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Review

Functions of DNA methyltransferase 3-like in germ cells and beyond

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DNA methyltransferase 3-like (DNMT3L) is one of the key players in *de novo* DNA methylation of imprinting control elements and retrotransposons, which occurs after genome-wide epigenetic erasure during germ cell development. In this review, we summarise the biochemical properties of DNMT3L and discuss the possible mechanisms behind DNMT3L-mediated imprinting establishment and retrotransposon silencing in germ cells. We also discuss possible connections between DNMT3L and non-coding RNA-mediated epigenetic remodelling, the roles of DNMT3L in germ cell development and the implications in stem cell and cancer research.

Introduction

In mammals, all cells in the body come from two germ cells–one sperm and one oocyte, which are contributed by each parent. Germ cells undergo a dramatic epigenetic reprogramming to confer totipotency for the next generation, which is crucial for subsequent embryo development. The reprogramming processes in germ cells include reactivation of the inactivated X chromosome, as well as the re-establishment of epigenetic marks at parent-of-origin-specific imprinted genes, single copy genes and repeat sequences, such as transposable elements, microsatellites and centromere repeats (Seydoux and Braun, 2006; Sasaki and Matsui, 2008; Figure 1). When analysing the expression patterns of various epigenetic regulators throughout reprogramming, the protein DNA methyltransferase 3-like (DNMT3L) stands out, as its cell-specific and stage-specific expression patterns overlap perfectly with the timing of genome-wide *de novo* DNA methylation, following the erasure of the majority of DNA methylation marks in germ cells (Sasaki and Matsui, 2008; Figure 1).

DNMT3L belongs to the DNA methyltransferase (DNMT) 3 family, based on its sequence similarity to DNMT3A and DNMT3B. However, DNMT3L lacks several critical components in the C-terminal catalytic domain and therefore does not have DNMT activity. Instead, DNMT3L interacts with DNMT3A or DNMT3B to facilitate *de novo* DNA methylation. The importance of DNMT3L in germ cell development was demonstrated by the phenotype of *Dnmt3l* knockout (*Dnmt3l*-KO) mice. Although female and male *Dnmt3l*-KO mice are viable, male *Dnmt3l*-KO mice show a complete azoospermia phenotype (Hata et al., 2002, 2006; Bourc'his and Bestor, 2004). This may be primarily due to the failure to silence endogenous retroviruses and retrotransposons in *Dnmt3l*-KO male germ cells (Bourc'his and Bestor, 2004; Hata et al., 2006; Webster et al., 2005). In this context, lack of DNA methylation and associated repressive epigenetic marks on retroelements correlates with severe meiotic defects at the pachytene stage when homologous chromosomes pair. Male germ cells in the *Dnmt3l*-KO mice cannot develop beyond the

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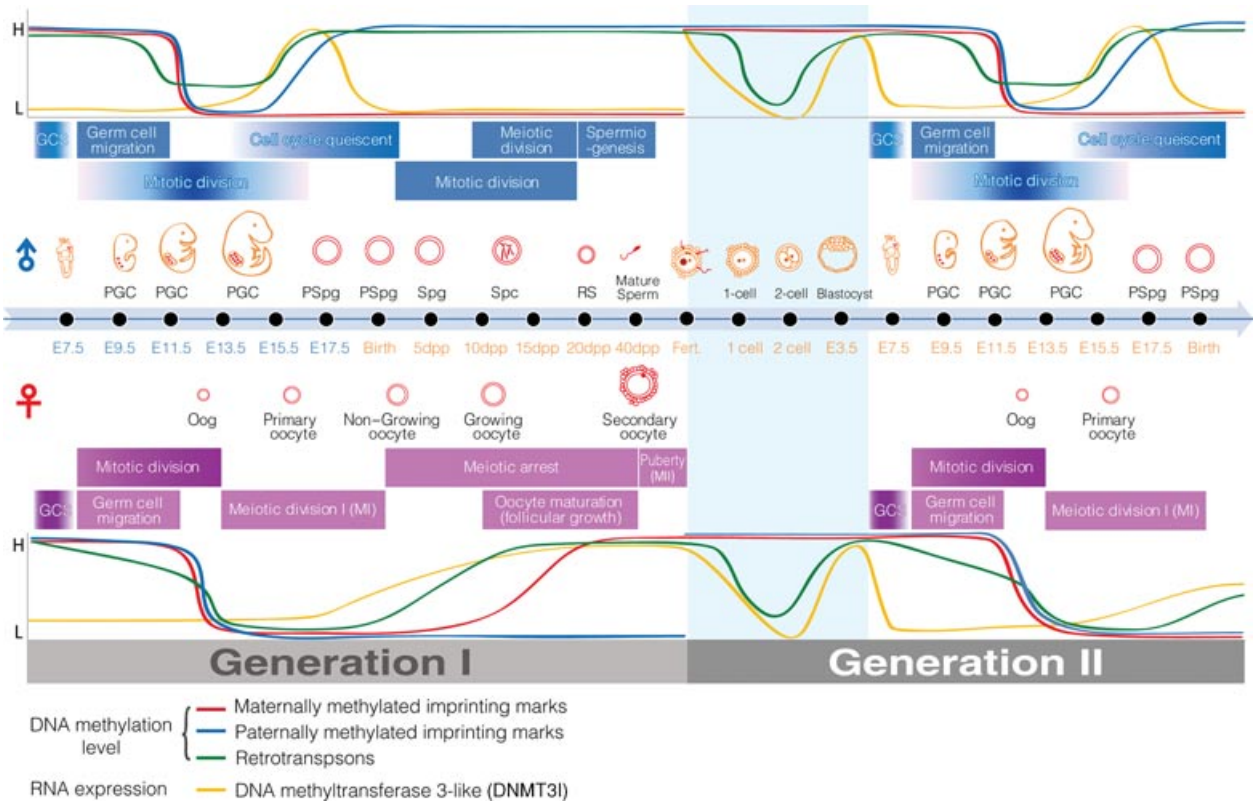
Key words: DNA Methyltransferases, Genomic Imprinting, Histone, piRNA, Retrotransposons.

