

DNA methylation in mouse embryonic stem cells and development

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Abstract Mammalian development is associated with considerable changes in global DNA methylation levels at times of genomic reprogramming. Normal DNA methylation is essential for development but, despite considerable advances in our understanding of the DNA methyltransferases, the reason that development fails when DNA methylation is deficient remains unclear. Furthermore, although much is known about the enzymes that cause DNA methylation, comparatively little is known about the mechanisms or significance of active demethylation in early development. In this review, we discuss the roles of the various DNA methyltransferases and their likely functions in development.

Keywords DNA methylation · Development · Imprinting · Chromatin · Embryonic stem cells

Introduction

Mammalian development is associated with considerable changes in global DNA methylation levels at times of genomic reprogramming. Normal DNA methylation is essential for development but, despite considerable advances in our understanding of the DNA methyltransferases, the reason that development fails when DNA methylation is deficient remains unclear. Furthermore, although much is known about the enzymes that cause DNA methylation, comparatively little is known about the mechanisms or significance of active

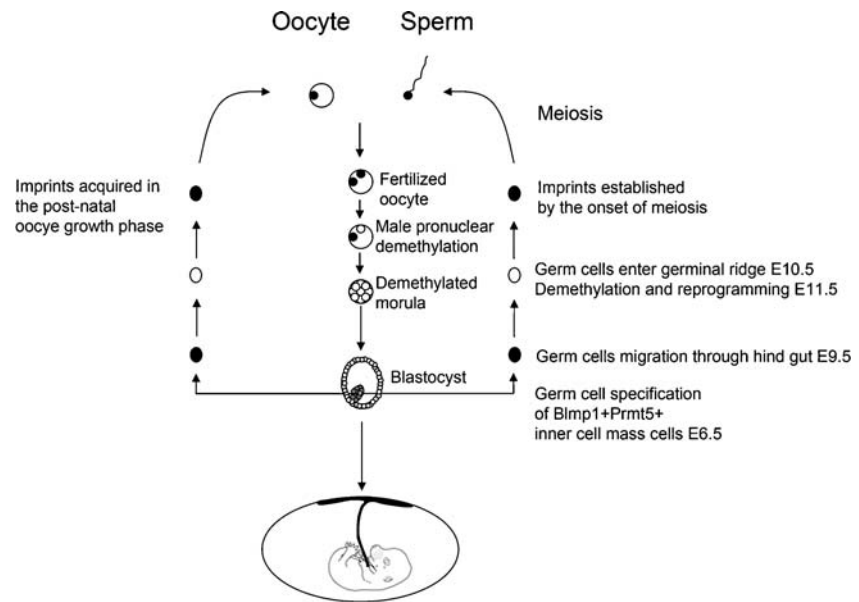
demethylation in early development. In this review, we discuss the roles of the various DNA methyltransferases and their likely functions in development.

The methylation of cytosine at position 5 of the pyrimidine ring is the only naturally occurring modification in mammalian DNA. Methylation occurs predominantly, but not exclusively, in the sequence CpG. In tissues, approximately 4% of cytosines are methylated (1% of all nucleotides in DNA), which corresponds to methylation at 70%–80% of all CpG dinucleotides in the genome (Gruenbaum et al. 1981). However, methylation levels are considerably lower during gametogenesis and in early development, times of genomic reprogramming (Kafri et al. 1992; Monk et al. 1987; Santos et al. 2002; Fig. 1). As the DNA methylation of promoters represses transcription and recruits other repressive chromatin-modifying activities to the chromatin (Bird 2002), reprogramming might be incomplete if the methyl marks are not removed from the DNA during these stages of development.

In mice, imprints that have been established in the germline are largely spared demethylation in the early embryo (Brandeis et al. 1993; Howell et al. 2001). Post-implantation development sees the restoration of DNA methylation, starting in the inner cell mass of the embryo and subsequently in the extra-embryonic tissues (Watanabe et al. 2002). Cells that will become the germ cells of the developing embryo are selected in the epiblast at approximately embryonic day 6.25 (E6.25) in mice. The expression of *Blimp1* complexed with the histone arginine methyltransferase *Prmt5* in these cells prevents their somatic development and commits them to a germ cell fate (Ancelin et al. 2006; Hayashi et al. 2007). These primordial germ cells migrate along the hind gut at E9.5 and enter the germinal ridge at E10.5. Imprint erasure and reprogramming occur at E11.5 and is associated with genome-wide DNA

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Fig. 1 Global changes in DNA methylation during pre-implantation development and gametogenesis



hypomethylation and translocation of Blimp1-Prmt5 from the nucleus to the cytoplasm. In males, imprints begin to be re-established pre-natally starting as early as E12.5 and are complete by the onset of meiosis in the adult. In females, imprints are acquired in the post-natal oocyte growth phase (Trasler 2006).

This complex series of events involves the co-ordinated regulation of DNA methyltransferases and other proteins that affect chromatin structure during specific phases of development. Much of what is known about the biology of the DNA methyltransferases and the function of DNA methylation has come from the study of mouse embryonic stem (ES) cells, which have pluripotent differentiative potential and are amenable to mutation by homologous recombination. ES cells have always been an enigma to DNA methylation biologists, mainly because of their distinctive ability to inactivate and methylate proviral DNA elements rapidly (Jahner et al. 1982). The ability to methylate proviral DNA elements de novo was long thought to be a feature of the only methyltransferase that was known at the time, the maintenance methyltransferase now called Dnmt1. This enzyme showed a marked preference for methylating hemi-methylated DNA in vitro, although cleavage of the regulatory N-terminal domain could stimulate de novo activity (Bestor 1992). Indeed, the enzyme was purified based on its ability to methylate DNA de novo (Bestor and Ingram 1983). However, when a targeted deletion of Dnmt1 in ES cells left the targeted cells with considerable residual DNA methylation and their ability to methylate de novo was preserved, it became clear that the genome coded for other enzymes capable of methylating the genome (Lei et al. 1996). The de novo DNA methyltransferases Dnmt3a and Dnmt3b, which are

highly expressed in ES cells, are now known to be largely responsible for de novo methyltransferase activity in vivo (Dodge et al. 2002; Lyko et al. 1999; Okano et al. 1998a, 1998b, 1999), but as will be seen in this review, their biological activities are not confined to the early embryo and ES cells.

ES cells have been a valuable tool in the study of DNA methylation and its biological effects but perhaps we would be wise to remember that, whereas in vitro cultured ES cells have a similar differentiative potential to early embryonic cells (in vivo), some differences are apparent in relation to DNA methylation. In normal development, the embryo is extremely demethylated prior to embryonic implantation. Remethylation of the mouse embryo starts in the inner cell mass with the expression of the de novo methyltransferase Dnmt3b. This is the stage at which embryos are harvested to generate ES cell lines. However, by the time that an ES cell line is established in vitro, it expresses high levels of both Dnmt3a and Dnmt3b, and the genome has been remethylated. The cellular features of pluripotency (retained during growth by leukaemia inhibitory factor, LIF), the high levels of DNA methyltransferase expression, and a fully methylated genome probably have no cellular correlates in vivo. In the mouse, remethylation and development are seamless parallel processes.

Targeted deletion of the DNA methyltransferases in the mouse has confirmed that normal methylation is essential for development (Lei et al. 1996; Li et al. 1992; Okano et al. 1999). This review will focus on the biology of DNA methylation in ES cells and development, including influences on chromatin structure and gene regulation but will also discuss DNA methylation in germ cells in which it has a particular role in the establishment of imprints.

Mammalian DNA cytosine-5 methyltransferases

The DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from S-adenosylmethionine to carbon-5 of deoxycytidine residues that have been incorporated into DNA. Mammalian genomes encode four Dnmts, the maintenance methyltransferase Dnmt1 (Bestor et al. 1988; Yoder et al. 1996), the de novo methyltransferases Dnmt3a and Dnmt3b (Okano et al. 1998a, 1998b; Xie et al. 1999), and Dnmt2 (Yoder and Bestor 1998), a DNA methyltransferase homologue with minimal DNA methyltransferase activity (Figs. 2, 3, 4, 5). All enzymes are expressed in ES cells. They share 10 motifs in their catalytic C-termini domains, which are conserved among the eukaryotic and prokaryotic DNA methyltransferases. These domains (I–X) are important for the activity of the methyltransferases (Figs. 2, 3, 5).

Dnmt1 protein

Dnmt1 is a large protein (190 kDa) encoded by 39 exons. The gene uses three promoters with sex-specific 5' exons (Fig. 2). The most 5' promoter is active in oocytes and codes for a 175-kDa protein (Dnmt1o) that is necessary for maintaining maternal and paternal methylation imprints in the early embryo. Dnmt1o has 118 distinct N-terminal amino acids that probably increase protein stability and the protein appears to be predominantly located in the cytoplasm. However, Dnmt1o also contains a nuclear localisation signal (NLS) and, together, these features facilitate the maintenance of imprints during cleavage (Ding and Chaillet 2002). A second promoter, lying 6 kb 3' to the oocyte-specific promoter, produces the 190-kDa protein common to ES cells and all somatic cells. This protein has distinct N-terminal amino acids, retains the NLS and is localised in the nucleus. A further promoter just 3' of

the somatic promoter produces a non-translated transcript in pachytene spermatocytes. During sperm development, switching promoter usage to this promoter is thought to have the effect of down-regulating Dnmt1 protein levels (Mertineit et al. 1998).

The Dnmt1 protein consists of a large N-terminal domain that comprises 2/3 of the entire enzyme and that is separated from the C-terminal catalytic domain by 13 alternating glycyl and lysyl residues. The centre of the N-terminal domain contains a cluster of cysteinyl residues (CX₂ CX₂ CX₄ CX₂ CX₂), which bind zinc ions (Bestor 1992). A peptide region (TRQTTITSHFAKG) in the human enzyme binds to PCNA (proliferating cell nuclear antigen) targeting the enzyme to the replication machinery (Chuang et al. 1997). This is likely to help to concentrate Dnmt1 activity at replication forks. The processivity of Dnmt1 is, however, an inherent property of the enzyme and is not dependent on the PCNA-binding site (Vilkaitis et al. 2005). The sequence specificity of Dnmt1 for CpG dinucleotides is a property of the C-terminal domain. This has been established by domain-swap experiments in which the N-terminal domain of Dnmt1 is fused to the catalytic domains of prokaryotic DNA methyltransferases with different target specificities (Pradhan and Roberts 2000). The target specificity of the prokaryotic enzyme is retained in these swap experiments but the N-terminal domain of Dnmt1 imparts a specificity for hemi-methylated substrates, a characteristic not usually present in the prokaryotic enzymes. This property is localised to amino acids 122–417 of the human enzyme in the proximity of the PCNA-binding site (Araujo et al. 2001).

