Genetic Studies on Mammalian DNA Methyltransferases

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Abstract

Cytosine methylation at the C5-position, generating 5-methylcytosine (5mC), is a DNA modification found in many eukaryotic organisms, including fungi, plants, invertebrates, and vertebrates, albeit its levels vary greatly in different organisms. In mammals, cytosine methylation occurs predominantly in the context of CpG dinucleotides, with the majority (60–80 %) of CpG sites in their genomes being methylated. DNA methylation plays crucial roles in the regulation of chromatin structure and gene expression and is essential for mammalian development. Aberrant changes in DNA methylation levels and patterns are associated with various human diseases, including cancer and developmental disorders. DNA methylation is mediated by three active DNA methyltransferases (Dnmts), namely, Dnmt1, Dnmt3a, and Dnmt3b, in mammals. Over the last two decades, genetic manipulations of these enzymes, as well as their regulators, in mice have greatly contributed to our understanding of the biological functions of DNA methylation in mammals. In this chapter, we discuss genetic studies on

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mammalian Dnmts, focusing on their roles in embryogenesis, cellular differentiation, genomic imprinting, and X-chromosome inactivation.

Abbreviations

5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
ADCA-DN	Autosomal dominant cerebellar ataxia deafness and narcolepsy
ADD	ATRX-Dnmt3-Dnmt3L
AML	Acute myeloid leukemia
BAH	Bromo-adjacent homology
DKO	Double knockout
DMR	Differentially methylated region
DNMT	DNA methyltransferase
ES	Embryonic stem
EST	Expressed sequence tag
HP1	Heterochromatin protein 1
HSAN IE	Hereditary sensory and autonomic neuropathy with dementia and hearing loss type IE
ICF	Immunodeficiency centromeric instability and facial anomalies
ICM	Inner cell mass
ICR	Imprinting control region
KAP1	KRAB-associated protein 1
KRAB	*Krüppel*-associated box
lncRNA	Long non-coding RNA
MBD3	Methyl CpG-binding domain protein-3
MEF	Mouse embryonic fibroblast
MTA2	Metastasis tumor antigen 2
NLS	Nuclear localization signal
NuRD	Nuclear remodeling and histone deacetylation
PBD	PCNA-binding domain
PCNA	Proliferating cell nuclear antigen
PGC	Primordial germ cell
PHD	Plant homeodomain
PRC2	Polycomb repressive complex 2
PWWP	Proline-tryptophan-tryptophan-proline
RFTS	Replication foci-targeting sequence
RING	Really Interesting New Gene
SRA	SET- and RING-associated
TDG	Thymine DNA glycosylase
TTD	Tandem tudor domain
UBL	Ubiquitin-like

Uhrf1	Ubiquitin-like with PHD and RING finger domains 1
Xa	Active X chromosome
XCI	X-chromosome inactivation
Xi	Inactive X chromosome
Xic	X-inactivation center
Xist	X-inactive-specific transcript
Xm	Maternal X chromosome
Xp	Paternal X chromosome

1 Distinct Roles of Dnmt1 and Dnmt3 Families in DNA Methylation

In 1975, long before the identification of any mammalian DNA methyltransferase, Holliday and Pugh and Riggs independently proposed a theory that DNA methylation could serve as a heritable epigenetic mark for cellular memory. Recognizing that the CpG dinucleotide is self-complementary, they postulated that methylated and unmethylated CpG sites could be copied when cells divide so that DNA methylation patterns would be replicated semiconservatively like the base sequence of DNA itself (Holliday and Pugh 1975; Riggs 1975). A prediction of the theory was the existence of at least two DNA methyltransferase activities: *de novo* methyltransferase(s) would methylate unmodified DNA and establish DNA methylation patterns, and maintenance methyltransferase(s) would recognize hemimethylated sites and "copy" the methylation patterns from the parental strand onto the daughter strand at each round of DNA replication.

1.1 Dnmt1: The Major Maintenance Methyltransferase

The first mammalian DNA methyltransferase gene, *Dnmt1*, was cloned from murine cells (Bestor et al. 1988). The *Dnmt1* locus has several transcription start sites and produces two major protein products (Mertineit et al. 1998; Rouleau et al. 1992). Transcription initiation within a somatic cell-specific exon (exon 1 s) results in the Dnmt1s isoform (generally referred to as Dnmt1) which consists of 1620 amino acids. Transcription initiation within an oocyte-specific exon (exon 1o) produces a transcript that utilizes a downstream AUG as the translation initiation codon. As a result, the oocyte-specific isoform, Dnmt1o, lacks the N-terminal 118 amino acids of Dnmt1s (Mertineit et al. 1998). Although Dnmt1o is more stable than Dnmt1s, genetic evidence suggests no functional difference between these isoforms (Ding and Chaillet 2002). Human DNMT1, consisting of 1616 amino acids, is nearly 80 % identical to the mouse Dnmt1 at the amino acid level.

Dnmt1 contains a C-terminal catalytic domain containing characteristic amino acid sequence motifs that are homologous to bacterial DNA methyltransferases and an N-terminal regulatory region that is not present in bacterial enzymes (Bestor

et al. 1988). The N-terminal regulatory region contains several functional domains, including a proliferating cell nuclear antigen (PCNA)-binding domain (PBD) responsible for the interaction with the DNA replication machinery, a nuclear localization signal (NLS), a replication foci-targeting sequence (RFTS) that mediates the association with late replicating heterochromatin, a zinc-finger CXXC domain that recognizes unmethylated CpG-containing DNA, and a pair of bromo-adjacent homology (BAH) domains (Fig. 1a). Recent structural data revealed that the RFTS domain binds to the catalytic domain and blocks the catalytic center, suggesting an autoinhibitory role in the regulation of enzymatic activity (Takeshita et al. 2011).

In vitro biochemical assays revealed that, although Dnmt1 is capable of methylating both unmethylated and hemimethylated CpG dinucleotides, its activity toward hemimethylated substrates is far more efficient (Pradhan et al. 1999). Dnmt1 is ubiquitously expressed through development, with high levels in proliferating cells. Dnmt1 associates with the DNA replication machinery at S phase and with heterochromatin at late S and G2 phases (Chuang et al. 1997; Easwaran et al. 2004; Leonhardt et al. 1992; Schneider et al. 2013), suggesting that Dnmt1-mediated methylation is coupled to DNA replication. These findings supported the notion that Dnmt1 functions as a maintenance enzyme (Fig. 1b). However, because Dnmt1, the only known DNA methyltransferase at the time, also has *de novo* methylation activity *in vitro*, it was initially debated whether *de novo* methylation and maintenance methylation are carried out by Dnmt1 alone or by two or more distinct enzymes (Bestor 1992).