Abbreviations used: DNMTs, DNA methyltransferases; dpp, days post-partum; E, embryonic day; ESC, embryonic stem cell; FF, phenylalanine-phenylalanine; H3K4me0, unmethylated histone 3 lysine 4; ICR, imprinting control region; PGC, primordial germ cell; PHD, plant homeodomain; piRNA, PIWI-interacting RNA; RD, arginine-aspartic acid; TE, transposable element.

Figure 1 | Epigenetic reprogramming in germ cells and pre-implantation embryos correlates well with the expression of mouse DNMT3L

In germ cell and pre-implantation embryo development, DNA reprogramming is essential in maintaining the biological functions of cells. The expression windows of *Dnmt3l* are consistent with these two major DNA reprogramming events. The expression level of *Dnmt3l* is high in fertilised eggs, but drops dramatically after the cleavage stage. *Dnmt3l* expression level climbs up again at the blastocyst stage. *Dnmt3l* transcripts cannot be detected during either germ cell specification or their migration. The red/blue lines and green line indicate the DNA methylation level of maternally/paternally methylated imprinted regions and retrotransposons in germ cells, respectively. DNA demethylation in germ cells begins during PGC migration and the global methylation level declines rapidly when PGCs move into the gonads. In both female and male germ cells, *de novo* DNA methylation of imprinting regulatory regions and retrotransposon sequences coincides with *Dnmt3l* mRNA levels (yellow line). In female germ cells, *Dnmt3l* transcripts can be strongly detected around 6 dpp and are continuously expressed in all developing oocytes. For each new wave of folliculogenesis, *de novo* DNA methylation takes place in the growing oocytes. Comparatively, the *de novo* DNA methylation process in male mouse germ cells begins from E15.5 and reaches its peak at around E17.5. The DNA methylation and *Dnmt3l* expression profile from the blue shadowed region were generated from pre-implantation embryos. PGC, primordial germ cell; PSsg, prospermatogonia; Spg, spermatogonia; Spc, spermatocyte; RS, round spermatid; Oog, oogonia; E, embryonic day; dpp, day post-partum (Ginsburg et al., 1990; Huntriss et al., 2004; La Salle et al., 2007; Lees-Murdock et al., 2005; Lees-Murdock and Walsh, 2008; Li et al., 2004; Lucifero et al., 2007; Sasaki and Matsui, 2008; Shovlin et al., 2007; Xie et al., 2006).



pachytene checkpoint (Bourc'his and Bestor, 2004; Hata et al., 2006).

Although female *Dnmt3l*-KO mice can produce morphologically normal and fertilisable oocytes, their offspring rarely live past 9.5 days post-coitum (dpc), primarily due to failures in establishing oocyte-derived genomic imprinting methylation marks (Arnaud et al., 2006; Bourc'his et al., 2001). Genomic imprinting is an epigenetic phenomenon in flowering plants, eutherian mammals and marsupials, dictated by a parent-of-origin-specific fashion. This type of control is achieved, at least in part, through the

presence of differentially methylated regions on the DNA sequence of each allele. The establishment of genomic imprinting marks during male and female gametogenesis plays a significant role in development, metabolism, foetal growth and behaviour (Ferguson-Smith et al., 2004).

The contribution of DNMT3L-associated epigenetic regulation in establishing parental origin-specific genomic imprinting marks as well as silencing marks on retrotransposon-related elements is discussed in the following sections.