A further unexpected function of Dnmt1 turns out to be in DNA mismatch repair (MMR). This function was first identified in Bloom syndrome protein (blm)-deficient ES cells (Guo et al. 2004). The cells have an enhanced propensity for mitotic recombination, a feature that

Fig. 2 **a** Dnmt1 gene showing development stage-specific promoters and transcripts. **b** Dnmt1 proteins derived from somatic and oocyte-specific (*Oocyte spec*) transcripts (NLS nuclear localisation signal, BAH bromo-adjacent homology domain, GK13 13 alternating glycyl and lysyl residues, KEN KEN box motif [KENxxxN/D]). **c** Dnmt2 protein

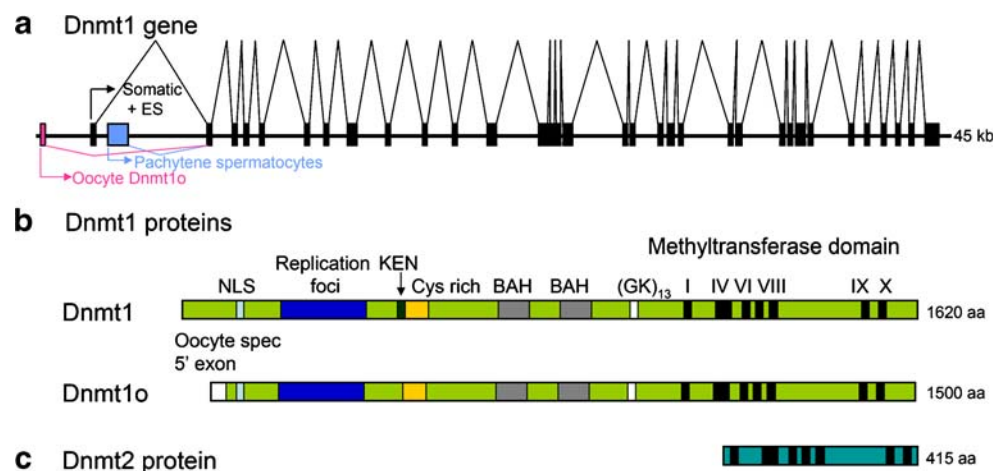
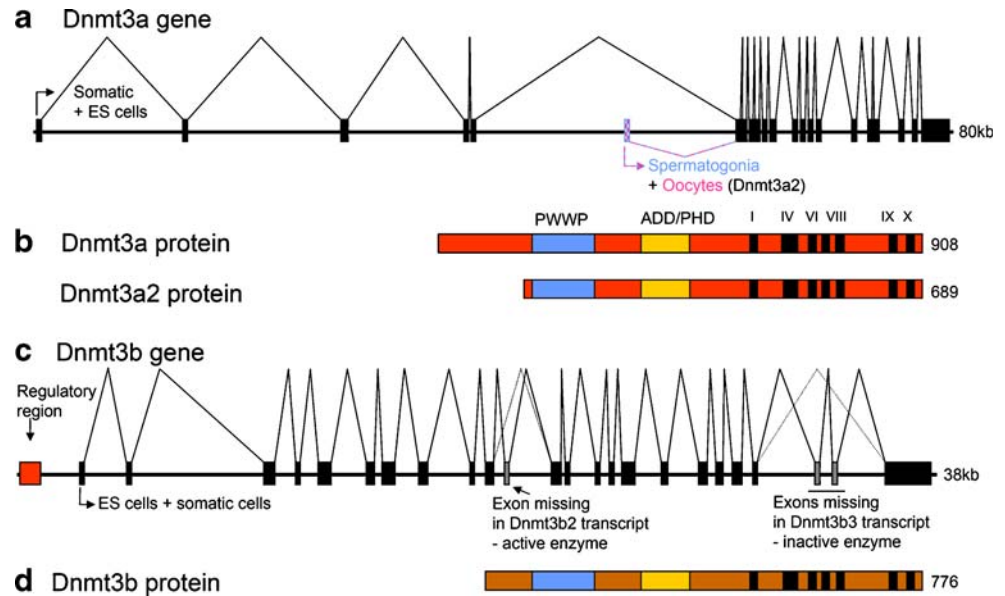


Fig. 3 a Dnmt3a gene showing development stage-specific promoters and transcripts.

b Dnmt3a proteins (*PWWP* Pro-Trp-Trp-Pro motif, *ADD* Atrx Dnmt3a Dnmt3b domain, *PDH* promoter). **c** Dnmt3b gene with transcripts coding for active/inactive protein. **d** Dnmt3b protein



increases the rate at which heterozygous mutations duplicate to homozygosity. A gene trap library was generated in *Blm*-deficient ES cells and the integrants were put under selective pressure to duplicate (to homozygosity) any mutant alleles that permitted growth in 6-thioguanine (6-TG). This enriches for MMR deficient cells, because 6-TG incorporation results in the death of MMR-competent cells. Under these conditions, homozygous *Dnmt1*-deficient ES cells were selected for, and subsequent studies have confirmed that *Dnmt1*-deficient cells are indeed MMR-deficient (Kim et al. 2004; Wang and James Shen 2004). The mechanism by which *Dnmt1* is involved in MMR is unknown but *Dnmt1* might be involved in determining which DNA strand should be repaired when a mismatch arises, as *Dnmt1* is exclusively active on the newly synthesised strand (T. Chen et al. 2007).

Dnmt2 protein

Dnmt2 is a small 415-amino-acid protein, similar in size to the prokaryotic DNA methyltransferases and lacking a large N-terminal domain (Fig. 2). Despite *Dnmt2* having a conserved catalytic domain, difficulties have been experienced with regard to ascribing DNA methyltransferase activity to this protein (Dong et al. 2001; Van den Wyngaert et al. 1998). Orthologues exist in humans, mouse, *D. melanogaster* and *Saccharomyces pombe*. Homozygous gene deletion has no obvious effects in mice, and *Dnmt2*-deficient ES cells have no decrease in their DNA methylation or ability to methylate proviral DNA (Okano et al. 1998a, 1998b; Yoder and Bestor 1998). A study of the human protein suggests that DNMT2 has a restricted substrate specificity (consensus target site: *tnCGga(g/a)*)

Fig. 4 Mechanism of action of DNA cytosine-5 methyltransferases (*SAM* S-adenosylmethionine, *SAH* S-adenosyl-homocysteine)

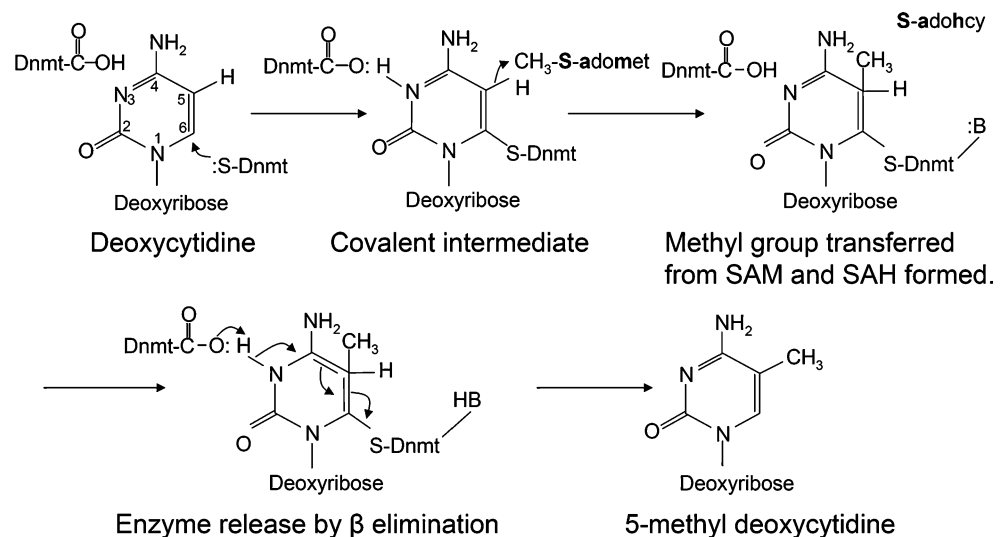
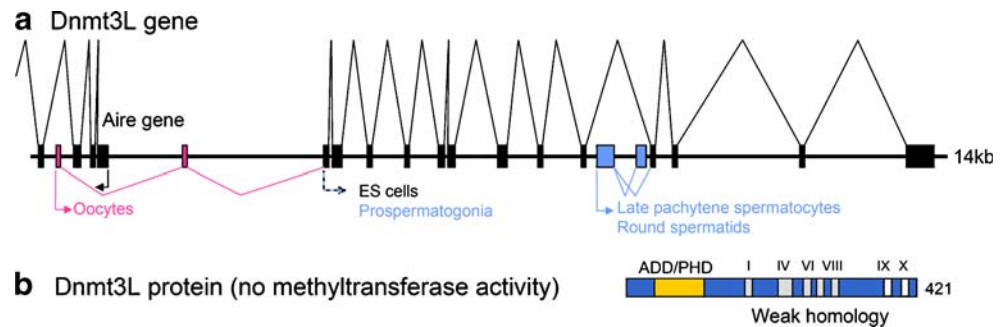


Fig. 5 a Dnmt3L gene showing development stage-specific promoters and transcripts.
b Dnmt3L protein (ADD Atrx Dnmt3a Dnmt3b domain)



which may explain why detection of its DNA methyltransferase activity has been difficult (Hermann et al. 2003) but the most important biological function of this enzyme may be as an RNA methyltransferase. A recent study has demonstrated that Dnmt2 is an RNA methyltransferase with specificity for aspartic acid tRNA (Goll et al. 2006) and morpholino-induced depletion in zebrafish results in reduced RNA methylation and differentiation defects in the liver, retina and brain (Rai et al. 2007).

De novo methyltransferases (Dnmt3 enzymes)

The de novo methyltransferases Dnmt3a and Dnmt3b show the same propensity for methylating unmethylated duplex DNA as for hemi-methylated DNA. Intriguingly, when these proteins were first discovered, their de novo methyltransferase activities in vitro appeared weaker than that of the maintenance enzyme Dnmt1 (Okano et al. 1998a, 1998b). Subsequent studies have indicated that the full methyltransferase activities of these proteins may be dependent on other proteins such as Dnmt3L (Gowher et al. 2005). The de novo methyltransferases Dnmt3a and Dnmt3b have N-terminal domains that are distinct from Dnmt1. The enzymes are highly homologous to each other in their C-terminal catalytic domains but diverge at their N-termini. The N-terminal domains are responsible for targeting the proteins to chromatin. Dnmt3a has two predominant isoforms, which are transcribed from separate promoters (T. Chen et al. 2002; Fig. 3a,b). The longest isoform, Dnmt3a (130 kDa), localises to heterochromatin. The short isoform, Dnmt3a2 (100 kDa), is transcribed from an internal CpG island promoter, lacks the N-terminal amino acids that are present in Dnmt3a and has no sequences that are unique to it. A green fluorescent protein (GFP)-tagged version of Dnmt3a2 has been shown to localise to euchromatin in NIH3T3 cells (fibroblasts).

Dnmt3b localises to heterochromatin and has several different splice variants all transcribed from the same promoter (Fig. 3c). A region 7–8 kb 5' of the Dnmt3b minimal promoter confers repression of the transcript in NIH3T3 cells but not in ES cells (Ishida et al. 2003). The full length protein Dnmt3b1 is catalytically active, as is

Dnmt3b2. However, certain transcripts, such as Dnmt3b3, 4 and 5, lack exons that code for crucial conserved C-terminal domains and are therefore inactive (T. Chen et al. 2003; Fig. 3d). The function, if any, of these catalytically inactive transcripts is unknown.

Dnmt3a, Dnmt3a2 and Dnmt3b are highly expressed in male ES cells, although Dnmt3a is more weakly expressed in female ES cells. This may account for the lower levels of methylation observed in female ES cells compared with those of males (35% vs. 70% CpG methylation in male ES; Zvetkova et al. 2005). Whatever the mechanism for hypomethylation in females ES cells, it seems also to be associated with having two X chromosomes, because spontaneous loss of an X chromosome, a frequent occurrence in female ES cells, leads to an increase in methylation. Increased methylation may give XO ES cells a selective advantage over XX ES cells in culture.

