial time-point in childhood when gene regulation patterns are being set in the brain. This would mean treating children to prevent a chronic condition. However, not all children who suffer early abuse or neglect go on to develop long-term neuropsychiatric conditions, and it is not clear how the most appropriate patients would be identified. Safe and appropriate dosing regimens are likely to be challenging.

Inhibition of active DNA demethylation could also be of use in other therapeutic areas, namely oncology. As already stated, many cancers are associated with hypermethylation of specific genes (e.g. tumour suppressors) against a background of hypomethylation. There are two clinically licensed inhibitors of the DNMT1 maintenance methyltransferase – the nucleoside analogues 5-aza-cytidine (Vidaza®) and 5-aza-2′-deoxycytidine (Dacogen®). These are used as therapies in myelodysplastic syndrome [56]. In proliferating cancer cells the methylation and repression of the tumour suppressors are maintained by DNMT1 and the inhibitors relieve this gene repression, promoting reactivation of tumour suppressor activity [57].

Until recently, the global hypomethylation of the majority of the genome in many cancers has received less attention and there has been an almost tacit assumption that this is a passive process with little functional significance. But if hypermethylation of tumour suppressors has a functional effect in cancer progression then so might hypomethylation of genes that promote cell cycle proliferation, inhibit apoptosis or enhance migration. Examples of such genes include Transketolase-like 1 (*TLKT1*) in head and neck squamous cell carcinomas [58] and the c-ROS receptor tyrosine kinase in malignant glioma [59]. In addition, hypomethylation of structural components of the genome such as centromeres impairs their function, leading to mis-segregation of chromosomes and karyotypic abnormalities [60]. If this hypomethylation is simply a passive process, perhaps a consequence of mistargeting DNMT1 in the genome, then the therapeutic opportunities are limited; but what if at least some of this hypomethylation is a consequence of active DNA demethylation?

One argument against this is that TET2 mutations have been implicated in several cases of haematological malignancies [61]. Bone marrow samples from patients with mutations in the catalytic domain of TET2 have decreased levels of 5-hydroxymethylcytosine compared with controls [62]. The prognostic implications of these findings are currently unclear.

The existing data suggest that not all proteins involved in demethylation of DNA are created equally, at least in relationship to cancer. Mutations in TET2 are relatively common in haematological tumours and AID has also been proposed to be involved in the development and progression of several cancers, although this has usually been ascribed to its somatic mutator function, rather than its role in DNA demethylation (reviewed in Ref. [63]). Conversely, neither TET1 nor TET3 appears to be a commonly mutated gene in cancer [61], suggesting that the TET family members might have different cellular roles despite their apparent biochemical similarities. Owing to technical issues it is also relatively unclear at the moment whether the hypomethylation that has been reported many times in cancer reflects an over-abundance of unmethylated cytosine relative to 5-methylcytosine, or if it is a combination of unmethylated cytosine and 5-hydroxymethylcytosine.

Intriguing new data suggest that active DNA demethylation could indeed play a part in tumour development and progression [64]. Zebrafish embryos were generated that were homozygous for a mutation in the *apc* gene. This mutation creates a truncated protein similar to that found in many cases of human colon cancer. Various genes implicated in active DNA demethylation increased in expression in these mutants. These included the orthologues of GADD45 (various family members), AID, APOBEC and MBD4. This increased expression was associated with decreased DNA methylation which could be reversed by specific morpholino treatment to knockdown candidate gene expression. AID, GADD45a and MBD4 were also upregulated in colon adenoma samples from patients with homozygous APC mutations who presented with the familial adenomatous polyposis (FAP) phenotype. The active DNA demethylation was implicated in maintaining the mutant cells in a relatively non-differentiated state, which is characteristic of several cancers [64].

These findings suggest that the active DNA demethylation pathway(s) could represent a potential new epigenetic therapeutic intervention point in oncology. The data from the zebrafish experiment suggest that multiple proteins could all play an important part in mediating this active demethylation. This could be significant from a drug discovery aspect because it provides a variety of opportunities. Targets can be prioritized by their chemical tractability and by their biological attractiveness. For example, if their normal role in healthy cells is relatively restricted they could be more attractive targets than proteins that intersect with many cellular pathways and functions. Until recently this would have focused predominantly on the enzymatic candidates but progress in developing small-molecule inhibitors of proteins that bind to epigenetic modifications might offer alternative possibilities [65].

Concluding remarks

Our understanding of DNA methylation might be about to undergo the same revolution as has already occurred in the field of histone methylation. Histone methylation was believed to be irreversible until 2004 when Yang Shi demonstrated that the Lysine-specific demethylase 1 (LSD1) protein is a histone demethylase [66]. Over 20 histone demethylases have now been identified in the human genome and this class is an active source of drug discovery targets

(reviewed in Ref. [67]). The same might be about to occur with DNA demethylation. Researchers have identified several proteins that take part in this process and we are beginning to recognize that active DNA demethylation is not restricted to selected tightly specified events in very early development but has a more global impact that we are just starting to understand and exploit.