Genetic studies in mouse models and murine cells helped settling the debate. Several *Dnmt1* mutant alleles were generated by gene targeting. The $Dnmt1^{n}$ allele (n stands for N-terminal disruption) was reported in 1992 (Li et al. 1992). This allele, in which a genomic region coding 60 amino acids near the N-terminal end was replaced by a neomycin resistance cassette, is a partial loss-of-function (hypomorphic) mutation. $Dnmt1^{n/n}$ embryos have a ~70 % reduction in global DNA methylation and show mid-gestation lethality (Li et al. 1992). Subsequently, the $Dnmt1^{s}$ allele (s stands for *Sal*I site) was reported, which had a neomycin resistance cassette inserted into a *Sal*I site in exon 17, disrupting the RFTS (Li et al. 1993). The $Dnmt1^{s}$ allele is functionally more severe than the $Dnmt1^{n}$ allele, as $Dnmt1^{s/s}$ embryos show lower levels of DNA methylation and earlier lethality (Lei et al. 1996). However, it was unclear whether the $Dnmt1^{s}$ allele was a null mutation, because the C-terminal catalytic domain was intact. To completely inactivate *Dnmt1*, Lei et al. generated the $Dnmt1^{c}$ allele (c stands for C-terminal disruption) by disrupting the catalytic domain, including the highly conserved PC and ENV motifs that are essential for enzymatic activity (Lei et al. 1996). The development of $Dnmt1^{c/c}$ embryos is arrested prior to the 8-somite stage, significantly earlier than the developmental phenotype of $Dnmt1^{n/n}$ embryos, while the viability and proliferation of *Dnmt1* null embryonic stem (ES) cells are not affected (Lei et al. 1996). Inactivation of Dnmt1 by mutating the cysteine (C1229) residue at the catalytic center (PC motif) results in similar developmental defects (Takebayashi et al. 2007), suggesting that the phenotype is largely due to the loss of catalytic activity. DNA methylation analyses revealed that *Dnmt1* null embryos and ES cells contain low but stable levels of

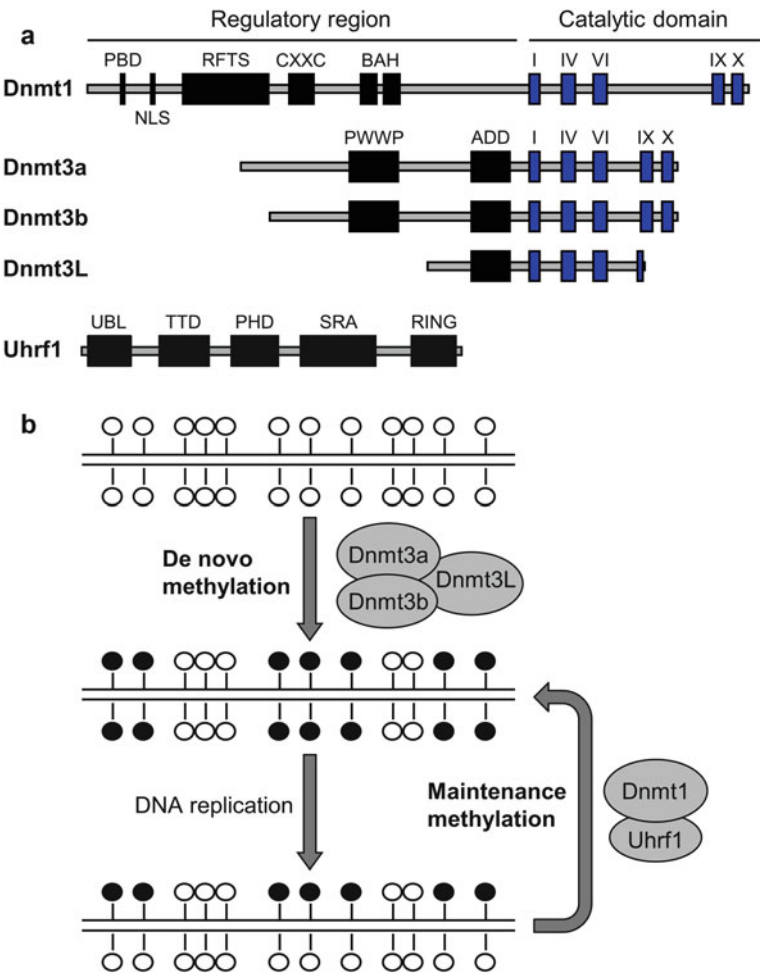

Fig. 1 DNA methyltransferases and major regulatory proteins involved in DNA methylation. (**a**) Schematic diagrams of Dnmt1, Dnmt3a, Dnmt3b, Dnmt3L, and Uhrf1. The C-terminal catalytic domains of the Dnmt1 and Dnmt3 families are conserved (the highly conserved signature motifs I, IV, VI, IX, and X are shown), but their N-terminal regulatory regions are distinct. Functional domains of the proteins are indicated. *PBD* PCNA-binding domain, *NLS* nuclear localization signal, *RFTS* replication foci-targeting sequence, *CXXC* a cysteine-rich domain implicated in binding CpG-containing DNA sequences, *BAH* bromo-adjacent homology domain, *PWWP* proline-tryptophan-tryptophan-proline domain, *ADD* ATRX-Dnmt3-Dnmt3L domain, and *UBL* ubiquitin-like domain; *TTD* tandem tudor domain, *PHD* plant homeodomain, *SRA* SET- and RING-associated domain, and *RING* Really Interesting New Gene domain. (**b**) *De novo* and maintenance methyltransferase activities. The *de novo* methyltransferases Dnmt3a and Dnmt3b, in complex with their accessory factor Dnmt3L, methylate unmodified DNA and establish methylation patterns. At each round of DNA replication, the maintenance methyltransferase Dnmt1, aided by its accessory factor Uhrf1, "copies" the methylation pattern from the parental strand onto the daughter strand. Open circles represent unmethylated CpG dinucleotides, and filled circles represent methylated CpG dinucleotides

5-methylcytosine (5mC) and methyltransferase activity. Moreover, the *de novo* methylation activity is not impaired by Dnmt1 loss, as integrated provirus DNA in MoMuLV-infected *Dnmt1* null ES cells becomes methylated at a similar rate as in wild-type ES cells (Lei et al. 1996). Taken together, these studies provided compelling evidence for the existence of one or more DNA methyltransferases that are important for *de novo* methylation.

1.2 Dnmt2/Trdmt1: A tRNA Methyltransferase

Results from genetic studies of *Dnmt1* prompted the search for more DNA methyltransferase genes. In 1998, several groups reported the identification of a second putative DNA methyltransferase gene, named *Dnmt2*, which encodes a protein of 391 amino acids in human or 415 amino acids in mouse (Okano et al. 1998b; Van den Wyngaert et al. 1998; Yoder and Bestor 1998). Despite the presence of all the conserved motifs shared by known prokaryotic and eukaryotic DNA cytosine methyltransferases, Dnmt2 has no detectable DNA methyltransferase activity in standard *in vitro* assays. Furthermore, inactivation of *Dnmt2* in mouse ES cells by gene targeting has no effect on preexisting genomic methylation patterns or on the ability to methylate newly integrated retrovirus DNA *de novo* (Okano et al. 1998b). Indeed, a subsequent study demonstrated that Dnmt2 is a tRNA methyltransferase, specific for cytosine 38 in the anticodon loop of aspartic acid tRNA, and has been renamed tRNA aspartic acid (D) methyltransferase 1 (Trdmt1) (Goll et al. 2006).

1.3 Dnmt3a and Dnmt3b: The *De Novo* Methyltransferases

By searching an expressed sequence tag (EST) database using full-length bacterial type II cytosine-C5 methyltransferase sequences as queries, Okano *et al*. identified two additional homologous genes, *Dnmt3a* and *Dnmt3b*, in both mouse and human. Their protein products contain the highly conserved DNA methyltransferase motifs in their C-terminal regions, but their N-terminal regulatory regions are unrelated to that of Dnmt1 (Okano et al. 1998a). The N-terminal regions of Dnmt3a and Dnmt3b contain a variable region and two conserved domains, the proline-tryptophan-tryptophan-proline (PWWP) domain and the ATRX-Dnmt3-Dnmt3L (ADD) domain (Fig. 1a). Both domains are implicated in chromatin binding. The PWWP domain is required for heterochromatin localization and mediates H3K36me3 binding (Baubec et al. 2015; Chen et al. 2004; Dhayalan et al. 2010). The ADD domain interacts with the N-terminal tail of histone H3, and the interaction is disrupted by various posttranslational modifications of H3, including di- and trimethylation of K4, acetylation of K4, and phosphorylation of T3, S10, or T11 (Otani et al. 2009; Noh et al. 2015; Zhang et al. 2010).