In male ES cells, the deletion of Dnmt1 leads to a dramatic reduction in DNA methylation levels from 70% of CpGs being methylated to 20% (Ramsahoye et al. 2000). The residual DNA methylation is the result of Dnmt3 activity; these enzymes methylate at asymmetrical sites (non-CpG) and symmetric sites (CpG). Studies of DNA methylation in Dnmt1-deficient ES cells have revealed that 60% of residual methylation is at CpG, although a considerable fraction (40%) is at CpA and CpT dinucleotides (Dodge et al. 2002; Ramsahoye et al. 2000). Wild-type ES cells also have higher levels of non-CpG methylation than somatic cells and tissues, but the fraction of methylation that is at non-CpG sites is lower than in Dnmt1-deficient cells because the methylation contributed by Dnmt1 is all at CpG. The propensity for the de novo methyltransferases to methylate at non-CpG targets has been detected in transgenic flies expressing Dnmt3a (Lyko et al. 1999) and in ES cells by reduced representation bisulphite sequencing (Meissner et al. 2005). In vitro methylation experiments with purified Dnmt3a have also demonstrated that this enzyme methylates at non-CpG and CpG sites (Gowher and Jeltsch 2001).

The biological significance of non-CpG methylation in ES cells is unknown. A tempting speculation is that, in the early embryo, the repression of certain genes is dependent

on de novo methyltransferase activity alone, with methylation at non-CpG sites conferring these effects. This form of methylation would provide for temporary transcriptional control, while the de novo enzymes were being expressed, but repression would be lost when the de novo enzymes were subsequently down-regulated in later development. This is in contrast to de novo methylation at CpG, which would be maintained by Dnmt1 when the de novo enzymes were no longer expressed.

Non-CpG methylation may be a consistent feature of Dnmt3 orthologues. The *Arabidopsis* orthologues Drm1 and Drm2 are involved in RNA-directed asymmetric (non-CpG) DNA methylation (Cao et al. 2003). In the tobacco plant, the de novo methyltransferase NrDRM1 also methylates at asymmetric cytosines (Wada et al. 2003). Could these enzymes have a role in RNA-directed gene silencing in mammals? A small number of reports in the literature suggest that DNA methylation may occur during RNA interference (RNAi)-mediated silencing in mammalian cells; this would be consistent with the phenomenon observed in plants (Castanotto et al. 2005; Kawasaki and Taira 2004; Morris et al. 2004). Kawasaki and Taira (2004) have shown RNAi-mediated suppression of E-cadherin to be dependent on DNMT1 and DNMT3B but not on DNMT2. Morris et al. (2004) have revealed that siRNA reduces transcription from the EF1A promoter of a GFP reporter, an effect that is abolished by treatment with the histone deacetylases inhibitor trichostatin A and 5-azacytidine. These studies indicate that de novo methylation is integral to RNAi-mediated suppression, although the findings have been questioned by others. Ting et al. (2005) have found that knock-down of the CDH1 gene by siRNA is not accompanied by promoter methylation. A subsequent retraction by one of the authors of Kawasaki and Taira (2004) has also cast doubt as to whether RNAi-mediated DNA methylation exists in mammalian cells (Taira 2006).

DNA methyltransferase reaction mechanism

The active site of all DNA cytosine-5 methyltransferases is a conserved prolylcysteiny motif (PC box) in domain IV. The mechanism of action of DNA cytosine-5 methyltransferases, first proposed by Wu and Santi in 1987 (Wu and Santi 1987) and modified by Verdine (Bestor and Verdine 1994), involves nucleophilic attack at carbon-6 (C6) of the pyrimidine ring by the reactive cysteine in the PC box (Fig. 4). This leads to the activation of C5 and the transfer of the methyl group of S-adenosylmethionine (the methyl donor in the reaction). A 5'-6' dihydro intermediate is thus formed and the enzyme is subsequently released by β -elimination (Fig. 4). One intriguing aspect of this reaction is

the mechanism by which the enzyme gains access to the cytosine base that is buried in the DNA helix. In 1994, the crystal structure of a prokaryotic DNA cytosine-5 methyltransferase bound to its DNA substrate revealed an unexpected feature of the mechanism. By incorporating 5-fluorocytosine into the CpG target site of a DNA duplex, the enzyme could be trapped during catalysis and the crystal structure of the reaction intermediate could be studied. Analysis of this structure has revealed that, during catalysis, the enzyme causes the target cytosine to be completely flipped out of the double helix (Klimasauskas et al. 1994). This mechanism explains the manner in which the base is delivered to the active site of the enzyme.

5-Azacytidine, a nucleoside analogue frequently used to induce DNA demethylation, is also thought to exert its effects by covalently trapping DNA cytosine-5 methyltransferases to DNA, thus leading to a reduction in the amount of enzyme available for methylation at other sites (Taylor and Jones 1982). In 5-azacytidine, a nitrogen replaces carbon at the C5 position of the pyrimidine ring. Once reduced to 5-aza-2' deoxycytidine and incorporated into the DNA, this analogue becomes a target for the DNA methyltransferase at 5-aza-substituted CpG sites. The first phase of the methylation reaction, during which a covalent bond is formed between the enzyme and the C6 position of the nucleotide, proceeds as normal. However, failure to methylate the nitrogen at position 5 means that the enzyme cannot be eliminated and it remains covalently trapped to the DNA. If there is sufficient incorporation of 5-azacytidine into the DNA, then this results in a significant reduction in the amount of enzyme available for methylating normal cytosines at CpG sequences. In cell lines, the marked effect on DNA methylation levels is a consequence of their dependence on Dnmt1 for maintaining methylation levels. Cell lines require constant "maintenance" methylation because they divide continuously and are constantly methylating their newly synthesised DNA. Dnmt1 shows a marked preference for methylating the hemi-methylated CpG duplexes that result from DNA synthesis and is highly processive (Bestor and Ingram 1983; Vilkaitis et al. 2005). This activity ensures that the methylated parental strand serves as a template for methylation of the newly synthesised daughter strand. Because of its high processivity, the covalent entrapment of DNMT1 by 5-aza 2' deoxycytidine residues in the DNA causes a rapid decline in the availability of DNMT1. There is a subsequent fall in methylation levels as synthesis proceeds without methylation of the newly synthesised DNA. This covalent attachment to DNA may in large part contribute to the toxicity of 5-azacytidine (Juttermann et al. 1994); evidence also exists that it contributes to the high rate of C to G transversions in 5-azacytidine-treated animals (Jackson-Grusby et al. 1997).

Recent studies question whether the active site cysteine is as important to the activity of the mammalian enzymes as it is to the prokaryotic enzymes. Reither et al. (2003) have shown that mutation of the active site cysteine in Dnmt3a only reduces activity two- to six-fold, whereas mutation of the glutamate in the ENV domain (VI), which is predicted to be important in holding the flipped base in place for methyl transfer, almost completely abolishes activity. Similarly, some have questioned whether covalent entrapment of the enzyme by 5-azacytidine is the sole mechanism leading to demethylation. 5-Aza 2'-deoxycytidine has been shown to induce the degradation of Dnmt1 by a proteosomal pathway. Mutation of the active site cysteine appears not to influence this degradation indicating that the formation of a covalent intermediate is not necessary for the effect, although the N-terminal KEN box motif, bromo-adjacent homology domain and NLS are required (Ghoshal et al. 2005). Proteosomal degradation has no apparent effect on Dnmt3a or Dnmt3b in the studies of Ghoshal et al. (2005) but other authors have found that the cytotoxic effects of 5-aza 2'-deoxycytidine depend on the expression of Dnmt3a and Dnmt3b and on Dnmt1 (Oka et al. 2005). As Dnmt1 targets hemi-methylated DNA, the toxicity (of 5-aza 2'-deoxycytidine) that is mediated by Dnmt1 adduct formation probably depends on the level of DNA methylation, as this in turn would determine the number of hemi-methylated Dnmt1 target sites generated during synthesis.

Protein “facilitators” of DNA methylation

Deficiencies of a number of proteins are now known to be associated with DNA hypomethylation, indicating that their activities are required either to target the methyltransferases to chromatin, to increase the methyltransferase activities of the enzymes, or else to make the chromatin sensitive to methylation. The proteins known to exhibit this function include lymphoid-specific helicase-1 (Lsh1), Dnmt3L and Atrx. Lsh and Atrx have homology to the SWI/SNF family of ATP-dependent helicases, suggesting that chromatin-remodelling activity is necessary for the efficient de novo methylation of chromatin. Dnmt3L has an ADD (Atrx, Dnmt3 and Dnmt3L) domain located near the imperfect PHD domain in the cysteine-rich region. The ADD domain is also present in Atrx and the Dnmt3 enzymes and may be a site of interaction with other chromatin-associated proteins. Although facilitation of de novo methyltransferase activity by a chromatin-remodelling ability is a tantalising idea, there is no evidence to support it. Indeed, in the best characterised case, that of Dnmt3L, the mechanism appears to be one in which Dnmt3L induces a conformational change in the Dnmt3 methyltransferase active site, increasing its methyltransferase activity. Lsh1 deficiency has the

most global effects on DNA methylation but the detailed mechanism of its interaction with the DNA methyltransferases has not been reported.

Dnmt3L protein

Dnmt3L shows significant sequence homology to the cysteine-rich N-terminal domains of Dnmt3a and Dnmt3b but has only weak homology to the C-terminal methyltransferase catalytic domain (Aapola et al. 2000). *Dnmt3L* has three promoters (Fig. 5a,b). The most 5' promoter is oocyte-specific and is located within an intron of the neighbouring *Aire* gene. A second promoter located approximately 5 kb 3' to this promoter is active in prospermatogonia and ES cells. A third promoter in intron 9 of the gene is active in late pachytene spermatocytes and produces three truncated non-coding RNAs (Shovlin et al. 2007).

Interestingly, in mice, Dnmt3L is not necessary for zygotic development but is essential for the establishment of maternal and paternal genomic imprints. The progeny of Dnmt3L-deficient females lack imprints both in the embryo and in the extra-embryonic tissues and die in mid-gestation (Arima et al. 2006; Bourc'his et al. 2001a, 2001b; Hata et al. 2002; Webster et al. 2005). These embryos have neural tube defects and a small chorion-placenta possibly attributable to the de-regulation of genes such as *Mash2*, the spongiotrophoblast-specific marker 4311, and *Gcm1* (differentiation marker of labyrinthine trophoblast), all of which are markedly down-regulated in the progeny of Dnmt3L-deficient mice. Dnmt3L-deficient males have impaired spermatogenesis. The male germ cells arrest and die in early meiosis; Dnmt3L deficiency causes hypomethylation and the aberrant expression of interspersed repeated sequences in these cells (Bourc'his and Bestor 2004; Hata et al. 2006; Webster et al. 2005).

Whereas Dnmt3L is essential for establishing methylation imprints in the female germline, this protein has no DNA methyltransferase activity. The maternal methylation marks are a function of Dnmt3a activity (Hata et al. 2002). More recent studies of conditional *Dnmt3a* alleles have demonstrated that Dnmt3a is also required for the establishment of paternal imprints (Kaneda et al. 2004) and deficiency of Dnmt3a leads to impaired spermatogenesis. Indeed, the features of conditional Dnmt3a deficiency are identical to Dnmt3L deficiency, whereas the conditional deletion of Dnmt3b appears to have no effect on imprinting.