The biochemical properties of DNMT3L predicts its function

DNMT3L's role in facilitating *de novo* DNA methylation

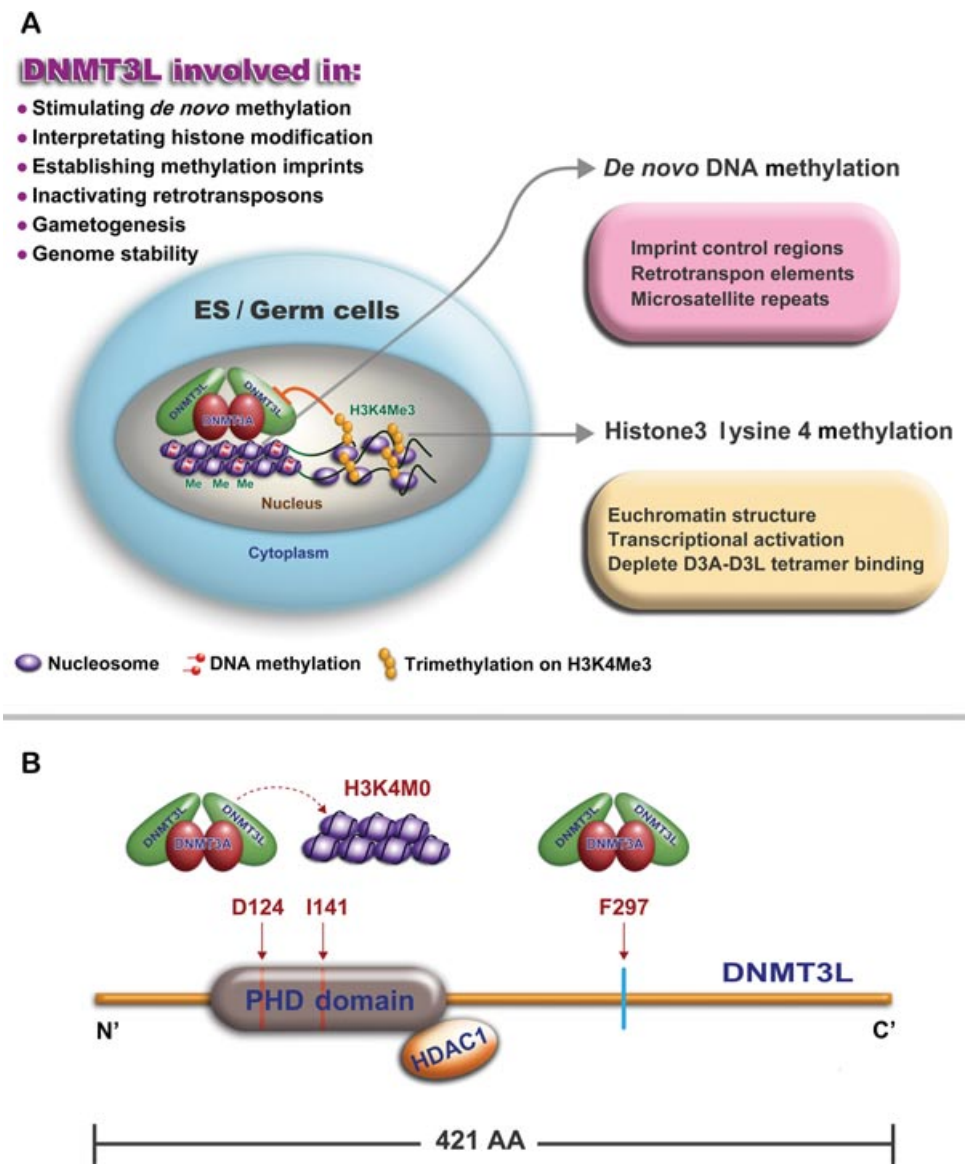
De novo DNA methylation is crucial for the establishment of gene expression patterns during embryonic development and gametogenesis (Chen et al., 2003; Chen and Li, 2006; Klose and Bird, 2006). Two conserved DNMTs, encoded by DNMT3A and DNMT3B, have been identified as the primary catalytic components of the *de novo* methylation machinery (Hsieh, 1999; Okano et al., 1999). Recent reviews consolidating the structure and function of DNMT3A and DNMT3B, as well as their regulatory pathways can be found in Cheng and Blumenthal, 2008; Chedin, 2011; and Denis et al., 2011. Briefly, both DNMT3A and DNMT3B contain the proline–tryptophan–tryptophan–proline (PWWP) domain, required for chromatin binding (Chen et al., 2004; Dhayalan et al., 2010; Ge et al., 2004; Shirohzu et al., 2002); the ATRX–DNMT3–DNMT3L (ADD) domain, which contains a C2–C2 zinc finger and an atypical plant homeodomain (PHD) finger (Hashimoto et al., 2010); and the catalytic domain on the C-terminal responsible for the DNMT activity (Gowher and Jeltsch, 2002). The PWWP domain in DNMT3B interacts non-specifically with DNA, but in DNMT3A, this domain displays lower DNA-binding activity (Chen et al., 2004). Deletion of both *Dnmt3a* and *Dnmt3b* genes results in a lack of *de novo* methylation, but not maintenance of C-type endogenous retroviral DNA during early post-implantation embryo development. In contrast, $Dnmt3b^{-/-}$ and $Dnmt3a^{-/-}$ single knockout embryos have little and no effect on the methylation level of C-type retroviral DNA, respectively (Okano et al., 1999). These results indicate some redundancy between DNMT3A and DNMT3B in the *de novo* methylation of proviral and endogenous retroviral sequences (Okano et al., 1999). Furthermore, Smallwood et al. (2011) recently demonstrated the gross reduction of DNA methylation in $Dnmt3a^{-/-}$ oocytes, indicating the requirement of DNMT3A for appropriate DNA methylation during oogenesis.

The third member of the DNMT3 family, DNMT3L, is closely related to DNMT3A and DNMT3B structurally. Although it contains the PHD/ADD domain, DNMT3L does not have the N-terminal PWWP domain and does not interact with DNA directly. DNMT3L also lacks some critical components in the C-terminal catalytic domain (Figure 2) and thus has no methyltransferase activity (Aapola et al., 2001, 2002; Chedin et al., 2002; Hata et al., 2002). Nevertheless, DNMT3L is known to facilitate *de novo* DNA methylation (Kareta et al., 2006; Suetake et al., 2004). Using an episomal system in a human cell culture model, DNMT3L was reported as a stimulatory factor for *de novo* DNA methylation mediated by active DNMT3A and DNMT3B (Chedin et al., 2002; Chen et al., 2005). A recent study also demonstrated that DNMT3L increased DNMT3A's catalytic activity by 20-fold (Kareta et al., 2006).