Dnmt3a produces two major isoforms, Dnmt3a and Dnmt3a2, driven by different promoters (Chen et al. 2002). The full-length Dnmt3a protein, consisting of

908 amino acids in mouse and 912 amino acids in human, is expressed ubiquitously at relatively low levels. The *Dnmt3a2* transcript is initiated in intron 6 of the *Dnmt3a* gene and encodes a protein that lacks the N-terminal 219 (in mouse) or 223 (in human) amino acids of Dnmt3a. Dnmt3a2, which is catalytically active, is the predominant form in mouse ES cells, early embryos, and developing germ cells, as well as human embryonal carcinoma cells, and is also detectable in spleen and thymus (Chen et al. 2002). The *Dnmt3b* gene produces multiple alternatively spliced isoforms, some of which encode catalytically inactive protein products. The longest isoform, Dnmt3b1, consists of 859 amino acids in mouse and 853 amino acids in human, respectively. Both active and inactive Dnmt3b isoforms appear to co-express in most, if not all, cell types. For example, Dnmt3b1, an active form, and Dnmt3b6, an inactive form, are the predominant forms in mouse ES cells, whereas Dnmt3b2, an active form, and Dnmt3b3, an inactive form, are expressed at low levels in many somatic cells (Chen et al. 2002). There is evidence that catalytically inactive Dnmt3b protein products may play regulatory roles in DNA methylation. For example, overexpression of human DNMT3B7, a truncated isoform frequently found in cancer cells, leads to higher levels of total genomic methylation and altered gene expression in both transgenic mice and human cancer cells (Ostler et al. 2012; Shah et al. 2010).

Several lines of evidence suggest the involvement of Dnmt3a and Dnmt3b in *de novo* DNA methylation (Fig. 1b). First, Dnmt3a and Dnmt3b are highly expressed in early embryos (and ES cells) and developing germ cells, where an active *de novo* methylation takes place, but are downregulated in somatic tissues and when ES cells are induced to differentiate (Okano et al. 1998a). Second, recombinant Dnmt3a and Dnmt3b proteins methylate unmethylated and hemimethylated DNA with equal efficiency (Okano et al. 1998a). Genetic studies provided definitive evidence that Dnmt3a and Dnmt3b were the long-sought *de novo* methyltransferases. Inactivation of both *Dnmt3a* and *Dnmt3b* by gene targeting blocks *de novo* methylation in ES cells and early embryos but has no effect on maintenance of imprinted methylation patterns (Okano et al. 1999). Dnmt3a deficiency also leads to failure to establish DNA methylation imprints in developing germ cells (Kaneda et al. 2004).

It is worth noting that the *de novo* DNA methyltransferase activity of Dnmt3a and Dnmt3b is not only essential for the establishment of new DNA methylation patterns but also important for the faithful maintenance of these patterns. In culture, *Dnmt3a/3b* double knockout (DKO) ES cells exhibit gradual loss of global DNA methylation and, after multiple passages, show severe hypomethylation (Chen et al. 2003), suggesting that Dnmt1 and Dnmt3 enzymes have distinct and nonredundant functions but act cooperatively in the maintenance of global DNA methylation. Based on the kinetics of DNA methylation loss, it was proposed that Dnmt1 is the major maintenance methyltransferase that, upon DNA replication, methylates hemimethylated CpG sites with high efficiency but not absolute fidelity, whereas Dnmt3a and Dnmt3b, as *de novo* methyltransferases, act as "proofreading" enzymes that methylate the hemimethylated CpG sites missed by Dnmt1 (Chen et al. 2003).

1.4 Dnmt3L: A Regulator of *De Novo* Methylation

A third member of the Dnmt3 family, *Dnmt3*-like (*Dnmt3L*), was originally isolated by database analysis of the human genome sequence (Aapola et al. 2000). Its murine homolog was subsequently identified (Aapola et al. 2001; Hata et al. 2002). The human and mouse Dnmt3L proteins consist of 387 and 421 amino acids, respectively. Dnmt3L contains an ADD domain, but lacks a PWWP domain, in the N-terminal region. Its C-terminal region is highly related to the catalytic domains of Dnmt3a and Dnmt3b, but lacks some motifs essential for enzymatic activity, including the PC dipeptide at the active site and the sequence motif involved in binding of the methyl donor *S*-adenosyl-L-methionine (Aapola et al. 2000, 2001; Hata et al. 2002) (Fig. 1a). Therefore, Dnmt3L has no methyltransferase activity. However, Dnmt3L has been shown to interact with Dnmt3a and Dnmt3b, stimulate their enzymatic activities, and target them to chromatin (Hata et al. 2002; Jia et al. 2007; Ooi et al. 2007; Suetake et al. 2004). The expression pattern of Dnmt3L during development is also strikingly similar to that of Dnmt3a and Dnmt3b, including high expression in developing germ cells, early embryos, and ES cells (Hata et al. 2002). These findings indicate that Dnmt3L may regulate Dnmt3a/3b functions (Fig. 1b). Genetic studies indeed demonstrate that Dnmt3L is an essential accessory factor of Dnmt3a in the germ line. *Dnmt3L* homozygous null mice are viable and grossly normal, but both male and female mice are infertile (Bourc'his et al. 2001; Hata et al. 2002). Male mice show activation of retrotransposons in spermatogonia and spermatocytes, due to failure to establish methylation at these elements, and are azoospermic (Bourc'his and Bestor 2004). Female mice fail to establish maternal methylation imprints in oocytes, and, as a result, embryos derived from these oocytes cannot survive beyond mid-gestation (Bourc'his et al. 2001; Hata et al. 2002). The phenotype is indistinguishable from that of mice with conditional *Dnmt3a* deletion in germ cells (Kaneda et al. 2004). Recently, Dnmt3L was shown to antagonize DNA methylation at H3K4me3/K27me3 bivalent promoters, which are often associated with developmental genes, and favor DNA methylation at gene bodies in ES cells. It was suggested that Dnmt3L, via its ADD domain, interacts with Polycomb repressive complex 2 (PRC2) in competition with Dnmt3a and Dnmt3b to maintain low methylation levels at regions with H3K27me3, thus maintaining hypomethylation at promoters of bivalent developmental genes (Neri et al. 2013). The physiological relevance of this finding remains to be determined, given that zygotic Dnmt3L is not required for embryonic development and postnatal survival (Bourc'his et al. 2001; Hata et al. 2002).