The Dnmt3a2 isoform is probably responsible for the generation of imprints in the germ-line. It is co-ordinately expressed with Dnmt3L in the male gonocytes at day 14–18 post-coitum and at around the time of birth in the growing oocytes (Lees-Murdock et al. 2005; Sakai et al. 2004). Much evidence has now accumulated for the existence of a complex involving the Dnmt3 enzymes and Dnmt3L (Hata

et al. 2002). Both Dnmt3a and Dnmt3b promote the nuclear localisation of Dnmt3L but, in ES cells, Dnmt3L is found in foci that partially overlap with DAPI (4,6-diamidino-2-phenylindole) bright chromocentres, with recruitment to these foci being a specific function of the Dnmt3a2 isoform (Nimura et al. 2006). This is somewhat surprising given the absence of localisation of GFP-tagged Dnmt3a2 to chromocentres in NIH3T3 cells (T. Chen et al. 2002) and suggests that a chromatin protein or modification, present in ES cells but not in NIH3T3 cells, is necessary for the localisation of Dnmt3a2 to chromocentres.

The physical association between the de novo methyltransferases and Dnmt3L also stimulates the methyltransferase activities of the former. The C-terminal domain of Dnmt3L binds to the catalytic domains of Dnmt3a and Dnmt3b increasing their catalytic activities 15-fold, presumably by inducing a conformational change in the active site. Complex formation between Dnmt3a and Dnmt3L accelerates DNA binding 20-fold and lowers the K_m for DNA. Complex formation also increases the binding of S-adenosylmethionine and lowers the K_m of Dnmt3a for S-adenosylmethionine (Gowher et al. 2005). The binding of Dnmt3L to Dnmt3a is transient. After binding of the complex to DNA, Dnmt3L dissociates but a change in the conformation of Dnmt3a leads to a slow rate of release of the enzyme from the DNA. Human DNMT3A and DNMT3L have been found to interact in a similar manner (Kareta et al. 2006).

Lsh protein

Lsh was originally cloned in 1996 from a T-cell precursor library (Jarvis et al. 1996); it has 45%–53% homology to a number of proteins involved in chromatin remodelling, specifically SNF2 from *S. cerevisiae*, ISWI, Brahma from *D. melanogaster*, and human BRG-1, CHD3 and 4. Homozygous deletion of the helicase domains I, Ia and part of II in mice causes low birth weight, renal lesions and perinatal death (Geiman et al. 2001). This also causes genome-wide demethylation (including hypomethylation at major and minor satellite sequences), interspersed repeat sequences (IAP, Sine B1 and Line 1 elements) and single copy genes (Dennis et al. 2001). Lsh has been shown to control imprinting at the p57Kip2 locus (*Cdkn1c*) but not at *H19*, *Igf2*, *Igf2r*, *Zac1* or *Meg9*. The protein also binds to the 5' differentially methylated region (DMR) of *Cdkn1c* (Fan et al. 2005). In the female gonad, Lsh is required for meiotic chromosome synapsis and the transcriptional repression of retrotransposons (De La Fuente et al. 2006). Nearest neighbour analysis has demonstrated that just 34% of all CCGG sites are methylated in homozygous deleted animals, compared with 57% methylation in wild-type mice. DNA methyltransferase expression is not affected and so the loss of Lsh appears to affect DNA methylation by

influencing the activities of the methyltransferases. Interestingly, these changes are associated with an increase in histone H3 lysine-4 acetylation; this may be a direct effect of hypomethylation because 5-azacytidine treatment is able to induce similar effects (Yan et al. 2003). Histone H3 lysine-9 methylation is unaffected. Despite the impressive global decrease in DNA methylation, the proportion of genes significantly deregulated is of the order of 0.5%. Intriguingly, the majority of these genes contain LTR, SINE and LINE sequence elements indicating that they may have been deregulated because of the activity of Lsh against retrotransposons (Huang et al. 2004). Lsh has been shown to bind directly to these elements in addition to major and minor satellite repeats, is also essential for the de novo methylation of episomal vectors but not for the maintenance of their DNA methylation (Zhu et al. 2006) and is essential for retroviral gene silencing in fibroblasts and in ES cells. In fibroblasts, Dnmt3a or Dnmt3b is also required for silencing proviral elements but, in ES cells, these enzymes are not required (Pannell et al. 2000).

Atrx protein

The *ATRX* gene belongs to the SNF2/SWI family and shares homology with Dnmt3L, Dnmt3a and Dnmt3b through the ADD domain in the cysteine-rich region. Mutations in *ATRX* lead to mental retardation and alpha thalassaemia and induce changes in the pattern of methylation of several highly repeated sequences including the rDNA arrays, a Y-specific satellite and subtelomeric repeats (Gibbons et al. 2000). Both gains and reductions in DNA methylation are observed. *ATRX* interacts with HP1 at heterochromatin (Berube et al. 2000) and is recruited to promyelocytic leukemia nuclear bodies via an interaction with DAXX (Ishov et al. 2004). *ATRX* has been shown to remodel chromatin (Xue et al. 2003) but whether this property is essential for normal DNA methylation is not known. Intriguingly, *ATRX* has recently been shown to be dependent on the methyl-CpG-binding protein MECP2 for its localisation to heterochromatin, indicating that, in addition to facilitating methylation, *ATRX* binding may itself also be dependent on DNA methylation. Mutations in the methyl-CpG-binding domain, which cause mental retardation in Rett syndrome, disrupt the interaction between MECP2 and *ATRX* (Nan et al. 2007). As mutations in both proteins cause mental retardation, this suggests that the interaction may be essential for normal brain function.

Maintenance methylation

One model for the establishment of DNA methylation patterns is that the de novo methyltransferases establish

methylation marks in gametogenesis and early development, with these marks subsequently being maintained by the maintenance methyltransferase activity of Dnmt1 (Ramsahoye et al. 2000). This hypothesis requires that all CpG sites in the genome are accessible to the Dnmt3 enzymes and to Dnmt1. A study examining methylatable *NotI* sites by restriction landmark genomic scanning has shown that this is indeed the case (Hattori et al. 2004). However, it may be overly simplistic to assume that, once Dnmt1 has maintained methylation at a site, it will continue to maintain it in perpetuity. Strong evidence suggests that maintenance methylation by Dnmt1 alone is insufficient for “perfect” maintenance. For example, in ES cells, methylation at the *Xist* promoter is apparently dependent on reiterative de novo methylation by Dnmt3a and Dnmt3b and on maintenance methylation by Dnmt1 (Okano et al. 1999). Presumably, either a demethylase activity is active at this site or, for some reason, maintenance methylation is imperfect at this locus. Indeed, this requirement for de novo methylation for the maintenance of methylation levels may apply to a greater or lesser extent throughout the genome. ES cells deficient in Dnmt3a and Dnmt3b but retaining Dnmt1 gradually lose methylation in culture. By passage 70, *Dnmt[3a^{-/-},3b^{-/-}]* ES cells have just 0.6% methylation at CpG dinucleotides (compared with 70% in wild-type ES cells; Jackson et al. 2004). Whatever the cause, these methylation losses appear only to be recoverable by the de novo methyltransferases Dnmt3a and Dnmt3b. Liang et al. (2002) have used an assay for detecting hemi-methylated sites in ES cells and demonstrated that certain sequences, such as murine LINE-1 promoters, have a high level of hemi-methylation, indicating a failure or defect in their maintenance methylation by Dnmt1. By reducing methylation levels with 5-aza 2'-deoxycytidine, these authors have been able to demonstrate that Dnmt1 activity on its own cannot restore methylation to these sites but that methylation can be restored if Dnmt3a and Dnmt3b are present (Liang et al. 2002).

The finding that maintenance methylation is actually dependent on de novo methyltransferase activity might have implications for all dividing cells, as it indicates that a de novo methyltransferase is needed to be expressed to some extent in all proliferative tissues to prevent DNA methylation from being gradually lost. Hence, the frequently observed expression of de novo methyltransferases in tumours and cell lines may reflect an innate requirement of all dividing cells to express these activities in support of maintenance methylation, rather than indicating an epigenetic cause for the transformation. Furthermore, the finding of extremely low methylation levels in the presence of Dnmt1 indicates that, despite the observed de novo activity of this enzyme in vitro (with unmethylated oligonucleotides as substrate; Pradhan et al. 1999) and despite the observa-

tion that certain human CpG island sequences can be methylated by mammalian Dnmt1 transgenic flies (Jair et al. 2006), there is no significant de novo methyltransferase activity of this enzyme towards endogenous sequences in ES cells in vivo. Dnmt1 over-expression does increase CpG island methylation in somatic cells (Vertino et al. 1996), at IAP elements and at the *Igf2/H19* locus in ES cells (Biniszkiwicz et al. 2002). Over-expression in embryos also causes developmental failure but the cause is not known. The methylation effects could be a direct result of Dnmt1 over-expression inducing aberrant de novo methylation in these cases, although, at high levels of Dnmt1, the enzyme might cause a higher level of methylation by increasing the level of maintenance activity at de novo methyltransferase-initiated sites of DNA methylation.

Function of DNA methylation in ES cells and embryos

One of the most striking features about ES cells in relation to DNA methylation is their higher tolerance of Dnmt1 deficiency compared with somatic cells, as long as the cells are retained in the undifferentiated state by growth in the presence of LIF or feeders (Lei et al. 1996; Li et al. 1992). Combined Dnmt3a and Dnmt3b deficiency is also well tolerated, even when the cells become severely hypomethylated (T. Chen et al. 2003; Jackson et al. 2004; Okano et al. 1999), and the evidence so far indicates that deficiencies of these enzymes in somatic cells, at least in the short term, are also well tolerated (Tadokoro et al. 2007). Undifferentiated triple-knockout ES cells (*Dnmt[1^{-/-},3a^{-/-},3b^{-/-}]*) are also oblivious to DNA hypomethylation (Tsumura et al. 2006). Recent studies have demonstrated that ES cells also have a unique epigenetic signature, with genes important for development being marked by both repressive (polycomb) and activating marks of transcription (Boyer et al. 2006; Lee et al. 2006). Differences in transcriptional control mechanisms could be at the heart of explaining the tolerance of ES cells to DNA hypomethylation. Control by polycomb might be the predominant mechanism through which genes are repressed in ES cells, making DNA methylation a relatively redundant control mechanism over any genes other than imprinted genes (Li et al. 1993).