Co-crystallisation experiments confirmed that DNMT3L interacts with the catalytic domain of DNMT3A through its carboxy-terminal pseudocatalytic domain to form a tetrameric complex (DNMT3L–$(DNMT3A)_2$–DNMT3L) (Jia et al., 2007). Structural studies showed that DNMT3A has two interfaces for protein–protein interaction. The arginine-aspartic acid (RD) interface is required for the formation of DNMT3A homodimer, while the phenylalanine-phenylalanine (FF) interface can mediate both the DNMT3A–DNMT3A and the DNMT3A–DNMT3L contact (Jia et al., 2007; Jurkowska et al., 2008). Both RD and FF interfaces are required for DNMT3A to bind to its co-factor, S-adenosyl-L-methionine (SAM) and for its catalytic activity (Jia et al., 2007). In the absence of DNMT3L, DNMT3A molecules concatemerise through the FF and RD bindings and form DNMT3A oligomers. The DNMT3A oligomers are often found in heterochromatin regions and can bind multiple DNA molecules (Jurkowska et al., 2008, 2011). Because of the absence of an RD interface, DNMT3L prevents the oligomerisation of DNMT3A by binding

Figure 2 | Domains and functions of DNMT3L

(**A**) Capabilities of DNMT3L in development and epigenetic regulation. Documented functions of DNMT3L include facilitation of *de novo* DNA methylation on imprinting control regions and transposons. This is crucial for germ cell development and genome stability. DNMT3L can interact with DNMT3A to form a tetramer complex (DNMT3L–$(DNMT3A)_2$–DNMT3L). The complex can target H3K4me0 to induce *de novo* DNA methylation. Methylation on the H3K4 residue abolishes the interaction with DNMT3L. (**B**) Schematic diagram of the key residues and protein–protein interaction in DNMT3L. DNMT3L interacts with unmethylated lysine 4 of histone H3 (H3K4me0) and HDAC1 through its N-terminal PHD-like domain. Its carboxy-terminal domain can interact with the catalytic domain of DNMT3A to form a dimeric complex, which further forms a tetrameric complex for *de novo* methylation. The side chains of aspartic acid (D124) and isoleucine acid (I141) in the PHD domain are known as the crucial sites for interaction with H3K4me0. The phenylalanine (F297) in the C-terminal of DNMT3L plays a role in the stimulatory effect on DNMT3A activity, although the F297A mutation alone does not disrupt the interaction between DNMT3L and DNMT3A (Aapola et al., 2002; Jia et al., 2007; Ooi et al., 2007).



with it via the FF interface and blocking the binding of another DNMT3A. Formation of DNMT3A–DNMT3L tetramers is associated with a reduction in heterochromatin-localised DNMT3A oligomers (Jurkowska et al., 2011). This is one of the likely mechanisms behind the role of DNMT3L, namely, releasing DNMT3A from heterochromatic regions to less favourable targets, including imprinting control elements and certain retrotransposons.

DNMT3A and DNMT3B have both overlapping and distinct target sequences. Although DNMT3A is required for the establishment of parental origin-specific imprinted marks and methylation of repeat elements, DNMT3B mainly methylates pericentric satellite repeats (Chen et al., 2004; Kaneda et al., 2004a; Xu et al., 1999). These preferences may be partly mediated by the base composition flanking the CpG sites, as demonstrated by the episomal DNA methylation assay (Wienholz et al., 2010). For DNMT3A, selection of target sites is mediated by the base composition at the −2 and +2 positions flanking CpG sites. In contrast, DNMT3B selects high efficiency sites based on the −1 and +1 positions flanking CpG sites (Wienholz et al, 2010) Recently, Xie et al. (2012) also correlated the immediate flanking sequences with CG methylation in mouse frontal cortex. These flanking sequence preferences apply to both allele-specific methylation sides as well as the genome-wide scale, and may be conserved between mice and human (Xie et al., 2012). In addition, the interactions of DNMT3A and DNMT3B with specific transcription factors provide possible regulatory machineries for targeted promoter hypermethylation (Brenner et al., 2005; Hervouet et al., 2009) in a tissue- and stage-specific manner.

It is well documented that DNMT3L does not affect the intrinsic sequence preference for DNMT3A and DNMT3B (Wienholz et al, 2010; Van Emburgh and Robertson, 2011). Instead, DNMT3L increases DNMT3A's affinity with AdoMet (Kareta et al., 2006). DNMT3L also increases the processive catalytic activity of DNMT3A (i.e. the ability to carry out multiple cycles of *de novo* DNA methylation on the same piece of DNA), most likely by increasing the binding stability of the DNMT3A–DNA complex (Holz-Schietinger and Reich, 2010). Indeed, disruption of the DNMT3A–DNMT3L interface results in faster dissociation from DNA (Holz-Schietinger et al., 2011).

On the basis of the DNMT3A–DNMT3L tetramer structure and the distance between the two catalytic domains of DNMT3A, CpGs located 8–10 bp apart have been predicted to be the preferential targets. Although CpGs spaced 8 bp apart are relatively common in human and mouse genomes, this sequence feature is indeed enriched in differentially methylated imprinted regions and retrotransposons (SINE, Alu) (Glass et al., 2009). These sequences are primary targets of DNMT3A–DNMT3L tetramers as deduced from the methylation analysis of DNMT3A and DNMT3L knockout animals (Kato et al., 2007). However, LINE elements, another type of retrotransposons that require DNMT3L for transcription silencing, do not have particular CpG periodicity (Glass et al., 2009).