1.5 Uhrf1: A Regulator of Maintenance Methylation

Besides Dnmts, a number of DNA methylation regulators have been identified, including the multi-domain protein Uhrf1 (ubiquitin-like with PHD and RING finger domains 1), also known as NP95 (mouse) and ICBP90 (human) (Fig. 1a). Genetic studies demonstrated an essential role for Uhrf1 in maintaining DNA

methylation (Fig. 1b). Uhrf1 deficiency leads to embryonic lethality and global DNA hypomethylation (Bostick et al. 2007; Muto et al. 2002; Sharif et al. 2007), resembling the phenotype of Dnmt1 deficiency. Cellular and biochemical evidence suggested functional interactions between Uhrf1 and Dnmt1. Uhrf1 co-localizes with Dnmt1 at DNA replication foci and heterochromatin, and Dnmt1 fails to enrich at these regions in the absence of Uhrf1 (Bostick et al. 2007; Liu et al. 2013; Sharif et al. 2007). These findings suggest that Uhrf1 is a key accessory factor for directing Dnmt1 to hemimethylated CpG sites. However, it remains somewhat controversial as to whether Uhrf1 directly recruits Dnmt1 or indirectly controls Dnmt1 localization by affecting chromatin structure. Uhrf1 harbors five known functional domains: a ubiquitin-like domain (UBL) at the N-terminus, followed by a tandem tudor domain (TTD), a plant homeodomain (PHD), a SET- and RING-associated (SRA) domain, and a Really Interesting New Gene (RING) domain (Fig. 1a). All the domains, with the exception of UBL, have been shown to be important for Dnmt1 subnuclear localization and maintenance of DNA methylation. Biochemical and structural evidence revealed that the SRA domain preferentially binds hemimethylated DNA and is thought to play an important role in loading Dnmt1 onto newly synthesized DNA substrates (Arita et al. 2008; Avvakumov et al. 2008; Bostick et al. 2007; Hashimoto et al. 2008; Sharif et al. 2007). The association of Uhrf1 with heterochromatin is also mediated by TTD, which contains an aromatic cage for binding of the heterochromatic H3K9me3 mark. The PHD acts in combination with TTD to read the H3K9me3 mark and, additionally, interacts with histone H3 tails with unmethylated R2 (H3R2me0) (Cheng et al. 2013; Liu et al. 2013; Rothbart et al. 2012, 2013; Rottach et al. 2010). Recent studies suggested that Uhrf1, via the E3 ligase activity of its RING domain, mediates ubiquitylation of H3K23 and H3K18, creating binding sites for Dnmt1 (Nishiyama et al. 2013; Qin et al. 2015). It is worth noting that Uhrf1 has also been shown to control Dnmt1 ubiquitylation and stability (Du et al. 2010; Qin et al. 2011). Indeed, a recent study revealed that Uhrf1 overexpression results in DNA hypomethylation, due to destabilization and delocalization of Dnmt1, which led the authors to propose that Uhrf1 overexpression, which is frequently observed in cancer cells, is a mechanism underlying global DNA hypomethylation in cancer (Mudbhary et al. 2014).

2 Dnmts in Embryonic Development and Cellular Differentiation

2.1 Dynamic Changes of DNA Methylation During Early Embryogenesis

DNA methylation is relatively stable in somatic tissues but exhibits dynamic changes in early embryos. During preimplantation development, both the maternal and paternal genomes undergo global DNA demethylation, albeit the mechanisms involved are distinct. The maternal genome is demethylated mainly through DNA replication-dependent passive dilution because of deficient maintenance

methylation, presumably due to the exclusion of Dnmt1 from the nucleus (Hirasawa et al. 2008; Howell et al. 2001). In contrast, demethylation of the paternal genome involves both active and passive mechanisms. Shortly after fertilization and before the first cell division, the 5mC dioxygenase Tet3 converts the majority of 5mC in the male pronucleus to 5-hydroxymethylcytosine (5hmC) (Gu et al. 2011; Wossidlo et al. 2011). 5hmC can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can be excised by thymine DNA glycosylase (TDG) and replaced by unmodified cytosine (He et al. 2011; Ito et al. 2011). 5hmC, 5fC, and 5caC can also be passively diluted during cleavage divisions (Inoue et al. 2011; Inoue and Zhang 2011). As a result of these processes, DNA methylation marks inherited from gametes are largely erased by the blastocyst stage, with the exception of imprinting control regions (ICRs) and some retroelements, which resist this wave of global demethylation (Borgel et al. 2010; Smith et al. 2012). Around the time of implantation, *de novo* methylation takes place when the inner cell mass (ICM) starts to differentiate to form the embryonic ectoderm. Lineage-specific DNA methylation patterns are then stably maintained.

2.2 Embryonic and Adult Phenotypes of Dnmt Mutant Mice

Most of our knowledge about the significance of DNA methylation in mammalian development came from genetic manipulations of *Dnmt* genes in mice. Results from characterization of *Dnmt* mutant mice demonstrated that the establishment of embryonic methylation patterns requires both *de novo* and maintenance Dnmts and that maintaining genomic methylation above a threshold level is essential for embryonic development (Lei et al. 1996; Li et al. 1992; Okano et al. 1999). Complete inactivation of Dnmt1 results in the arrest of embryonic development between presomite and 8-somite stage around E9.5 (Lei et al. 1996). DNA methylation analysis showed that embryos deficient for Dnmt1 undergo dramatic decreases in global DNA methylation (Lei et al. 1996; Li et al. 1992), in agreement with its role as the major maintenance Dnmt. Disruption of *Dnmt3b* also leads to embryonic lethality after E12.5, with multiple defects, including growth impairment and rostral neural tube defects (Okano et al. 1999). In contrast, *Dnmt3a*-deficient mice develop to term and appear to be normal at birth but become runted and die at about 4 weeks (Okano et al. 1999). Consistent with the developmental phenotypes, DNA methylation analysis of E9.5 embryos revealed that germ line-specific genes, pluripotency genes, hematopoietic genes, and eye genes are severely hypomethylated in the absence of Dnmt3b but not of Dnmt3a (Borgel et al. 2010). This suggested that Dnmt3b is the main enzyme responsible for *de novo* methylation during embryogenesis. Dnmt3b shows a dynamic expression change during pre- and early postimplantation development, with preferential expression in the trophectoderm at the mid-blastocyst stage and subsequent transition of expression in the embryonic lineage (Hirasawa and Sasaki 2009). Notably, DNA methylation at certain genes such as *Brdt*, *Dpep3*, *Cytip*, and *Crygd* is only partially reduced in *Dnmt3b*$^{-/-}$ embryos (Borgel et al. 2010), suggesting that Dnmt3a cooperates with Dnmt3b to methylate some loci. Indeed,

Dnmt3a/*3b* DKO embryos exhibit more severe defects than *Dnmt3b*$^{-/-}$ embryos. Specifically, DKO embryos show smaller size, lack somites, do not undergo embryonic turning, and die before E11.5, indicating that their growth and morphogenesis are arrested shortly after gastrulation (Okano et al. 1999).

Conditional knockout (KO) studies have also demonstrated that Dnmts and DNA methylation are essential in various organs and tissues. For example, disruption of both *Dnmt1* and *Dnmt3a* in forebrain excitatory neurons leads to abnormal synaptic plasticity and deficits in learning and memory (Feng et al. 2010). Conditional deletion of *Dnmt1* at sequential stages of T cell development has also revealed a critical role for DNA methylation in T cell development, function, and survival. Specifically, deletion of *Dnmt1* in early double-negative thymocytes leads to an impaired survival of TCRαβ(+) cells and the generation of atypical CD8(+) TCRγδ(+) cells and deletion of Dnmt1 in double-positive thymocytes impairs activation-induced proliferation but differentially enhanced cytokine mRNA expression by naive peripheral T cells (Lee et al. 2001).