The requirement for Dnmt1 in somatic cells is context-dependent. Conditional deletion of Dnmt1 in mouse brain has demonstrated that Dnmt1 is required for maintaining methylation during the proliferative phase of development but not during the post-mitotic phase of neural development (Fan et al. 2001). Furthermore, deletion of Dnmt1 in proliferating neural cells causes functional impairment and death in early post-natal life. Dnmt1-deficient cells show poor survival and are selected against in mosaic animals that survived to 3 weeks (Fan et al. 2001). Dnmt1-

deficient fibroblasts are also known to senesce prematurely through a p53-dependent mechanism (Jackson-Grusby et al. 2001). Naïve *Dnmt1*^{-/-} CD8⁺ T-cells have been reported to have limited cell growth after activation in vitro (less than five population doublings; Lee et al. 2001). However, a subsequent study has shown that short-term growth of *Dnmt1*^{-/-} CD8⁺ T-cells is not limited in vitro but that extensive proliferation is limited in vivo (Chappell et al. 2006). *Dnmt1* deficiency in *Xenopus* is also associated with p53-dependent apoptosis and developmental defects (Stancheva et al. 2001; Stancheva and Meehan 2000). A previous report appears to indicate that the human somatic cell line (HCT116) tolerates DNMT1 deficiency well, with only 20% loss of DNA methylation (Rhee et al. 2000), but recent results have indicated that the DNMT1 locus is not effectively targeted. It turns out that DNMT1 is indeed essential for the survival of HCT116 cells (Egger et al. 2006; Spada et al. 2007). An independent targeting approach has demonstrated that DNMT1 is essential for viability in HCT116 cells, as they undergo an early mitotic catastrophe in the absence of DNMT1 and arrest in G2 of the cell cycle (T. Chen et al. 2007). This indicates that DNMT1 protein is required to transit a G2 checkpoint in somatic cells. In contrast to *Dnmt1* deficiency, combined *Dnmt3a* and *Dnmt3b* deletion in haematopoietic stem cells does not result in a loss of viability and does not appear to affect differentiation, although self renewal is affected in long-term reconstitution assays (Tadokoro et al. 2007). Thus, ES cells tolerate deletion of all DNA methyltransferases, whereas proliferating somatic cells have a requirement for *Dnmt1*.

Dnmt1 deficiency causes early lethality shortly after gastrulation in mouse embryos (Lei et al. 1996). Hypomorphic alleles of *Dnmt1* are better tolerated and can even result in live-born mice, albeit mice that are smaller than wild-types and that have a propensity to lymphoid tumours (Gaudet et al. 2003). An elegant explanation for the failure of development in *Dnmt1*-null embryos has been proposed based on the de-regulation of *Xist* expression (Panning and Jaenisch 1996). *Xist* mediates the silencing in *cis* of a single X chromosome in females. *Xist* expression is imprinted in the extra-embryonic tissues of mice, being repressed on the maternal X and expressed on the paternal X in females. Thus, in the extra-embryonic tissues of mice, the maternal X chromosome is always expressed, whereas in the embryo, X-inactivation is random. In *Dnmt1*-deficient ES cells, all imprints are lost and *Xist* is demethylated. However, demethylation does not induce aberrant expression of *Xist* until these cells are induced to differentiate. Differentiation induction in vitro has been associated with apoptosis and an apparent reduction in the expression of X-linked genes in male ES cells (Panning and Jaenisch 1996). Therefore, *Xist* de-regulation has been proposed to have

caused the aberrant silencing of the active X chromosome in female *Dnmt1*^{-/-} mice and of the only X chromosome in male *Dnmt1*^{-/-} mice.

Early passage ES cells from *Dnmt3a*^{-/-},*3b*^{-/-} males retain most methylation imprints but, like *Dnmt1*^{-/-} cells, they also lose methylation of *Xist*. This indicates that the *Xist* locus requires de novo methyltransferase activity and *Dnmt1* activity to retain DNA methylation, presumably because maintenance methylation by *Dnmt1* is inefficient at this locus or because the locus is actively demethylated (Okano et al. 1999). Aberrant *Xist* expression is seen in 0%–4.9% of cells in male E9.5 *Dnmt3a*^{-/-},*3b*^{-/-} embryos, and in 3.9%–17.7% of cells in female embryos, but the vast majority of cells do not mis-express *Xist* despite severe hypomethylation at the *Xist* locus (Sado et al. 2004). Markers of aberrant X chromosome inactivation, such as late replication, hypoacetylation and down-regulation of X-linked genes, are also not observed. In ES cells from *Dnmt3a*^{-/-},*3b*^{-/-} males, *Xist* accumulates progressively on the induction of the ES cells to differentiate in vitro, affecting 68% of cells at day 12 of differentiation but, again, this is not associated with the silencing of the X chromosome or cell loss. These data have been interpreted as indicating that a narrow window of time exists in early development during which *Xist* expression can induce X-inactivation and that, in *Dnmt*-deficient ES cells, the up-regulation of *Xist* on differentiation induction does not happen in the correct time-frame significantly to influence X-inactivation. Thus, developmental failure might not be explained by *Xist* de-regulation in combined deficient *Dnmt3a* and *Dnmt3b* embryos. The extent to which these findings can be extrapolated to *Dnmt1*^{-/-} embryos is not clear. Because of timing differences, *Xist* might have more severe effects on the X chromosome in differentiating *Dnmt1*-deficient cells. The developmental failure in *Dnmt1* deficiency might also be attributable to some other aspect of *Dnmt1* function, as indicated by the early activation of the G2 checkpoint function in DNMT1-deficient human HCT116 cells (T. Chen et al. 2007).

Combined *Dnmt3a* and *Dnmt3b* deficiency is also embryonic lethal in early development (equivalent to the *Dnmt1*^{-/-} phenotype) demonstrating that both de novo and maintenance methylation are essential for development (Okano et al. 1999). Homozygous deficiency of either of the de novo methyltransferases also has a milder phenotype than combined deficiency. *Dnmt3a* null mice are live-born but are runted and die within 1 month of life, whereas *Dnmt3b* null mice die in late gestation (Okano et al. 1999). *Dnmt3b*^{-/-} hypomorphs survive to adulthood and exhibit many of the features of ICF syndrome (for immunodeficiency, centromere instability and facial anomalies) in man (Ueda et al. 2006). As *Dnmt3a* and *Dnmt3b* have some over-lapping activities, for example in respect of the de

novo methylation of proviral DNA sequences, this redundancy presumably explains why individual deficiencies of these enzymes are better tolerated than combined deficiency. However, as the *Dnmt3b*^{-/-} phenotype is more severe than that of *Dnmt3a*^{-/-}, *Dnmt3b* can be reasonably concluded to have the more important role in embryonic development. Consistent with this, *Dnmt3b* turns out to be the first de novo methyltransferase to be expressed in the embryo, with activity appearing in the inner cell mass at the time of implantation (Watanabe et al. 2002).

Differentiation in methylation-deficient mouse ES cells

As both *Dnmt1*^{-/-} and *Dnmt[3a^{-/-},3b^{-/-}]* embryos are profoundly hypomethylated (Lei et al. 1996; Okano et al. 1999), it is not possible to determine whether a low level of DNA methylation per se (caused by *Dnmt1* or *Dnmt3* deficiency) or the specific inability either to maintain DNA methylation or to methylate de novo (or both) causes their developmental failure. Some insight into this could be gained by studying the in vitro differentiating abilities of ES cells with these genotypes. ES cells can be differentiated in a number of ways. In the simplest protocol, ES cells can be plated at low concentration on gelatinised plastic in the absence of LIF. Wild-type ES cells should differentiate completely and form colonies under these conditions. The retention of any undifferentiated cells in the colonies can be determined by staining for alkaline phosphatase, a marker of undifferentiated ES cells. ES cells can also be encouraged to differentiate down specific pathways by using specific protocols. One protocol for cardiomyocyte differentiation is to allow the ES cells to aggregate into embryoid bodies (EB) in the presence of LIF (the hanging drop technique) and to plate the EBs onto gelatinised plastic. After LIF withdrawal, the EBs differentiate and the majority should show rhythmic contractions after 10 days without LIF. ES cells (-LIF) can also be plated directly into methylcellulose in the presence of haematopoietic growth factors (IL-3 and erythropoietin). After a number of days, myeloid and erythroid colonies emerge.

Jackson et al. (2004) have taken advantage of the fact that *Dnmt[3a^{-/-},3b^{-/-}]* ES cells gradually lose methylation in culture and have looked at the consequences for differentiation during this period of methylation loss. They have found that the capacity to differentiate spontaneously at low density and to differentiate into cardiomyocytes and haematopoietic progenitors by using specific protocols is relatively well preserved until the ES cells are highly deficient in DNA methylation after prolonged passage. However, when severely hypomethylated (0.6% of CpGs methylated), a fraction of the culture simply fails to differentiate on LIF withdrawal, remaining viable and

continuing to express alkaline phosphatase. Thus, severe hypomethylation appears to induce a block to the initiation of differentiation. Restoring methylation back to these cells also restores their ability to differentiate, demonstrating that the failure to differentiate is not simply a feature of prolonged passage. In contrast, *Dnmt1*-deficient cells (which have 20% of CpGs methylated) can form alkaline-phosphatase-negative colonies at low density on LIF withdrawal but are incapable of differentiating into erythroid cells, myeloid cells or cardiomyocytes. This is interpreted as indicating that the cells have the ability to initiate differentiation (i.e. to stop being alkaline-phosphatase-positive ES cells) but lack the capacity to differentiate down specific lineages. These data are consistent with a putative *Dnmt3*-dependent methylation mark or marks being required before ES cells can initiate differentiation in vitro. Early passage *Dnmt[3a^{-/-},3b^{-/-}]* ES cells retain such marks because they are derived from wild-type ES cells and still possess *Dnmt1* (which retains the marks until late passage). *Dnmt1*-deficient cells express the de novo methyltransferases and so retain the mark by re-iterative de novo methylation.

Despite being able to initiate differentiation, *Dnmt1*-deficient cells are unable to differentiate further into cardiomyocytes or haematopoietic progenitors. Indeed, at similar overall levels of DNA hypomethylation, *Dnmt[3a^{-/-},3b^{-/-}]* ES cells differentiate into cardiomyocytes and haematopoietic progenitors far more effectively than *Dnmt1*^{-/-} ES cells. This indicates that, once differentiation has been successfully initiated, the quality rather than the level of DNA methylation is important for successful terminal differentiation. Maintenance methylation (by *Dnmt1*) at certain sites may therefore be necessary for successful differentiation (Jackson et al. 2004).

DNA methylation and transcriptional control

Whereas sequence-specific transcription factors are essential for transcription, chemical modifications of the chromatin (including DNA methylation) can influence the transcriptional potential of genes, promoting repression even when specific transcription factors are present. These chromatin modifications, and the repression that they induce, can be stably propagated through cell division leading to the epigenetic inheritance of transcriptionally repressed states. As will be discussed below, these effects are responsible for the stable propagation of imprinted gene expression patterns during development, a phenomenon in which only one parental allele of a gene is expressed (despite the presence of transacting factors capable of activating both alleles). Epigenetic mechanisms are also predicted to be involved in the production and propagation of stable tissue phenotypes.

Indeed, the expression of only a small proportion of genes appears to be directly controlled by DNA methylation. More than half of all human genes have promoters that lie in CpG islands (Davuluri et al. 2001; Marino-Ramirez et al. 2004). These are 0.6–2 kb segments of GC-rich DNA that contain a high frequency of CpG dinucleotides, but islands are usually unmethylated at all CpGs in all tissues, irrespective of expression (Bird et al. 1985). Many tissue-specific genes have promoters that do not lie within CpG islands. In these cases, promoter methylation frequently correlates with repression but the consensus of opinion is that the methylation status reflects the activity state of the gene in these cases, rather than determining it (Burch and Weintraub 1983; Sullivan et al. 1989).