DNMT3L fine-tunes the interaction between *de novo* DNMT and chromatin

The PHD finger, composing part of the ADD domain that is shared amongst DNMT3A, DNMT3B and DNMT3L, has been suggested for the interaction with nucleosomes (Aapola et al., 2000; Hashimoto et al., 2010; Mellor, 2006). Indeed, all DNMT3 family members bind to the N-terminus of histone H3, and these binding activities are disrupted when the H3K4 residue has di- or tri-methyl modifications (Hu et al., 2009; Ooi et al., 2007; Otani et al., 2009; Zhang et al., 2010). DNMT3L is most sensitive to H3K4 methylation, and its affinity with the extreme N-terminus of H3 can be significantly reduced with mono-methylation of H3K4 (Ooi et al., 2007, Zhang et al., 2010). Recent genome-wide epigenetic analysis also revealed that the epigenetic mark correlating to DNA methylation is the absence of H3K4 methylation in chromatin (Edwards et al., 2010; Meissner et al., 2008). The fact that the H3K4 demethylase, KDM1B, is required for the correct establishment of several imprinted DNA methylation marks (Ciccone et al., 2009) also pinpoints the importance of H3K4 demethylation for attracting *de novo* methylation.

DNMT3L is also very sensitive to chromatin composition and it directs *de novo* methylation towards well-chromatinised DNA templates. The association between DNMT3L and chromatin is important for the biological function of DNMT3A (Wienholz et al., 2010). Apart from H3K4 methylation, binding of DNMT3A with nucleosomes is also disrupted when histone 3 is phosphorylated at T3, S10 or T11 and

acetylated at K4 (Zhang et al., 2010). On the contrary, H3K36me3 attracts DNMT3A and guides *de novo* DNA methylation (Dhayalan et al., 2010). These data demonstrate the complexity of the cross-talk between histone modifications, chromatin composition and *de novo* DNA methylation. Although DNMT3A itself interprets various histone modifications, DNMT3L further fine-tunes the selection of target sequences based on chromatin composition and specific histone modifications. As mentioned before, DNMT3L can also reduce the oligomerisation and heterochromatin localisation of DNMT3A (Jurkowska et al., 2011). Taken together, these findings may help to explain the loss of methylation at imprinted loci and retrotransposon sequences in *Dnmt3l*-KO germ cells (Bourc'his and Bestor, 2004; Bourc'his et al., 2001; Hata et al., 2002, 2006; Webster et al., 2005).

DNMT3L's potential role in influencing histone modification through HDAC1

DNMT3L can guide and modulate DNMT3A to more specific chromatin regions by reading the histone modifications and chromatin context. In addition, the ability of DNMT3L to interact with HDAC1 suggests the active role of DNMT3L in facilitating transcriptional repression at the chromatin level (Aapola et al., 2002; Deplus et al., 2002). The ADD domain of DNMT3L is suggested to associate with HDAC1 (Aapola et al., 2002; Deplus et al., 2002). The transcriptional repression activity of DNMT3L *in vitro* can be partly explained by binding with catalytically active HDAC1. Administration of histone deacetylase inhibitor trichostatin A relieves the transcriptional repression activity by DNMT3L–HDAC1 complex (Aapola et al., 2002; Deplus et al., 2002). Whether DNMT3L also influences the level of histone acetylation through HDAC1 in germ cells and pluripotent stem cells remain to be further elucidated.

DNMT3L and the establishment of the DNA methylation landscape in germ cells

The PIWI-interacting RNA–DNMT3L network safeguards germ cells from genomic parasites

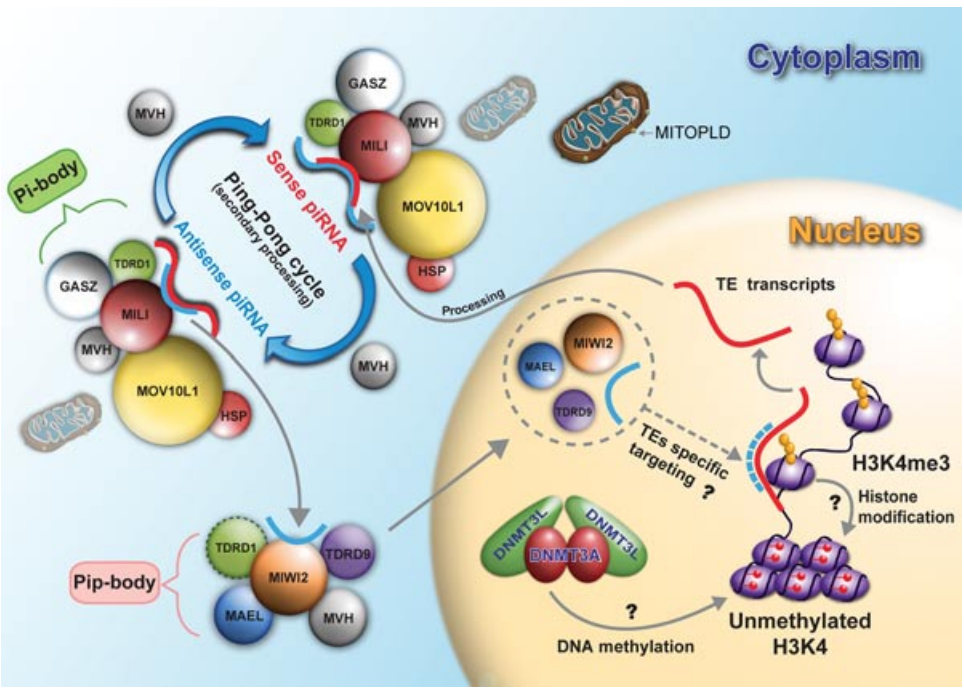
Retroviral infections have been linked to numerous diseases in humans and domestic animals (Jern and Coffin, 2008). Surprisingly, approximately 40% of mammalian genomes are composed of retroelements that include endogenous retroviruses and retrotransposons (Faulkner et al., 2009; Stocking and Kozak, 2008). Retroelements are maintained in a silenced state most of the time in the host organism. If not silenced by the epigenetic machinery, retroelements can replicate via a 'copy and paste' mechanism and damage the host genome. For example, replicated retrotransposons can insert into important regulatory sequences or exons, causing major disturbances to the host genome and triggering serious genome instabilities (Goodier and Kazazian, 2008). In order to prevent these events, host cells have developed a large number of safeguarding silencing machineries, including different levels of epigenetic regulatory mechanisms (Maksakova et al., 2008).