References

1 Miller, J.R. *et al.* (1978) The nucleotide sequence of oocyte 5S DNA in *Xenopus laevis* II. The GC-rich region. *Cell* 13, 717–725
2 Lister, R. *et al.* (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462, 315–322
3 Ramsahoye, B.H. *et al.* (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc. Natl Acad. Sci. USA* 97, 5237–5242
4 Lopez-Serra, L. *et al.* (2008) Unmasking of epigenetically silenced candidate tumor suppressor genes by removal of methyl-CpG-binding domain proteins. *Oncogene* 27, 3556–3566
5 Watt, F. and Molloy, P.L. (1988) Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. *Genes Dev.* 2, 1136–1143
6 Hawkins, R.D. *et al.* (2010) Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. *Cell Stem Cell* 6, 479–491
7 Reik, W. *et al.* (1987) Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature* 328, 248–251
8 Mohandas, T. *et al.* (1981) Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science* 211, 393–396
9 Miura, A. *et al.* (2001) Mobilization of transposons by a mutation abolishing full DNA methylation in Arabidopsis. *Nature* 411, 212–214
10 Farthing, C.R. *et al.* (2008) Global mapping of DNA methylation in mouse promoters reveals epigenetic reprogramming of pluripotency genes. *PLoS Genet* 4, e1000116
11 Feinberg, A.P. and Vogelstein, B. (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 301, 89–92
12 Driscoll, D.J. *et al.* (1992) A DNA methylation imprint, determined by the sex of the parent, distinguishes the Angelman and Prader-Willi syndromes. *Genomics* 13, 917–924
13 Schneid, H. *et al.* (1993) Parental allele specific methylation of the human insulin-like growth factor II gene and Beckwith–Wiedemann syndrome. *J. Med. Genet.* 30, 353–362
14 Okano, M. *et al.* (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, 247–257
15 Borgel, J. *et al.* (2010) Targets and dynamics of promoter DNA methylation during early mouse development. *Nat. Genet.* 42, 1093–1100
16 Bachman, K.E. *et al.* (2001) Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. *J. Biol. Chem.* 276, 32282–32287
17 Hansen, R.S. *et al.* (1999) The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proc. Natl Acad. Sci. USA* 96, 14412–14417
18 Xu, G.L. *et al.* (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* 402, 187–191
19 Kaneda, M. *et al.* (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429, 900–903
20 Aapola, U. *et al.* (2000) Isolation and initial characterization of a novel zinc finger gene, DNMT3L, on 21q22.3, related to the cytosine-5-methyltransferase 3 gene family. *Genomics* 65, 293–298
21 Pradhan, S. *et al.* (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J. Biol. Chem.* 274, 33002–33010
22 Rottach, A. *et al.* (2010) The multi-domain protein Np95 connects DNA methylation and histone modification. *Nucleic Acids Res.* 38, 1796–1804
23 Feng, S. *et al.* (2010) Epigenetic reprogramming in plant and animal development. *Science* 330, 622–627
24 Murgatroyd, C. *et al.* (2009) Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nat. Neurosci.* 12, 1559–1566
25 Elliott, E. *et al.* (2010) Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. *Nat. Neurosci.* 13, 1351–1353
26 Kim, M.S. *et al.* (2009) DNA demethylation in hormone-induced transcriptional derepression. *Nature* 461, 1007–1012
27 Bruniquel, D. and Schwartz, R.H. (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat. Immunol.* 4, 235–240
28 Gehring, M. *et al.* (2006) DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. *Cell* 124, 495–506
29 Choi, Y. *et al.* (2002) DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in arabidopsis. *Cell* 110, 33–42
30 Gong, Z. *et al.* (2002) ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. *Cell* 111, 803–814
31 Ortega-Galisteo, A.P. *et al.* (2008) Arabidopsis DEMETER-LIKE proteins DML2 and DML3 are required for appropriate distribution of DNA methylation marks. *Plant Mol. Biol.* 67, 671–681
32 Zhu, B. *et al.* (2000) 5-methylcytosine-DNA glycosylase activity is present in a cloned G/T mismatch DNA glycosylase associated with the chicken embryo DNA demethylation complex. *Proc. Natl. Acad. Sci. USA* 97, 5135–5139
33 Zhu, B. *et al.* (2000) 5-Methylcytosine DNA glycosylase activity is also present in the human MBD4 (G/T mismatch glycosylase) and in a related avian sequence. *Nucleic Acids Res.* 28, 4157–4165
34 Barreto, G. *et al.* (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature* 445, 671–675
35 Muramatsu, M. *et al.* (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102, 553–563
36 Morgan, H.D. *et al.* (2004) Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. *J. Biol. Chem.* 279, 52353–52360
37 Petersen-Mahrt, S.K. and Neuberger, M.S. (2003) *In vitro* deamination of cytosine to uracil in single-stranded DNA by apolipoprotein B editing complex catalytic subunit 1 (APOBEC1). *J. Biol. Chem.* 278, 19583–19586
38 Popp, C. *et al.* (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature* 463, 1101–1105
39 Rai, K. *et al.* (2008) DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45. *Cell* 135, 1201–1212
40 Bhutani, N. *et al.* (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature* 463, 1042–1047
41 Tahiliani, M. *et al.* (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324, 930–935
42 Ito, S. *et al.* (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 466, 1129–1133
43 Wu, H. *et al.* (2011) Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. *Genes Dev.* 25, 679–684
44 Wu, H. *et al.* (2011) Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. *Nature* 473, 389–393
45 Ficz, G. *et al.* (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473, 398–402
46 Wossidlo, M. *et al.* (2011) 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. *Nat. Commun.* 2, 241
47 Guo, J.U. *et al.* (2011) Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell* 145, 423–434
48 Valinluck, V. *et al.* (2004) Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). *Nucleic Acids Res.* 32, 4100–4108
49 Takahashi, K. and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676
50 Yamanaka, S. (2009) Elite and stochastic models for induced pluripotent stem cell generation. *Nature* 460, 49–52
51 Wang, Y. *et al.* (2010) A transcriptional roadmap to the induction of pluripotency in somatic cells. *Stem Cell Rev.* 6, 282–296
52 Lagarkova, M.A. *et al.* (2010) Induction of pluripotency in human endothelial cells resets epigenetic profile on genome scale. *Cell Cycle* 9, 937–946
53 Nelson, E.D. *et al.* (2008) Activity-dependent suppression of miniature neurotransmission through the regulation of DNA methylation. *J. Neurosci.* 28, 395–406
54 Gilbert, R. *et al.* (2009) Burden and consequences of child maltreatment in high-income countries. *Lancet* 373, 68–81