Imprinted genes Imprinted genes are a special class of autosomal genes that are mono-allelically expressed in the embryo and/or adult in a process that is dependent on DNA methylation. Recent estimates indicate that there are several hundred imprinted genes, and allele-specific DNA methylation marks correlate with and appear to control allele-specific gene expression (Maeda and Hayashizaki 2006). Inherited parental imprints are erased during gametogenesis so that sex-specific imprints can be re-established in the germ cells and passed onto the next generation. Dnmt3a2 and Dnmt3L are both necessary for the establishment of imprints (Bourc'his et al. 2001a, 2001b; Hata et al. 2002), and Dnmt1 is required for their maintenance (Li et al. 1993). The Dnmt1o isoform ensures that imprints are maintained during cleavage when the global genome is progressively demethylated, although the mechanism for this is unclear. Deficiency of Dnmt1o in the oocyte leads to a loss of approximately one half of imprinted methyl marks in embryos derived from such oocytes (Howell et al. 2001), indicating that this activity is required during the fourth embryonic S phase.

The mechanism through which DNA methylation affects the expression of imprinted genes is often more complex than the simple differential methylation of allelic promoters and appears to vary at different loci. Methylation can increase or decrease gene transcription in *cis*, depending on the type of regulatory element that has been methylated. Whatever the mechanism employed, the imprints persist in the embryos to which the germ cells contribute, causing the allele-specific transcription of genes in diploid embryonic and adult tissues.

Imprinting frequently involves the mono-allelic methylation of imprinting control regions (ICRs), which are often many kilobases away from the genes that they regulate in *cis*. Certain CpG island ICR segments function as insulators, and their differential methylation controls the binding of factors such as Ctf (a zinc-finger protein). For example, at the *Igf2/H19* locus, Ctf binds to the unmethylated

ICR just upstream of the *H19* gene. On the maternal allele, this blocks access of *Igf2* (located 80 kb upstream) to an enhancer downstream of *H19*. This causes down-regulation of *Igf2* on the maternal allele and reciprocal up-regulation of *H19* (Hark et al. 2000; Szabo et al. 2000). The reciprocal case occurs on the methylated paternal allele. It turns out that a further two DMRs are located upstream of *Igf2*, both of which are methylated on the *Igf2*-active paternal allele. The most 5' (DMR1) is a repressor of *Igf2* in the unmethylated state but repression is eliminated by DNA methylation (Eden et al. 2001). The DMR2 is intragenic and enhances transcription in the methylated state (Murrell et al. 2001). Recent studies with chromosome conformation capture (3C method) demonstrate an interaction between the *Igf2* promoters and the downstream enhancer on the *Igf2*-active paternal allele but this interaction is absent on the maternal allele to which Ctf is bound (Kurukuti et al. 2006). Reciprocal patterns of imprinted gene expression have also been found at the *Gtl2/Dlk1* locus (Paulsen et al. 2001). Ctf is now known to have 20,000 binding sites in the human genome (Barski et al. 2007). The extent to which binding to these sites is controlled by DNA methylation is not known but Ctf is also known to act as a barrier against repressive forces from heterochromatin (Recillas-Targa et al. 2002) and may be involved in the methylation-dependent regulation of genes at the *DM1* locus (Filippova et al. 2001). Ctf deletion causes an early embryonic lethal phenotype and some of the deleterious effects of DNA hypomethylation and/or Dnmt1 deficiency might be mediated by the deregulatory effects of inappropriate Ctf binding in the genome.

Loss of imprinting is unlikely to be the cause of developmental failure in Dnmt1-deficient animals. Rescuing DNA methylation in *Dnmt1*^{-/-} ES cells with a Dnmt1 cDNA restores global methylation levels but not imprints, and the rescued ES cells can still contribute to the embryonic lineages in chimaeras. Interestingly, there is a subsequent predisposition to cancer when imprints have been lost (Holm et al. 2005; Tucker et al. 1996). Extra-embryonic development is known to be compromised when imprints are lost in the heterozygote progeny of *Dnmt3L*^{-/-} mothers but the early developmental failure of *Dnmt1*^{-/-} embryos makes placental insufficiency an unlikely cause of their demise.

Maintenance of X chromosome inactivation The promoters of genes on the inactive X chromosome are usually DNA methylated (Norris et al. 1991). This is an important mechanism in their transcriptional repression as conditional deletion of Dnmt1 leads to the derepression of genes on the inactive X chromosome in females (Sado et al. 2000). In the extra-embryonic tissues of mice, the female X chromosome is active and the male is inactive but, interestingly, methylation

appears not to be central for maintaining X-inactivation in extra-embryonic tissues (Sado et al. 2000). Presumably polycomb proteins, which bind to the histone H3 proteins methylated at lysine-27, are sufficient to maintain inactivation in the extra-embryonic tissues (Kalantry et al. 2006).

Pluripotency genes Genes such as *Oct4* and *Nanog*, which are required for the maintenance of pluripotency, are inactivated in association with DNA methylation when ES cells and embryos differentiate. As the de novo methyltransferases are highly expressed in ES cells, they appear to be poised for the inactivation of these genes but the signal for inactivation is dependent on repressors that act upstream of DNA methylation. Thus, in the case of *Oct4*, down-regulation is induced first by trans-acting repressors, such as *Gcnf*, in concert with the methyl-CpG-binding proteins *Mbd2* and *Mbd3* (Gu et al. 2006). The locus then becomes “heterochromatinised” in association with lysine-9 histone tri-methylation of histone H3, the binding of *Hp1beta* and DNA methylation (Feldman et al. 2006). However, the DNA methylation induced by *Dnmt3* is clearly a secondary event at the *Oct4* locus as the chromatin must first be modified by the *G9a* histone methyltransferase, which trimethylates lysine-9 of histone H3. Undifferentiated *G9a*-deficient ES cells can be more efficiently recovered from differentiated *G9a*^{-/-} colonies than can wild-type cells under the same conditions, indicating an important role for histone methylation in the permanent silencing of *Oct4* and the commitment of ES cells to terminal differentiation. *Rex1* has also been found to be histone H3K9 trimethylated during differentiation but, strikingly, other embryonic or homeotic genes, such as *Dppa3/Stella/Pgc7*, *Nanog*, *Sox-2*, *Hox-B5* and *Hox-D11*, have not been found to undergo histone methylation. A tissue-dependent DMR controlling gene expression has been identified upstream of *Nanog*. This segment is DNA hypomethylated in ES cells but methylated in trophoblast cells and NIH 3T3 cells (Hattori et al. 2007).

DNA methylation, induced by *Dnmt3a* and *Dnmt3b*, has also been shown to be important for the methylation of the X-linked homeobox gene cluster *Rhox* in ES cells. Methylation controls the lineage-specific expression of this cluster in the embryo, the genes being unmethylated and expressed in the trophoblast but methylated and repressed in the embryo at E9.5. Interestingly, using *Dnmt1*^{-/-}, *3a*^{-/-}, *3b*^{-/-} triple knockout ES cells, Oda et al. (2006) have shown that the full methylation of this locus is dependent on the concerted action of the *Dnmt1* and *Dnmt3* enzymes.

Germ-cell-expressed genes A small number of CpG island genes are expressed in the germ cells but repressed in somatic tissues. The MAGE genes (melanoma-associated genes) are the most prominent in this group (De Smet et al.

1999). Repression in tissues appears to be dependent on promoter methylation, because the genes become expressed in tumours in association with hypomethylation. Studies with 5-azacytidine indicate that the induction of hypomethylation results in the derepression of these genes. A number of other gene families with this characteristic have also been recognised (*BAGE*, *GAGE*). Because of their tumour-restricted expression, these genes have become the focus of attempts to induce host immunity to tumours. Other genes such as *Pgk2* (Zhang et al. 1998), *Pdha-2* (Iannello et al. 2000) and *Tact1* (Hisano et al. 2003) are also expressed in germ cells but repressed in tissues in association with DNA methylation. *Ant4* is expressed in mouse ES cells and germ cells but repressed in tissues (Rodic et al. 2005). Here again, methylation appears to be required for silencing, as the *Ant4* gene can be derepressed by 5-azacytidine treatment.

Tissue-specific genes The tissue-specific expression profiles of a small number of genes are also clearly controlled by CpG island methylation. The p53-inducible gene *maspin* (*SERPINB5*) was the first gene to be described with this property (Futscher et al. 2002) but another gene, viz. 14-3-3 sigma (also p53-inducible) is also controlled by tissue-specific methylation (Oshiro et al. 2005). As methods for detecting DNA methylation on a global scale improve, the number of identified tissue specific genes controlled by DNA methylation will most probably increase.

Transposable elements Despite DNA methylation being present across the genome, the number of genes that are apparently directly regulated by it are small. What then is the purpose of the vast majority of cellular DNA methylation? Bestor and Tycko (1996) have argued that one of the main functions of DNA methylation is to control the transcription of parasitic elements and prevent their transposition. The genome is littered with such elements, and reverse transcriptase appears to be one of the highest copy number genes in the genome. An analysis of the genome reveals that it is in large part (45%) comprised of mutated interspersed DNA transposons and retrotransposons (Smit and Riggs 1996). A marked increase occurs in the transcription of IAP elements during early development in *Dnmt1*-deficient embryos (Walsh et al. 1998), indicating that DNA methylation controls their repression. In a more recent study using the *Aiapy* locus as an in vivo reporter of IAP methylation (this locus has an IAP element inserted into the *Agouti* gene driving ectopic expression when hypomethylated), Gaudet et al. (2004) have been able to show that the oocyte-specific isoform of *Dnmt1* (*Dnmt1o*) is important for maintaining methylation of the IAP in the cleavage-stage embryo, whereas the longer isoform is important for maintaining repression after implantation.

Methylation mediated by Lsh and Dnmt3L also appears to be crucial for controlling the expression of interspersed repeats in germ cells; this activity may in part be attributable to secondary effects on DNA methylation.

Methyl-CpG-binding proteins DNA methylation is likely to exert its effects on transcriptional repression by a number of mechanisms. First, methylation of promoters may impair the binding of transcription factors to promoters - a direct effect. Second, DNA methylation may recruit proteins that have repressive effects to the DNA. An early observation that an in vitro methylated episomal vector transfected into cells is initially transcriptionally active but becomes repressed coincident with assembly of the vector into chromatin indicates that the repressive effects of DNA methylation require the presence of chromatin or chromatin-associated proteins (Buschhausen et al. 1987).

The first protein to be discovered with specific activity for binding to methylated CpG dinucleotides was MECP2 (Lewis et al. 1992), mutations of which are now known to cause Rett syndrome, the commonest cause of mental retardation in females (Amir et al. 1999). The methyl-CpG-binding domain of the MECP2 protein (MBD) has been found in a number of other proteins by homology searches (MBD1–4; Hendrich and Bird 1998). Whereas MeCP2, MBD1 and MBD2 have been found to have strong methyl-CpG-binding activity and transcriptional repression domains (Nan et al. 1996, 1998), MBD3 is part of a complex called NuRD with chromatin remodelling and repressive activities, but little methyl-binding activity of its own. The function of MBD4 has subsequently been uncovered through its homology to a DNA glycosylase. MBD4 repairs the product of mCpG deamination events that produce a T:G mismatch at CpG dinucleotides (Hendrich et al. 1999). Mbd3 is essential for life (Hendrich et al. 2001) but Mecp2 (Guy et al. 2001), Mbd 1 (Zhao et al. 2003), Mbd2 (Hendrich et al. 2001) and Mbd4 (Millar et al. 2002) are not. An unrelated protein, Kaiso, also binds to methylated DNA but is also not necessary for survival in mice (Prokhortchouk et al. 2001, 2006).