2.3 Cellular Defects of Dnmt Mutations

The mechanisms underlying the developmental defects observed in *Dnmt* mutant mice are not fully understood. Dnmt1, Dnmt3a, and Dnmt3b are all highly expressed in pluripotent ES cells, but disruption of these genes individually, both *Dnmt3a* and *Dnmt3b*, or even all three *Dnmts*, has no deleterious effects on mouse ES cells in the undifferentiated state (Lei et al. 1996; Li et al. 1992; Okano et al. 1999; Tsumura et al. 2006). However, *Dnmt1*$^{-/-}$ and *Dnmt3a*/*3b* DKO ES cells die upon induction of differentiation (Chen et al. 2003; Lei et al. 1996; Tucker et al. 1996). Interestingly, a recent study showed that, in contrast to mouse ES cells, human ES cells require *DNMT1*, but not *DNMT3A* and *DNMT3B*, for survival (Liao et al. 2015). It is well established that mouse and human ES cells represent different pluripotent states, with human ES cells resembling the more mature epiblast state (Tesar et al. 2007), which may explain the sensitivity of human ES cells to loss of DNA methylation. During development, the effects of DNA methylation deficiency become apparent during or after gastrulation, when the embryo differentiates to form the three germ layers (Lei et al. 1996; Li et al. 1992; Okano et al. 1999). Conditional inactivation of *Dnmt1* in mouse embryonic fibroblasts (MEFs) leads to severe hypomethylation and cell death, and *Dnmt3b*-deficient MEFs show modest hypomethylation, chromosomal instability, and abnormal cell proliferation (Dodge et al. 2005; Jackson-Grusby et al. 2001). Furthermore, although a hypomorphic mutation affecting the N-terminal region of human DNMT1 has no effect on the survival and proliferation of the colon cancer cell line HCT116 (Rhee et al. 2000), disruption of the DNMT1 catalytic domain in HCT116 leads to mitotic catastrophe and cell death (Chen et al. 2007). Taken together, these results suggest crucial roles for DNA methylation in cellular differentiation and in the viability and proper functioning of differentiated cells. Deregulation of gene expression likely plays a major role in the developmental and cellular defects associated with *Dnmt* mutations.

3 Dnmts in Genomic Imprinting

In early 1980s, elegant nuclear transplantation experiments using pronuclear stage embryos showed that mouse embryos constructed to contain only maternal or paternal diploid genome complements fail to develop beyond mid-gestation. This suggested that the parental genomes are functionally nonequivalent and marked or "imprinted" differently during male and female gametogenesis (Barton et al. 1984; McGrath and Solter 1984; Surani et al. 1984). Separate experiments using chromosome translocations in mice showed that specific chromosome segments function differently depending on the parental origin (Cattanach and Kirk 1985). In early 1990s, the first murine imprinted genes, *Igf2r*, *Igf2*, and *H19*, were discovered, which are expressed only from one parental allele (Barlow et al. 1991; Bartolomei et al. 1991; DeChiara et al. 1991). To date, approximately 150 imprinted genes, which exhibit monoallelic expression strictly according to the parental origin, have been identified in mouse (http://www.mousebook.org/mousebook-catalogs/imprinting-resource), and many of them are also imprinted in human. Imprinted genes are involved in diverse biological processes, including embryonic development, placental formation, fetal and postnatal growth, and adult behavior (Frontera et al. 2008; Reik and Walter 2001). In human, altered expression of imprinted genes, due to genetic and epigenetic changes, has been linked to infertility, molar pregnancy, and various congenital disorders such as Prader-Willi syndrome, Angelman syndrome, Beckwith-Wiedemann syndrome, and Silver-Russell syndrome (Butler 2009; Tomizawa and Sasaki 2012; Walter and Paulsen 2003). Loss of imprinting (biallelic expression or silencing of imprinted genes) is also frequently observed in cancer (Jelinic and Shaw 2007).

The majority of imprinted genes are arranged in chromosomal clusters, which usually span hundreds to thousands of kilobases. Each of the imprinting clusters is controlled by an ICR, an essential regulatory sequence that contains one or more differentially methylated regions (DMRs) between the two alleles. Thus, allele-specific DNA methylation is believed to be the primary epigenetic mark that controls the monoallelic expression of imprinted genes.

The life cycle of DNA methylation imprints consists of three major steps: establishment, maintenance, and erasure (Fig. 2).

3.1 Establishment of Methylation Imprints

DNA methylation imprints are acquired in the germ line, with the majority being established during oogenesis (maternally imprinted) and only four known loci (*H19*, *Dlk1-Gtl2*, *Rasgrf1*, and *Zdbf2*) being established during spermatogenesis (paternally imprinted). Conditional deletion of *Dnmt3a* in primordial germ cells (PGCs) disrupts both maternal and paternal imprinting. Embryos from crosses between conditional *Dnmt3a* mutant females and wild-type males die around E10.5, and conditional *Dnmt3a* mutant males are sterile due to impaired spermatogenesis (Kaneda et al. 2004). *Dnmt3L* KO mice show an identical phenotype, with the exception of

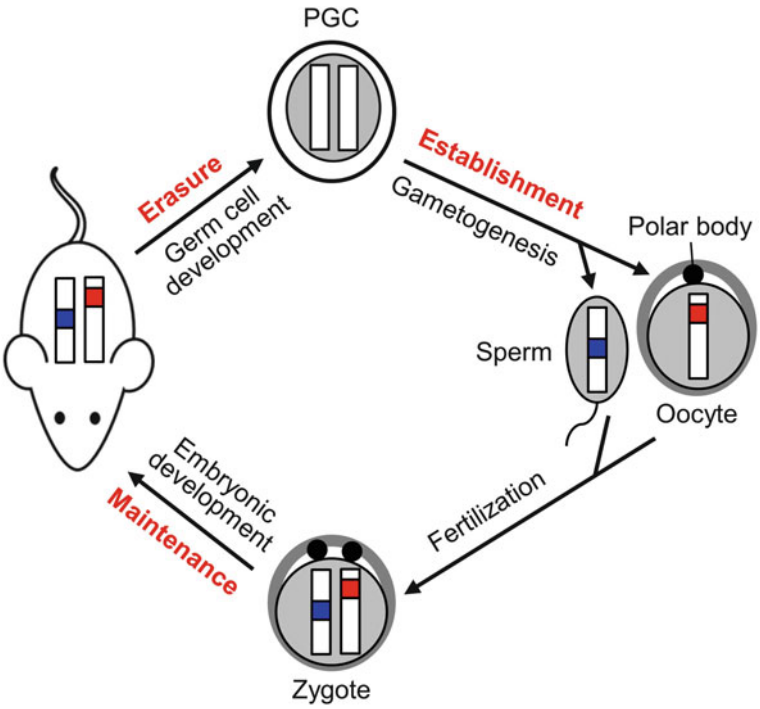

Fig. 2 Life cycle of DNA methylation imprints. The paternal (*blue*) and maternal (*red*) methylation imprints are established during gametogenesis and transmitted to the offspring through fertilization. These marks are maintained and control monoallelic expression of imprinted genes during embryogenesis and in somatic cells throughout adult life. However, they are erased in primordial germ cells (PGCs) before sex-specific methylation imprints are reestablished in later stages of germ cell development

one paternally methylated locus, *Dlk1-Gtl2*, which is methylated in *Dnmt3L* KO but not in *Dnmt3a* mutant spermatogonia (Bourc'his et al. 2001; Hata et al. 2002; Kaneda et al. 2004). In contrast, conditional deletion of *Dnmt3b* in PGCs shows no apparent phenotype (Kaneda et al. 2004). These results provide compelling genetic evidence that Dnmt3a is responsible for the establishment of germ line imprints, and Dnmt3L is an essential cofactor for Dnmt3a in this regard.