De-repression of retrotransposable elements in germ cells may be considered a side effect of epigenetic reprogramming, which is crucial for full germ cell function (Figure 1; reviewed in Smallwood and Kelsey, 2011). The expression of retrotransposons can be observed in the germ cells of male day 15–17 embryos (Aravin et al., 2009; Trelogan and Martin, 1995), right after the genome is demethylated. Loss of retrotransposon silencing has been shown to disrupt germ cell developmental potential (Bourc'his and Bestor, 2004; Siomi and Kuramochi-Miyagawa, 2009). To suppress retrotransposition activities, germ cells are equipped with several layers of epigenetic silencing (Aravin et al., 2008; Bourc'his and Bestor, 2004). Mammalian retrotransposon silencing in foetal male germ cells appears to depend on three linked networks: transposon detection, PIWI-interacting RNAs (piRNAs) amplification and DNA methylation (Aravin et al., 2008).

Ablation of DNMT3L leads to a failure in chromosomal synapsis in spermatocytes, which may result from the de-repression of retrotransposons (Bourc'his and Bestor, 2004, Webster et al., 2005). DNMT3L is known to be an essential factor for retrotransposon silencing, and *Dnmt3l*-KO testes have significantly different compositions of piRNAs (Aravin et al., 2008; Bourc'his and Bestor, 2004). piRNAs are a class of small RNAs of approximately 26–32 nucleotides in length found in a variety of animal species and predominantly expressed in germ cells. Biogenesis of piRNAs can be classified into primary and secondary processing. Two distinct complexes, named Pi-body (MILI, GASZ, TDRD1, MOV10L, MVH) and Pip-body (MIWI2, MAEL, TDRD9, MVH),

Figure 3 | Repression of transposons in mouse prospermatogonia

Genomic DNA methylation undergoes erasure and then re-establishment during germ cell development. Transposable element (TE) (including retrotransposon) silencing is one of the important events for male germ cell differentiation. The piRNA-related proteins are known as crucial factors to suppress active TEs. A fraction of retrotransposon-related transcripts will be processed into piRNAs of approximately 26–32 nucleotides in length and localise into the Pi-body and Pip-body. The Pi-body is composed of MILI, TDRD1, GASZ, MVH, HSP and MOV10L, and binds sense piRNAs preferentially. In contrast, the Pip-body, formed by MIWI2, MAEL, TDRD9, and MVH, binds the anti-sense piRNAs. MitoPLD, a protein located in the outer layer of mitochondria, is reported as a crucial factor for the localisation of the piRNA pathway components and piRNA biogenesis. Using Line1 retrotransposon as an example, Pi-bodies direct an amplification mechanism, named the ping-pong cycle, to maintain the piRNA pools for Line1 silencing at inter-mitochondrial cement. The components of Pip-body are also important for piRNA biogenesis and DNA methylation. Mutation of these proteins affected the piRNA profile. In addition to cytoplasmic localisation, the Pip-body components, MIWI2, MAEL and TDRD9, were also found in the nucleus. Anti-sense piRNAs are presumed to recognise nascent TE transcripts through reverse complementary binding. The proposed RNA–protein complex may further recruit histone modifiers to alter histone residues, possibly including the removal of the methyl groups from H3K4me3. The proposed modified chromatin may be more favourable targets for DNMT3A–DNMT3L complexes to perform *de novo* DNA methylation to repress the retrotransposon activity. This hypothetical pathway may explain how piRNAs facilitate *de novo* DNA methylation on retrotransposon-related sequences. This model indicates a potential regulatory network amongst DNMT3L, piRNA-related proteins and chromatin modification.



were demonstrated to mediate retrotransposon silencing (reviewed by van der Heijden et al., 2010; Figure 3). Recently, De Fazio et al. (2011) suggested that the endonuclease activity of MILI is essential for piRNA amplification for LINE1 retrotransposon silencing. Apart from the components of Pi-body and Pip-body, MITOPLD/Zuc, a protein localised at the mitochondrial outer membrane surface, is also involved in the primary pathway of piRNA biogenesis (Watanabe et al., 2011b). MITOPLD/Zuc belongs to the conserved phospholipase D superfamily. Although the exact biochemical mechanism is not yet clear, Watanabe et al. (2011b) demonstrated the requirement of MITOPLD/Zuc for the proper sub-cellular localisation of piRNA-associated proteins such as MILI, TDRD1 and MIWI2.

piRNAs and their associated proteins in the Pi-body and Pip-body have been implicated in the establishment of DNA methylation patterns on transposable elements (Thomson and Lin, 2009). MIWI2, MAEL and TDRD9 have independently been shown to move from the cytoplasm into the nucleus during the retroelement depression period (Aravin et al., 2009; Shoji et al., 2009, Soper et al., 2008). These findings have strengthened the hypothesis that during this critical period, some of the Pip complexes and anti-sense piRNA may target the (reverse complementary) nascent transposable element-related transcripts, and then probably attract epigenetic modifiers to the adjacent chromatin and DNA sequences. This is possibly why piRNA machinery is required for the proper DNA methylation of retrotransposons (Aravin et al., 2008; Girard and Hannon, 2008; Figure 3).