To what extent the biological functions of DNA methylation are dependent on methyl-CpG binding proteins or indeed the extent to which the functions of these proteins are dependent on DNA methylation is still not clear. In *Xenopus*, depletion of xKaiso matches xDnmt1 depletion phenotypically and functionally (Ruzov et al. 2004) but, in mice, there are no clear parallels between the phenotypes of MBD deficiency and DNA methyltransferase deficiency.

It is intriguing that mutations in MECP2, a protein capable of binding widely to mCpG dinucleotides, cause a specific neurological disorder (Rett syndrome), rather than a generalised syndrome affecting all of the organs of the body. Indeed, it is not certain that MeCP2 loss of function

causes a transcriptional defect rather than a structural problem leading to irreversible neurodegeneration. Recent studies have started to shed some light on this conundrum. In Rett syndrome, the mutations in MECP2 map to the methyl-CpG-binding domain, strongly suggesting that the phenotype of mental retardation is indeed caused by a loss of this activity. However, recent evidence indicates that Rett syndrome may be caused when this activity fails at specific loci. Detailed studies have now revealed that MECP2 binds to and represses the BDNF III promoter but that membrane depolarisation triggers a Ca-dependent phosphorylation of MECP2 that leads to release of MECP2 from the promoter and activation of the gene (W.G. Chen et al. 2003). Hence, in vivo, the methyl-binding capacity of MECP2 is controlled locally by other proteins that interact with it. One study has also shown that the promoter of *Bdnf* may also become demethylated upon depolarisation leading to the dissociation of the MECP2-mSin3a-HDAC1 complex (Martinowich et al. 2003). It is also now known that the binding of MECP2 to methylated DNA may be less non-specific than originally suspected. Methylation of CpG dinucleotides is necessary but not sufficient for the binding of MECP2. Binding seems to be dependent on an A/T run of four or more base pairs adjacent to the methylated CpG (Klose et al. 2005). Finally, the recent finding that the neurological deficit in *Mecp2*-deficient mice can be reversed by the restoration of *Mecp2* protein strongly supports the contention that *Mecp2* loss of function causes a defect in transcription rather than a structural defect. If *Mecp2* deficiency does result in a structural defect or in neurodegeneration, then the phenotype would not be reversible by the restoration of *Mecp2* function (Guy et al. 2007).

The MBD1 and MBD2 proteins have also been shown to repress methylated promoters in a histone-deacetylase-dependent manner. Interestingly, MBD1 has now been shown to associate with HDAC3 and is apparently necessary for the function of the PML-RAR α fusion oncoprotein (Villa et al. 2006). Despite MBD1 being a potent methylation-dependent repressor in vitro (Ng et al. 2000), MBD1 is recruited to both methylated and non-methylated CpGs via separate domains (Jorgensen et al. 2004). The MBD domain targets the protein to methylated CpGs, whereas the CXXC-3 domain targets the protein to unmethylated CpGs. MBD1 induces repression at both types of promoter.

MBD2 has been reported to be recruited to methylated CpG islands in cancer and may be involved in maintaining their repression (Magdinier and Wolffe 2001). It also has a role in the regulation of gene expression in the normal colon, as Mbd2 loss leads to the frequent activation of genes normally expressed in pancreas and duodenum in mice (Berger et al. 2007). Mbd2 also has a role in the

control of *Xist* expression (Barr et al. 2007). Mbd2-deficient mice have a subtle phenotype characterised by mild maternal behaviour deficits but have a more striking phenotype in mouse cancer models. Mice with the min mutation in the *Apc* gene develop bowel adenomas but this phenotype is strikingly reduced when the *Apc*^{min} mice are bred onto a Mbd2-deficient background (Sansom et al. 2003). As tumours in *Apc*^{min} mice are also reduced on a Dnmt1-deficient background (Laird et al. 1995), DNA methylation and Mbd2 may be required for transformation. Perhaps DNA methylation at other tumour suppressor loci could, through the binding of Mbd2, enhance tumour formation. However, conditional complete inactivation of *Apc* is known to cause tumours with a short latency (Shibata et al. 1997). This indicates that transformation is an inevitable consequence of *Apc* loss and that additional stochastic genetic or epigenetic events at other loci may not be required. Could it be, then, that Mbd2 is directly involved in conferring the effects of *Apc* loss, perhaps by being necessary for the transcriptional repression of genes downstream in the *Apc* pathway? Deficiency of Kaiso, a protein that binds to methylated CGCG sequences, is also known to reduce the frequency of adenomas in *Apc*^{min} mice, although the phenotype is less striking (Prokhortchouk et al. 2006).

Non-methylation dependent function of methyltransferases

DNMT1 is reported to form a repressor complex with Rb, E2F and HDAC1 (Fuks et al. 2000; Robertson et al. 2000); other studies report that de novo methyltransferases, through N-terminal interactions with other proteins, may function as repressors independent of their methyltransferase activities (Fuks et al. 2001). However, there is as yet no indication of the importance of these interactions in vivo. A recent investigation has indicated that, in the context of ES cell differentiation, the methyltransferase function of Dnmt1 is the critical function for differentiation, rather than any N-terminal interaction that the proteins might make with repressors (Damelin and Bestor 2007). Similar findings have been observed in zebrafish in which a catalytic mutant of Dnmt1 is less able to rescue the differentiation defect induced by knock-down of Dnmt1 than a wild-type version of the same protein (Rai et al. 2006). Similarly, hypomethylated *Dnmt*[3a^{-/-},3b^{-/-}] ES cells fail to form teratomas in mice; this defect can be rescued by a catalytically active form of Dnmt3b, but not by an active site mutant (T. Chen et al. 2003). Whether other biological processes are truly dependent on the non-catalytic activities of the DNA methyltransferases remains to be determined.

Chromatin structure and DNA methylation in ES cells

DNA-methylation-dependent histone modifications The re-establishment of histone modification states following replication requires a system whereby the modification can itself recruit more modifying activity to the chromatin in order to maintain the particular modification in question. The tri-methylated lysine-9 modification of histone H3 is maintained because HP1 proteins that bind to this mark also recruit the histone methyltransferase activities to the chromatin (Bannister et al. 2001). A potential problem arises when a de-modification must be maintained, such as the maintenance of histones in a deacetylated state. Here, as there is no acetyl mark, the recruitment of histone deacetylases to maintain deacetylation must be dependent on other marks. DNA methylation may provide such a mark, as methyl-CpG-binding proteins bind to methylated DNA and recruit histone deacetylase activities (Ng and Bird 2000). This provides one potential mechanism for the re-establishment of histone deacetylation following replication but there are likely to be others. The methyl-CpG-binding protein MECP2 is also known to complex with histone H3 methyltransferase activity (Fuks et al. 2002) and so DNA methylation could also help to maintain histone methylation.

In ES cells, profound DNA hypomethylation leads to modest global elevations in histone H4 and histone H3 acetylation as might be expected if methylation is able to direct the recruitment of histone deacetylases through methyl-CpG-binding proteins (Gilbert et al. 2007; Jackson et al. 2004). However, clearly, most of the histone deacetylase activity recruited to chromatin is not directly dependent on DNA methylation, because the inhibition of histone deacetylases activity with trichostatin A causes far higher levels of histone acetylation than are observed in severely hypomethylated *Dnmt*[3a^{-/-},3b^{-/-}] ES cells (Jackson et al. 2004). DNA hypomethylation also causes a significant loss (>50%) of histone H3 lysine-9 dimethylation (Gilbert et al. 2007) but has comparatively little effect on global histone H3 lysine-9 trimethylation levels. There is, however, a measurable reduction in histone H3 lysine-9 trimethylation levels at the minor satellite, with the effect at the major satellite being far less marked (Gilbert et al. 2007).

The maintenance of histone H3 methylation at lysine-9 by SETDB1 histone methyltransferase is dependent, at least in part, on the methyl-CpG-binding protein MBD1, which forms a stable complex with SETDB1 during replication (Sarraf and Stancheva 2004). However, the significance of this effect is questionable because Mbd1-deficient mice do not have a marked phenotype. Another study has demonstrated an interaction between Dnmt1 and G9a, an enzyme that catalyses histone H3 lysine-9 dimethylation during replication (Esteve et al. 2006). Knocking-down DNMT1 activity by using RNAi causes reduced DNA

methylation and a reduction in G9a loading and histone methylation.

DNA hypomethylation has other effects on the chromatin but these are less well understood. A study of *Dnmt1*-deficient ES cells has found a dramatic increase in the amount of MacroH2A at chromocentres in interphase cells and the peri-centromeric heterochromatin in mitotic cells (Ma et al. 2005). This abnormality can be reversed by rescuing the *Dnmt1*^{-/-} cells with a *Dnmt1* cDNA. Intriguingly, the abnormality has also been noted in male *Dnmt1*^{-/-} fibroblasts but not in female *Dnmt1*^{-/-} fibroblasts. The mechanism of this change and the significance for ES cell function is not known.

Biophysical properties of chromatin Experiments characterising the rates of sedimentation of chromatin through sucrose have demonstrated that the heterochromatin associated with satellite repeats has a compact structure at the level of the 30-nm fibre, compared with euchromatin (Gilbert and Allan 2001) and that gene-rich chromatin has a less compact folding irrespective of expression (Gilbert et al. 2004). As immunofluorescence studies with anti-5-methylcytosine antibodies also demonstrate that the peri-centromeric satellite-containing heterochromatin is rich in DNA methylation (Miller et al. 1974), Gilbert et al. (2007) have determined whether severe hypomethylation might affect the compaction of satellite chromatin. Surprisingly, chromatin compaction is not affected but the mobility of linker histones as assessed by photo-bleaching studies (fluorescence recovery after photo-bleaching; FRAP) does appear to be affected. Linker histone mobility increases in the presence of DNA methylation (Gilbert et al. 2007). Nuclear organisation is also affected, with increased nuclear clustering of chromocentres in the presence of severe hypomethylation.

DNA hypomethylation and telomere length A study of *Dnmt1*^{-/-} and *Dnmt1*[3a^{-/-},3b^{-/-}] ES cells has demonstrated that DNA methylation in some way limits telomere length (Gonzalo et al. 2006). Hypomethylated *Dnmt1*^{-/-} ES cells have telomeres that are almost twice as long as wild-type cells, similar to the increase found in early passage *Dnmt1*[3a^{-/-},3b^{-/-}] cells, which retain significant DNA methylation. However, after prolonged passage (passage 100), *Dnmt1*[3a^{-/-},3b^{-/-}] ES cells have average telomere lengths that are almost three times the length of wild-type cells. Gonzalo et al. (2006) have also noted an increase in sister-chromatid exchanges involving telomeric sequences. Indeed, this may be the mechanism through which telomere lengths are increased. Moreover, there may be a generally increased tendency to recombination in hypomethylated ES cells. One study has found that targeting by homologous recombination is two-fold more efficient in *Dnmt1*^{-/-} ES

cells than in wild-type ES cells (Dominguez-Bendala and McWhir 2004).