How Dnmt3L facilitates Dnmt3a function in the germ line is not fully understood. Dnmt3L, via its C-terminal domain, forms a tetrameric complex with Dnmt3a and, via its ADD domain, interacts with the N-terminal tail of histone H3 (Hata et al. 2002; Jia et al. 2007; Ooi et al. 2007; Suetake et al. 2004). These findings led to the hypothesis that Dnmt3L plays a critical role in targeting Dnmt3a to specific chromatin regions, including imprinted loci. A recent study showed that, similar to *Dnmt3L* null mutant mice, mice homozygous for an engineered point mutation (D124A) in the Dnmt3L ADD domain exhibit DNA methylation and spermatogenesis defects (Vlachogiannis et al. 2015), supporting a critical role of the ADD domain in Dnmt3L function in the male germ line. It would be interesting to

determine whether female mice homozygous for the D1124A mutation show defects in the establishment of maternal imprints. However, the Dnmt3a ADD domain also binds H3K4-unmethylated histone H3 (Otani et al. 2009; Zhang et al. 2010), which raises the question of the specific role of the Dnmt3L ADD domain. It is possible that Dnmt3L interacts with one or more proteins or histone marks that are not recognized by Dnmt3a.

The observation that H3K4 modifications disrupts the interaction between Dnmt3 proteins and histone H3 (Ooi et al. 2007; Otani et al. 2009; Zhang et al. 2010) suggests that chromatin organization may be an important determinant of the sites of *de novo* DNA methylation in the germ line. Indeed, genetic evidence indicated that the H3K4 demethylase KDM1B (also known as LSD2 and AOF1) is essential for the establishment of a subset of maternal imprints (Ciccone et al. 2009). KDM1B is highly expressed in growing oocytes, where maternal imprints are acquired, but shows little expression in most somatic tissues. *Kdm1b* KO mice are viable and show no defects in spermatogenesis and oogenesis, and male mice are fertile. However, oocytes from KDM1B-deficient females exhibit global accumulation of H3K4me2 and fail to establish DNA methylation imprints at a subset of imprinted loci. Consequently, embryos derived from these oocytes die around midgestation (Ciccone et al. 2009), similar to embryos derived from Dnmt3L- or Dnmt3a-deficient female mice (Bourc'his et al. 2001; Hata et al. 2002; Kaneda et al. 2004). These results strongly suggested that removal of H3K4me2 is a prerequisite for *de novo* DNA methylation. There is also evidence that transcription is an additional requirement in specifying DNA methylation, at least at some maternally imprinted loci. In the mouse *Gnas* locus, transcription initiated at the promoter of *Nesp55*, a gene upstream of the DMRs of the *Gnas* locus, occurs in growing oocytes, placing a large genomic region, including the DMRs, within an active transcription unit. Deletion of the *Nesp55* promoter or insertion of a transcription termination cassette downstream of *Nesp55* to ablate transcription results in failure to establish DNA methylation at the ICR of the *Gnas* locus (Chotalia et al. 2009; Frohlich et al. 2010; Williamson et al. 2011). Methylation of the DMR at the *Snrpn* locus has also been shown to depend upon transcription (Smith et al. 2011).

3.2 Maintenance of Methylation Imprints

The paternal and maternal imprints are transmitted to the zygote through fertilization, and despite extensive demethylation during preimplantation development (as described above), parental allele-specific DNA methylation imprints are faithfully maintained through development and adult life. Notably, recent genome-wide DNA methylation analyses revealed far more differentially methylated loci in oocytes and sperm than the number of imprinted genes (Kobayashi et al. 2012; Smallwood et al. 2011; Smith et al. 2012). Thus, the previous notion that imprinted loci are determined by distinct methylation patterns in gametes has been revised to the current view that genomic imprinting results from selective maintenance of germ line-derived allele-specific methylation. Genetic studies using conditional KO mice

demonstrated that Dnmt1, but not Dnmt3a or Dnmt3b, is responsible for maintaining methylation marks at imprinted loci during preimplantation development (Hirasawa et al. 2008). The oocyte-specific variant, Dnmt1o, is the predominant Dnmt1 isoform in preimplantation embryos (Hirasawa et al. 2008; Kurihara et al. 2008). However, offspring of females lacking Dnmt1o exhibit only a ~50% reduction of methylation at certain imprinted loci (Howell et al. 2001). While initial evidence suggested that the somatic form, Dnmt1s, does not express until the blastocyst stage (Ratnam et al. 2002), subsequent work showed that Dnmt1s is present at very low levels in the nucleus of oocytes and preimplantation embryos (Hirasawa et al. 2008; Kurihara et al. 2008). Conditional deletion of *Dnmt1* (both Dnmt1o and Dnmt1s) in growing oocytes leads to a partial loss of methylation imprints in the offspring (Hirasawa et al. 2008), resembling the effect of Dnmt1o loss (Howell et al. 2001). However, ablation of both maternal and zygotic Dnmt1 results in a complete loss of methylation at paternally and maternally methylated DMRs in embryos (Hirasawa et al. 2008). Therefore, both maternal and zygotic Dnmt1 proteins are necessary for the maintenance of methylation imprints in preimplantation embryos. Dnmt1 is also responsible for the maintenance of methylation imprints in postimplantation embryos (Li et al. 1993) and likely in adult somatic cells as well.

It is not well understood what confers the specificity of Dnmt1, such that methylation is maintained at imprinted genes but not at other sequences in preimplantation embryos. Genetic and epigenetic features may distinguish imprinted loci from other regions. Several other factors have been shown to be essential for the maintenance of DNA methylation imprints. PGC7 (also known as Stella and Dppa3), a DNA-binding protein, is highly expressed in oocytes and persists in preimplantation embryos. Genetic evidence suggested that, in early embryos, maternal PGC7 plays a crucial role in protecting the maternal genome against DNA demethylation. PGC7 also protects the paternally imprinted *H19* and *Rasgrf1* against demethylation (Nakamura et al. 2007). While the mechanisms involved remain to be determined, PGC7 has been shown to play a role in chromatin condensation during oogenesis and to protect the maternal genome against Tet3-mediated conversion of 5mC to 5hmC in early embryos (Bian and Yu 2014; Liu et al. 2012; Nakamura et al. 2012). Gene targeting experiments in mice have also implicated the involvement of the *Krüppel*-associated box (KRAB)-containing zinc-finger protein ZFP57 in the maintenance of genomic imprints (Li et al. 2008), and human ZFP57 mutations are associated with hypomethylation at multiple imprinted loci in patients with transient neonatal diabetes (Mackay et al. 2008). ZFP57 specifically binds the methylated allele of ICRs, recognizing a hexanucleotide sequence (TGCCGC) shared by all murine ICRs and some human ICRs (Quenneville et al. 2011). ZFP57 interacts with KRAB-associated protein 1 (KAP1, also known as TRIM28), which acts as a scaffold protein for various heterochromatin proteins, including heterochromatin protein 1 (HP1), the histone H3K9 methyltransferase Setdb1 (also known as ESET and KMT1E), the nuclear remodeling and histone deacetylation (NuRD) complex, and Dnmt proteins and Uhrf1 (Nielsen et al. 1999; Quenneville et al. 2011; Ryan et al. 1999; Schultz et al. 2001, 2002; Zuo et al. 2012). Ablation of either maternal or zygotic KAP1 causes partial loss of DNA

methylation imprints, and ablation of both maternal and zygotic KAP1 leads to a complete loss of imprinting (Lorthongpanich et al. 2013; Messerschmidt et al. 2012; Quenneville et al. 2011). Depletion of the NuRD components methyl CpG-binding domain protein-3 (MBD3) or metastasis tumor antigen 2 (MTA2) also results in reduction of methylation at some imprinted loci in preimplantation embryos (Ma et al. 2010; Reese et al. 2007). The current view is that the ZFP57/KAP1 complex specifically recruits the DNA methylation machinery, as well as other heterochromatin proteins, to the methylated allele of ICRs to maintain genomic imprints and control monoallelic expression of imprinted genes.