55 McGowan, P.O. *et al.* (2009) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat. Neurosci.* 12, 342–348

56 Cang, S. *et al.* (2010) Clinical advances in hypomethylating agents targeting epigenetic pathways. *Curr. Cancer Drug Targets* 10, 539–545

57 Bender, C.M. *et al.* (1998) Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res.* 58, 95–101

58 Sun, W. *et al.* (2010) TKTL1 is activated by promoter hypomethylation and contributes to head and neck squamous cell carcinoma carcinogenesis through increased aerobic glycolysis and HIF1alpha stabilization. *Clin. Cancer Res.* 16, 857–866

59 Jun, H.J. *et al.* (2009) Epigenetic regulation of c-ROS receptor tyrosine kinase expression in malignant gliomas. *Cancer Res.* 69, 2180–2184

60 Ji, W. *et al.* (1997) DNA demethylation and pericentromeric rearrangements of chromosome 1. *Mutat. Res.* 379, 33–41

61 Abdel-Wahab, O. *et al.* (2009) Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood* 114, 144–147

62 Ko, M. *et al.* (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* 468, 839–843

63 Maul, R.W. and Gearhart, P.J. (2010) AID and somatic hypermutation. *Adv. Immunol.* 105, 159–191

64 Rai, K. *et al.* (2010) DNA demethylase activity maintains intestinal cells in an undifferentiated state following loss of APC. *Cell* 142, 930–942

65 Filippakopoulos, P. *et al.* (2010) Selective inhibition of BET bromodomains. *Nature* 468, 1067–1073

66 Shi, Y. *et al.* (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941–953

67 Natoli, G. *et al.* (2009) The future therapeutic potential of histone demethylases: a critical analysis. *Curr. Opin Drug Discov. Devel.* 12, 607–615

68 Okada, Y. *et al.* (2010) A role for the elongator complex in zygotic paternal genome demethylation. *Nature* 463, 554–558

69 Hu, X.V. *et al.* (2010) Identification of RING finger protein 4 (RNF4) as a modulator of DNA demethylation through a functional genomics screen. *Proc. Natl Acad. Sci. USA* 107, 15087–15092

70 Bhattacharya, S.K. *et al.* (1999) A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* 397, 579–583

71 Ng, H.H. *et al.* (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat. Genet.* 23, 58–61

72 Hajkova, P. *et al.* (2010) Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway. *Science* 329, 78–82

73 Jin, S.G. *et al.* (2008) GADD45A does not promote DNA demethylation. *PLoS Genet.* 4, e1000013

74 Metivier, R. *et al.* (2008) Cyclical DNA methylation of a transcriptionally active promoter. *Nature* 452, 45–50

75 Ooi, S.K. and Bestor, T.H. (2008) The colorful history of active DNA demethylation. *Cell* 133, 1145–1148