Histone-methylation-dependent DNA methylation

Whereas DNA hypomethylation appears not to affect chromocentre staining for histone H3 tri-methylated at lysine-9, ES cells that are deficient in the histone methyltransferase Suv-39h1 and Suv-39h2 appear to have a defect in the methylation of major satellite DNA but not of minor satellite DNA or at C-type retroviral sequences (Lehnertz et al. 2003). The loss of HP1 from chromocentres in Suv-39h1- and Suv-39h2-deficient ES cells may explain this result. HP1 and *Dnmt3b* are known to interact. Defective histone H3 methylation may therefore lead directly to less HP1 and indirectly to less *Dnmt3b* recruitment at chromocentres. There is also an association in somatic cells between the polycomb protein EZH2 and DNA methylation. Recent studies have indicated that the propensity for CpG islands to become methylated in cancer may depend on the presence of polycomb complex proteins at the affected genes (Schlesinger et al. 2007). EZH2 methyltransferase establishes methylation on lysine-27 residues of histone H3, and chromodomain proteins, also components of the polycomb complex, bind to these marks and confer repression. Studies in HeLa cells and U2OS cells have demonstrated that the N-terminal domain of EZH2 interacts with the N- and C-terminal domains of DNMT1 and with the PHD domains of *DNMT3A* and *DNMT3B* to confer silencing and DNA methylation to EZH2 target genes. Promoter methylation of the EZH2 target *MYT1* is significantly reduced in the absence of EZH2 (Vire et al. 2006).

DNA demethylation

DNA demethylation can be achieved passively by the failure of the maintenance methylation during DNA synthesis or by the active removal of methyl groups from DNA. Passive demethylation caused by a failure of *Dnmt1*-associated maintenance methylation is known to occur during cleavage in pre-implantation development, although the literature contains a number of reports that argue for the existence of an active DNA demethylase. However, the mechanism of this demethylation and the enzymes responsible are still elusive. Active demethylation of the male pronucleus in mice is perhaps the best example of active demethylation. At 4 h following fertilisation, the paternal genome (male pronucleus) undergoes extensive active DNA demethylation (Mayer et al. 2000; Santos et al. 2002).

Another well-characterised example is the active DNA demethylation of the glucocorticoid-responsive unit located upstream of the rat tyrosine aminotransferase gene (*Tat*) in response to glucocorticoids (Thomassin et al. 2001).

Mechanisms of active DNA demethylation

An initial report that MBD2b might exhibit demethylating activity has not been verified by other groups (Bhattacharya et al. 1999). Although active demethylation might be the result of enzymatic activity that removes the methyl group from the cytosine base, this might not be energetically feasible and other mechanisms for active demethylation have been suggested.

The excision of the whole 5-methylcytosine base by a 5-methylcytosine DNA N-glycosylase (5MCDG) has been proposed (Jost 1993; Jost and Jost 1994). Further studies have suggested that the base-excising activity of the enzyme on a hemi-methylated substrate might be dependent on RNA complementary to the methylated DNA strand (Fremont et al. 1997; Jost et al. 1997). A DEAD box protein related to p68 RNA helicase is also highly associated with the 5MCDG-RNA complex and might also be necessary, although there is no proof of this (Jost et al. 1999). The 5-methylcytosine N-glycosylase activity found in extracts prepared from developing chicken embryos has later been found to be attributable to the chicken orthologue of thymidine DNA glycosylase (Zhu et al. 2000), an enzyme that excises thymines mispaired to guanine. Expressing the recombinant protein in *Escherichia coli* results in weak demethylation (Zhu et al. 2000). Morpholino-directed inhibition of 5MCDG results in higher levels of DNA methylation in differentiating myoblasts compared with controls, suggesting that the enzyme might have a global demethylating activity that is active on myoblast differentiation induction (Jost et al. 2001). The aforementioned demethylation of the glucocorticoid response unit of *Tat* is associated with strand breaks 3' to the 5-methylcytosine; this is consistent with a base-excision mechanism (Kress et al. 2006). The finding that DNA demethylation takes place via N-glycosylase activity in plants also supports the notion that such an activity might exist in mammals. Indeed, a family of 5-methylcytosine N glycosylases has been described in *Arabidopsis*, the founding enzyme being DEMETER (DME), which has a role in the regulation of imprinting in this species (Kinoshita et al. 2004; Penterman et al. 2007; Xiao et al. 2003).

Another potential mechanism of demethylation could be enzyme-induced deamination of 5-methylcytosine to thymine, followed by excision and repair of the G:T mismatches so produced. Whereas the Aid and Apobec1 proteins have now been shown to possess 5-methylcytosine

deaminase activity and are expressed in oocytes, embryonic germ cells and ES cells (Morgan et al. 2004), no evidence has been published that these are the enzymes responsible for pronuclear demethylation.

Passive DNA demethylation

Following fertilisation and demethylation of the male pronucleus, there follows a progressive demethylation of the early embryo during the cleavage divisions, because the oocyte-specific form of the maintenance enzyme Dnmt1 (Dnmt1o) is cytoplasmically located and therefore fails to maintain methylation. This isoform does apparently enter the nucleus transiently at the eight-cell stage, when it is necessary for maintaining methylation imprints through the fourth embryonic synthesis (Howell et al. 2001). The progeny of *Dnmt1o*^{-/-} females have no maternally derived Dnmt1o and this results in a loss of imprints and death in late gestation. The homozygous *Dnmt1o*^{-/-} animals are themselves apparently normal. The cause of death has not been established. Dnmt1o deficiency does not affect uterine function, although a defect in placental function cannot be ruled out, particularly in the light of evidence that defective imprinting in the progeny of *Dnmt3L*^{-/-} and *Dnmt3a*^{-/-} animals is associated with defects in placental growth (Arima et al. 2006).

Function of DNA demethylation in early development

Some degree of passive demethylation during cleavage has been observed in all methylated animals that have been studied, although the timing can vary considerably in different species. Demethylation is greatest at the midblastula transition in *Xenopus* (Stancheva et al. 2002) and occurs between the one- to two-cell stage and blastula formation in zebrafish (Mhanni and McGowan 2004). Maximal demethylation is observed at the eight-cell stage in sheep and cattle. In mice, maximal demethylation occurs later (in the morula) and is more pronounced (Beaujean et al. 2004a, 2004b, 2004c; Dean et al. 2001). Differences in timing may account for discrepancies in the literature as to whether hypomethylation is a genuine feature of early development in zebrafish (Macleod et al. 1999). Whereas some degree of genome-wide demethylation is seen during early development in most species studied, its role remains unknown. Interestingly, in *Xenopus*, which does not undergo active male pronuclear demethylation, loss of Dnmt1 activity is associated with the premature activation of developmentally decisive genes, thereby leading to the argument that DNA methylation has a role in controlling the transcription of these genes (Stancheva et al. 2002; Stancheva and Meehan 2000).

The function of active pronuclear demethylation is even more elusive, because it does not occur in all vertebrates. In mice, active demethylation of the male pronucleus occurs within 4 h of fertilisation (Mayer et al. 2000; Santos et al. 2002). This dramatic effect has also been observed in rats, pigs and humans and to a lesser extent in cows but has not been observed in frogs, rabbits or sheep (Beaujean et al. 2004a, 2004b, 2004c; Dean et al. 2001). Even more intriguingly, sheep oocytes possess an activity that demethylates mouse sperm nuclei introduced by intracytoplasmic injection (Beaujean et al. 2004a, 2004b, 2004c). As sheep sperm are not demethylated, this indicates that the extent of active demethylation is a property of the sperm and of the oocyte. Whether the property in sperm is a function of chromatin structure or gene sequence has not been established. Indeed, as a demethylase activity exists in the sheep oocytes, this indicates that pronuclear demethylation may be a feature of normal fertilisation in sheep after all but that its extent may be much more limited than it is in the mouse and therefore not detected by immunofluorescence detection of 5-methylcytosine. The same may be true of *Xenopus* oocytes, which appear to be able to reprogram mammalian nuclei and induce demethylation of the *Oct4* promoter, before inducing strong expression of Oct4 (Byrne et al. 2003; Simonsson and Gurdon 2004). A recent study has shown that Tpt1 (tumour translationally controlled protein 1) may be one of the proteins that is present in the oocyte cytoplasm and that is capable of binding to and activating Oct4 (and Nanog; Koziol et al. 2007).

Some indication as to the importance of demethylation in pre-implantation development comes from the study of cloned embryos in which demethylation appears to occur to some extent but is generally perceived to be sub-optimal. In one study examining methylation in cloned bovine embryos derived from fibroblasts, demethylation has been seen in the euchromatin during cleavage but does not occur in the heterochromatin (Bourc'his et al. 2001a, 2001b). In another study, active demethylation appeared to occur but precocious de novo methylation took place during cleavage starting at the four- to eight-cell stage (Dean et al. 2001). Sub-optimal demethylation may lead to the failure of Oct4 up-regulation and may be a major impediment to successful nuclear cloning. Successful up-regulation of the gene is associated with improved success in nuclear cloning (Boiani et al. 2002; Bortvin et al. 2003).

Concluding remarks

Enormous strides have been made in our understanding of the function of the DNA methyltransferases and their importance in development. Methylation is essential for the establishment and maintenance of allele-specific ex-

pression patterns and is important for maintaining X-inactivation in females. In both cases, the cell selects just one of the two parental alleles for expression and so DNA methylation has the capacity to maintain repression when trans-acting factors are present, which would otherwise induce transcription. Methylation is equally important for the suppression of parasitic elements. However, there is still no settled view as to why development fails when either the maintenance or the de novo methyltransferases are deleted. In both circumstances, the genome becomes markedly hypomethylated and the timing of developmental failure is similar. These similarities suggest that methylation itself is required, although whether this is through the regulation of *Xist* or other developmental regulators is unclear. DNA methylation clearly affects certain genes required for pluripotency but the evidence that the methylation of these genes is essential for their down-regulation during development is not compelling. DNA hypomethylation might also have global effects on the function of key regulatory elements, such as boundary elements and insulators. CTCF has been shown to bind to such elements in a hypomethylation-dependent manner, controlling the reciprocal expression of the imprinted genes *Igf2* and *H19*. In the context of global hypomethylation, promiscuous CTCF binding could conceivably disrupt the function of such elements at other loci in the genome.

Despite the advances made in our understanding of the importance of DNA methylation, we can only speculate on the importance of the global hypomethylation that occurs in pre-implantation development and during gametogenesis. The mechanism of active pronuclear demethylation has not been elucidated and so investigators are not yet able to test its importance or function. Global passive demethylation occurs during the cleavage divisions of pre-implantation development, because of a relative deficiency of nuclear Dnmt1. This is presumably required for the full reprogramming of the genome, but there is no formal proof for this. The establishment of the importance of global hypomethylation for reprogramming would inform efforts aimed at generating pluripotent cells from somatic cells, a key goal in therapeutic cloning.

Recently, mouse ES cells have also been generated from fibroblasts by the exogenous expression of Oct4, Sox2, c-myc and Klf4 (Takahashi and Yamanaka 2006). Whether global demethylation occurs when these cells are in the process of reprogramming is not clear but methylation patterns and other epigenetic marks acquired by the reprogrammed cells are similar to those observed in normal ES cells and strikingly different from the fibroblasts from which they were derived (Wernig et al. 2007). In the experimental situation, therefore, the simple expression of four transcription factors can reprogram the genome but the process takes 20 days and is of rather low efficiency

(0.05%–0.1% of cells are reprogrammed). We are tempted to speculate that a temporary induction of global hypomethylation during this process might increase the efficiency of reprogramming.

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