3.3 Erasure of Methylation Imprints

The last step of the imprint life cycle is the erasure of methylation imprints in PGCs, which ensures the establishment of sex-specific imprints in later stages of germ cell development. In mice, PGCs are specified around E7.25 in the epiblast of the developing embryo. Shortly afterwards, PGCs begin migrating along the embryonic-extraembryonic interface and eventually arrive at the genital ridge by E12.5. Recent genome-wide DNA methylation analyses reveal that PGCs undergo demethylation in two major phases (Guibert et al. 2012; Kobayashi et al. 2013; Popp et al. 2010; Seisenberger et al. 2012). The first phase takes place during PGC expansion and migration from ~ E8.5, which leads to a global demethylation affecting almost all genomic regions. Passive demethylation likely plays a major role in this phase, as Dnmt3a, Dnmt3b, and Uhrf1 are repressed in PGCs (Kagiwada et al. 2013; Kurimoto et al. 2008). The second phase occurs from E9.5 to E13.5 and affects specific loci including ICRs, germ line-specific genes, and CpG islands on the X chromosome (Guibert et al. 2012; Hackett et al. 2013; Popp et al. 2010; Seisenberger et al. 2012; Yamaguchi et al. 2013). Genetic studies suggested that Tet1- and Tet2-mediated 5mC oxidation is important in the second phase of demethylation (Zhao and Chen 2013).

4 Dnmts in X-Chromosome Inactivation

In mammals, sex determination is controlled by a pair of sex chromosomes, the X and the Y. Whereas the Y chromosome harbors very few protein-coding genes compared to other chromosomes, the X chromosome has a relatively high gene density. The balance of X-linked gene dosage between XX females and XY males is achieved through X-chromosome inactivation (XCI), whereby one of the two X chromosomes present in female mammals is inactivated. Marsupial mammals show imprinted XCI, with only the paternal X chromosome (Xp) being inactivated. Eutherian mammals exhibit two forms of XCI: imprinted Xp inactivation in the early embryo and extraembryonic tissues and random inactivation of either Xp or the maternal X chromosome (Xm) in the embryonic (epiblast) lineage (Payer and Lee 2008; Wutz 2011).

Our knowledge about XCI mostly came from studies in the mouse. In the female zygote, both X chromosomes appear active. Soon thereafter, a series of events result in the inactivation of Xp. This imprinted XCI occurs in a two-step manner, with Xp repeat elements first silenced at the two-cell stage followed by Xp genic silencing emerging at the eight- to sixteen-cell stage. Imprinted XCI is complete by the blastocyst stage. Whereas inactivation of Xp is maintained in extraembryonic tissues, it is reversed in the ICM of the blastocyst, resulting in the biallelic expression of X-linked genes. Shortly after implantation, epiblast cells undergo random XCI (Payer and Lee 2008). Murine ES cells, derived from the ICM, represent a useful model for the study of XCI. Undifferentiated murine ES cells, like ICM cells, have two active X chromosomes, and random XCI can be recapitulated during *in vitro* differentiation (Chaumeil et al. 2004).

The process of XCI, which converts an X chromosome from relatively open euchromatin to highly condensed heterochromatin (known as the "Barr body"), can be divided into three steps: initiation of X inactivation, spreading of heterochromatin to the entire chromosome, and maintenance of the inactive state (Fig. 3). XCI is controlled by the X-inactivation center (*Xic*), a complex locus on the X chromosome that determines how many (counting step) and which X chromosomes (choice step) will be silenced. A critical gene in *Xic* encodes the "X-inactive-specific transcript" (*Xist*), a 17-kb long, non-coding RNA (lncRNA). *Xist* is expressed only from the presumptive inactive X chromosome (Xi) and then coats the same chromosome in *cis* (Clemson et al. 1996). This step is necessary and sufficient for the initiation of XCI, as targeted disruption of the *Xist* gene abrogates XCI (Marahrens et al. 1997; Penny et al. 1996), and *Xist* transgenes on autosomes can induce autosomal gene inactivation (Jiang et al. 2013; Lee and Jaenisch 1997; Lee et al. 1996). The *Xic* also harbors several other genes encoding proteins and non-coding RNAs, including the *Xist* antisense RNA *Tsix*, the *Jpx* and *Ftx* RNAs, and the E3 ubiquitin ligase RNF12, that act as part of a sophisticated regulatory network to modulate *Xist* expression in *cis* and in trans (Gendrel and Heard 2014). *Xist* is also required for the spreading of XCI from the *Xic* to the rest of the chromosome. *Xist* RNA is able to recruit PRC2, a complex responsible for the deposition of H3K27me3, which contributes to chromatin and transcriptional changes during the initiation and spreading of XCI (Zhao et al. 2008; da Rocha et al. 2014; Cifuentes-Rojas et al. 2014). Once established, the globally silent state and heterochromatin structure of Xi are transmitted through somatic cell division and clonally inherited. Although *Xist* is not required for the maintenance of gene silencing on the Xi (Brown and Willard 1994; Csankovszki et al. 1999), it appears to be important for maintaining the heterochromatin structure of Xi, as deletion of *Xist* leads to refolding of the Xi into a structure resembling the active X chromosome (Xa) (Splinter et al. 2011).

The link between DNA methylation and XCI has been well established. In somatic tissues, the 5′ end of the *Xist* gene is fully methylated on Xa and completely unmethylated on Xi. Similarly, in tissues that undergo imprinted Xp inactivation, the paternal *Xist* allele is unmethylated, and the maternal allele is fully methylated (Norris et al. 1994). Studies of *Dnmt1*-deficient ES cells and embryos revealed that XCI can occur in the absence of DNA methylation, but maintenance of *Xist*