Dnmt3l-KO testes, similar to *Vasa*-KO and *Mael*-KO testes, display a disproportional ratio between sense and anti-sense piRNAs (Aravin et al., 2008, 2009; Kuramochi-Miyagawa et al., 2010). Retrotransposon reactivation was reported in these knockout testes. VASA, a DEAD RNA helicase protein that has been suggested to be an essential factor for proper Pi-body formation and piRNA processing, is essential for retrotransposon silencing (Kuramochi-Miyagawa et al., 2010). MAEL, a DNA-binding protein, was reported to interact with the chromatin remodelling protein SIN3B (Costa et al., 2006). In *Mael*-KO male germ cells, VASA fails to localise to Pip-body, while MIWI2 becomes unable to translocate into the nucleus (Aravin et al., 2009). However, the mechanisms connecting piRNAs, histone modification and DNA methylation remain to be elucidated. Further characterisation of the interactions between DNMT3L and piRNA-associated proteins would lead to an understanding of the epigenetic process for piRNA production and the stepwise mechanism of retrotransposon silencing.

DNMT3L and the establishment of imprinting control elements

Genomic imprinting is an epigenetic phenomenon found in eutherian mammals, marsupials and flowering plants. It involves differential epigenetic marking of the two parental chromosome homologs, which results in a small group of genes being regulated in a parent-of-origin-specific fashion. In order to ensure that imprinting marks are correctly established for each new generation, the inherited marks are erased during very early germ cell development in the embryos and re-established during gametogenesis based on the gender (Figure 1). Other key features of imprinting regulation include maintenance of such marks during pre-implantation embryo development (Figure 1) and interpretation of the germline-derived primary imprints (including the establishment of secondary/somatic imprints) (for review, please refer to Li and Sasaki, 2011). Human diseases associated with faulty imprinting include, but are not limited to, hydatidiform moles, ovarian teratomas, uniparental disomies (mUPD14, pUPD14, mUPD15, etc.), Prader–Willi, Angelman and Beckwith–Wiedemann syndromes (Lim and Maher, 2010). Furthermore, defects in a single imprinting control region (ICR) can cause lethality. Examples can be found in Lin et al. (2003, 2007) (reviewed in Hirasawa and Feil, 2010).

DNMT3L is one of the most important epigenetic regulators involved in the establishment of maternal imprints (Bourc'his et al., 2001; Hata et al., 2002; Kobayashi et al., 2012). Although oocytes from *Dnmt3l*-KO female mice can be normally fertilised, most of the resulting embryos die around 9.5 dpc. This maternal effect lethality is caused, at least in part, by the failure to establish oocyte-derived parental origin-specific imprinting marks during *Dnmt3l*-KO oogenesis (Bourc'his et al., 2001; Hata et al., 2002; Kobayashi et al., 2012; Lucifero et al., 2007). It has been suggested that DNMT3L may have co-evolved with the genomic imprinting phenomenon (Yokomine et al., 2006).

Interestingly, embryos derived from *Dnmt3l*-KO female mice show stochastic imprinting patterns at several imprinted loci including *Gnas*, *Peg3* and *Snrpn*, which might result from 'incomplete penetrance' of *Dnmt3l*-KO in the female germline or from the *de novo* DNA methylation wave in the embryos prior to implantation (Arnaud et al., 2006). A more recent study indicated that the zygotic contribution of the KRAB zinc-finger protein ZFP57 could facilitate the restoration of the imprinted methylation marks specifically on the maternal allele of the *Snrpn* region in embryos derived from $Zfp57^{-/-}$ females, which failed to establish *Snrpn* DMR methylation (Li et al., 2008). The stochastic acquisition of differential methylation at *Snrpn* DMR in *Dnmt3l*-KO

oocyte-derived embryos may be partly due to the ZFP57-mediated epigenetic network.

Initial studies suggested that the DNMT3L-related DNA methylation abnormalities in *Dnmt3l*-KO oocytes may be limited to imprinted regions, as the methylation levels of selected retrotransposons including IAP and LINE-1 were only moderately reduced (Lucifero et al., 2007). However, two recent studies using high-throughput DNA methylation analysis on wild-type and *Dnmt3l*-KO oocytes reveals a genome-wide role of DNMT3L in establishing DNA methylation pattern beyond genomic imprinting (Smallwood et al., 2011; Kobayashi et al., 2012). The lethality phenotype of the *Dnmt3l*-KO oocytes-derived embryos can therefore be attributed to more than just misregulation of imprinted genes (Kaneda et al., 2010; Smallwood et al., 2011; Kobayashi et al., 2012).