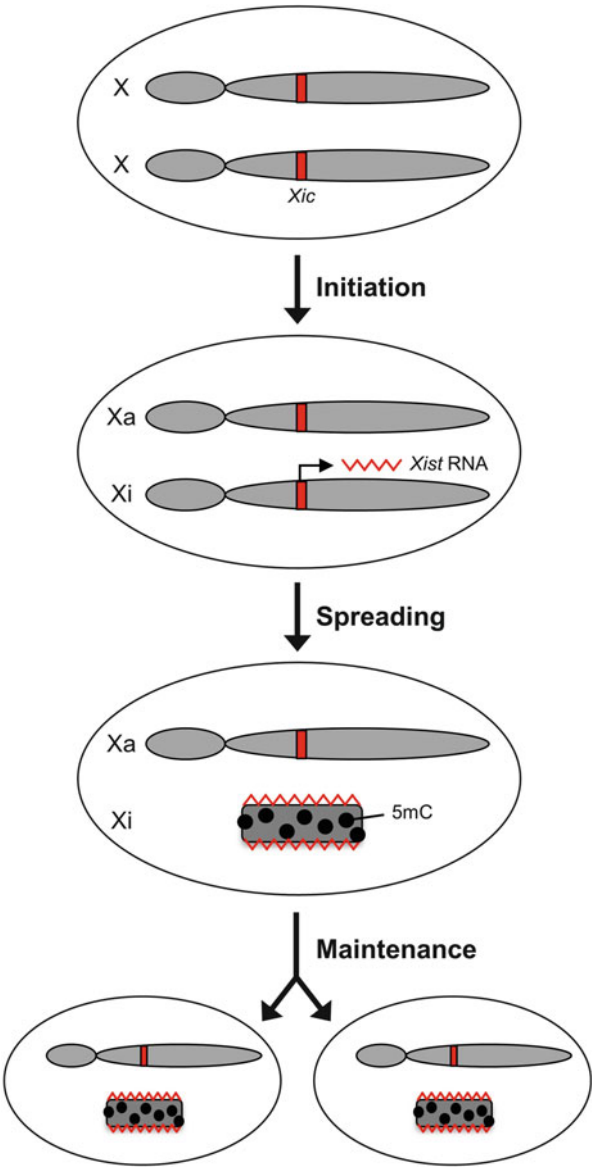

Fig. 3 Major steps of X-chromosome inactivation. The process of X-chromosome inactivation can be divided into three steps: (1) initiation (*Xist* RNA is expressed from the presumptive inactive X (Xi), but not the active X (Xa)), (2) spreading (*Xist* RNA coats the entire Xi chromosome, which recruits other factors (e.g., PRC2 complex, Dnmts) to induce heterochromatinization), and (3) maintenance (the highly compacted chromatin structure and most of the genes on Xi are stably maintained and clonally transmitted through somatic cell divisions). DNA methylation is required for the stable maintenance of Xi-linked gene silencing

promoter methylation is necessary for its stable repression in differentiated cells (Beard et al. 1995; Panning and Jaenisch 1996; Sado et al. 2000). A recent study showed that loss of Dnmt1o disrupts imprinted XCI and accentuates placental defects in females (McGraw et al. 2013). *De novo* DNA methylation is also dispensable for the initiation and propagation of XCI, as *Xist* expression is appropriately regulated and XCI occurs properly in female embryos deficient for both *Dnmt3a* and *Dnmt3b* (Sado et al. 2004). Interestingly, despite multiple mechanisms involved

in X-chromosome gene silencing, approximately 25 % of genes on Xi escape inactivation to some extent and exhibit biallelic expression in females (Carrel and Willard 2005; Yang et al. 2010). The promoter regions of these escapee genes are unmethylated (Weber et al. 2007). Furthermore, treatment of cells with the demethylating agents 5-azacytidine and 5-azadeoxycytidine has been shown to reactivate some genes on Xi (Haaf 1995). Collectively, these findings indicate that DNA methylation is not required for the initiation and propagation of XCI but is an essential component of the epigenetic mechanisms that stably maintain the silent state of Xi-linked genes.

5 Concluding Remarks

Since the discovery of mammalian Dnmts (Bestor et al. 1988; Okano et al. 1998a), great progress has been made in understanding the biological functions of DNA methylation in mammals. Genetic studies using *Dnmt* mutant mice and murine cells have provided important insights into the roles of DNA methylation in various developmental and cellular processes (Table 1). It is generally believed that DNA methylation, a relatively stable epigenetic mark, acts in concert with other epigenetic mechanisms such as histone modifications to stably maintain gene silencing and chromatin structure. It is well documented that aberrant DNA methylation patterns are associated with various human diseases. Studies in recent years have also identified genetic alterations affecting major components of the DNA methylation machinery, including DNA methylation "writers" (DNMTs), "erasers" (e.g., TETs), and "readers" (e.g., MeCP2), in cancer and developmental disorders (Hamidi et al. 2015). For example, *DNMT1* mutations are reported in two related neurodegenerative diseases (hereditary sensory and autonomic neuropathy with dementia and hearing loss type IE (HSAN IE), autosomal dominant cerebellar ataxia, deafness, and narcolepsy (ADCA-DN)), *DNMT3A* mutations are frequently found in acute myeloid leukemia (AML) and other hematologic malignancies, and *DNMT3B* mutations cause the immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome (Hamidi et al. 2015). The mechanisms by which these mutations contribute to the disease phenotypes are generally not well understood. Besides their values in elucidating the fundamental functions of DNA methylation, *Dnmt* mutant mice and cells provide important research tools for investigating the effects of *DNMT* mutations found in human patients. For instance, by expressing a Dnmt3a protein harboring a point mutation equivalent to human DNMT3A:R882H (the most prevalent *DNMT3A* mutation in AML) in *Dnmt3a* and *Dnmt3b* mutant murine ES cells, we recently demonstrated that this mutation, which occurs on only one allele in AML patients, not only leads to haploinsufficiency of DNMT3A enzymatic activity but also exhibits dominant-negative effect by forming functionally deficient complexes with wild-type DNMT3A and DNMT3B (Kim et al. 2013). Most of the *DNMT* mutations identified in patients are not null alleles, making *Dnmt* KO mice less ideal for modeling human diseases. With the development of new technologies such as CRISPR/Cas9-mediated gene editing, it now becomes more feasible to create genetically engineered animal

Table 1 Developmental phenotypes of *Dnmt* knockout mice

Gene	Mutations	Major developmental phenotypes	References
Dnmt1	*Dnmt1*$^{n/n}$	~70 % reduction in global DNA methylation, embryonic lethality at E12.5–15.5	Li et al. (1992)
	Dnmt1$^{c/c}$	~90 % reduction in global DNA methylation, developmental arrest at E8.5, embryonic lethality before 8-somite stage at ~ E9.5. Unstable random XCI	Lei et al. (1996); Sado et al. (2000)
	Dnmt1$^{s/s}$	~90 % reduction in global DNA methylation, developmental arrest at E8.5, embryonic lethality at ~ E9.5. Loss of methylation at *Xist* locus and abnormal *Xist* expression in male embryos	Beard et al. (1995); Lei et al. (1996)
	Dnmt1o$^{-/-}$	Maternal-effect phenotype: partial loss of DNA methylation imprints, defects in imprinted XCI, embryonic lethality at mid-gestation	Howell et al. (2001); McGraw et al. (2013)
	Maternal and zygotic *Dnmt3a*$^{-/-}$	Complete loss of paternal and maternal methylation imprints, embryonic lethality at mid-gestation	Hirasawa et al. (2008)
Dnmt3a	*Dnmt3a*$^{-/-}$	Gut malfunction, spermatogenesis defects, death at ~4 weeks of age	Okano et al. (1999)
	Dnmt3a$^{-/-}$ in PGCs	Failure to establish maternal and paternal methylation imprints, spermatogenesis defects	Kaneda et al. (2004)
Dnmt3b	*Dnmt3b*$^{-/-}$	Hypomethylation of minor satellite DNA, neural tube defects, embryonic lethality at E14.5–18.5	Okano et al. (1999)
	Dnmt3a$^{-/-}$, *Dnmt3b*$^{-/-}$	Failure to initiate *de novo* methylation after implantation, developmental arrest at E8.5	Okano et al. (1999)
Dnmt3L	*Dnmt3L*$^{-/-}$	Failure to establish maternal and paternal methylation imprints, spermatogenesis defects	Bourc'his et al. (2001); Hata et al. (2002)

and cellular models that better recapitulate the major features of human diseases associated with *DNMT* mutations. Genomic, epigenomic, transcriptomic, and proteomic analyses of these models will be powerful approaches for defining the molecular mechanisms and pathways involved in pathogenesis. Ultimately, such studies will likely lead to novel therapeutic and preventive strategies.

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