Although DNMT3L is indispensable for the establishment of maternal imprinting marks, the presence of overlapped transcripts across a DMR region may be a pre-requisite for the acquisition of maternally imprinted DNA methylation (Chotalia et al., 2009). It may be the nascent RNAs across the ICRs that recruit specific histone modifiers to alter the local chromatin signature (Nagano and Fraser, 2011; Santoro and Barlow, 2011; Wagschal et al., 2008), and could subsequently attract *de novo* DNA methylation. The methylated counterparts of these ICRs are indeed associated with the marks for repressive histone methylation, including H3K9me3, H4K20me3 and H2A/H4R3me2 (Henckel et al., 2009). It has been demonstrated that some of the long non-coding RNAs, such as HOTAIR, can recruit chromatin modifiers responsible for H3K27methylation (PRC2) and H3K4 demethylation (Gupta et al., 2010; Tsai et al., 2010). Many of the imprinted protein coding and non-coding RNAs, overlapped with germline or somatic-imprinted DMRs, are also associated with EZH2, one of the PRC2 components (Zhao et al., 2010). It would therefore be very interesting to study whether during the establishment of imprinting marks during germ cell development and early embryogenesis, these imprinted transcripts are responsible for attracting specific histone modifiers which subsequently prepare a regional chromatin context susceptible for establishing the DNA methylation imprints. The requirement of the histone deacetylase KDM1B for the establishment of the differential DNA methylation marks at some imprinted loci during oogenesis (Ciccone et al., 2009) suggests the importance of chromatin context in *de novo* DNA methylation. The exact order of the epigenetic events that establish and maintain genomic imprinting and the exact place of DNMT3L in that hierarchy still need to be elucidated.

Potential roles of DNMT3L and the piRNA pathway in paternal imprinting establishment

The involvement of DNMT3L in the establishment of paternal imprinting marks during spermatogenesis was demonstrated by the moderate to severe reduction of methylation levels in paternally methylated DMRs (H19, Dlk1/Gtl2 and Rasgrf1) in *Dnmt3l*-KO newborn prospermatogonia (Kato et al., 2007). There was a discrepancy in the severity of the effects at different paternally methylated imprinted marks in the *Dnmt3l*-KO male germ cells (Bouc'his and Bestor, 2004; Kaneda et al., 2004a, 2004b; Kato et al., 2007). This may partly be due to differences in genetic background or the stochastic imprint accumulation as demonstrated in *Dnmt3l*-KO oocyte-derived offspring (Arnaud et al., 2006; Kato et al., 2007). Alternatively, the differences may have arisen due to unique imprint mechanisms operating at the three imprinted loci.

Knowing that overlapped transcripts are required for the establishment of at least some maternally methylated imprinted regions (Ferguson-Smith, 2011; Koerner et al., 2009; Santoro and Barlow, 2011), it is tempting to speculate that the establishment of paternally methylated imprints requires a form of RNA-mediated machinery. Unlike imprinted loci with maternally methylated DMRs, paternally methylated imprinting control centres do not link with overlapped anti-sense transcripts. A recent publication has confirmed the link between piRNAs and the imprint establishment on one of the paternally methylated imprinted loci, *Rasgrf1* (Watanabe et al., 2011a). This is accomplished by analysis of mice with targeted deletion of a piRNA pathway protein, MITOPLD. *Mitopld*-KO females are fertile, whereas *Mitopld*-KO male germ cells are arrested at the zygotene stage (Watanabe et al., 2011b). Examination of four paternally methylated DMRs (H19, Dlk1-Gtl2, Gpr1-Zbdf2 and Rasgrf1-DMRs) in the prospermatogonia of MitoPLD mutants shows a strikingly reduced level of methylation at the

Rasgrf1 locus, compared with wild-type controls. In contrast, no significant differences in DNA methylation levels were observed in the H19, Dlk1–Gtl2 and Gpr1–Zdbf2 DMRs (Watanabe et al., 2011a). In addition, a non-coding RNA spanning the *Rasgrf1* DMR contains a retrotransposon sequence that is targeted by piRNAs generated from another chromosome. These data provide a link for piRNA-mediated imprint establishment in a sequence-specific manner. Since DNMT3L is also required for the full development of the methylation imprint at this locus, these results suggest a possible connection amongst DNMT3L, piRNAs and imprinted genes. In addition to piRNA-mediated imprint establishment, another possible model participated in male germ cells is the high levels of transcriptional read-through across the differentially methylated regions associated with the timing of imprint acquisition. These non-coding RNAs associated to H19-DMR of the Igf2-H19 locus and IgDMR of the Dlk1-Gtl2 locus may be indispensable in the acquisition of imprinted DNA methylation marks (Henckel et al., 2011).

The potential roles of DNMT3L in germline stem cells, cancers and the maintenance of DNA methylation in pluripotent stem cells

Apart from being highly expressed in germ cells at specific developmental stages and germline stem cells, DNMT3L is found in pre-implantation embryos and pluripotent cells, including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) (Bourc'his et al., 2001; Hata et al., 2002; Mikkelsen et al., 2008; Lin et al., unpublished data). Aberrant hypomethylation of the *DNMT3L* promoter region associated with DNMT3L expression can be found in certain cancer cell types (Gokul et al., 2007; Kim et al., 2010; Minami et al., 2010; Manderwad et al., 2010). In this section, we discuss the potential function of DNMT3L in germline stem cells, pluripotent stem cell differentiation and cancer.

DNMT3L and male germline stem cells

Spermatogenesis takes place in the seminiferous tubules of mouse testis where various developmental events occur, including spermatogonial stem cells (SSCs) formation, self-renewal/differentiation of SSCs, meiosis and spermiogenesis (Caires et al., 2010; reviewed by Oatley and Brinster, 2008). DNMT3L is an indispensable factor for mouse gametogenesis, from which *Dnmt3l* transcript is found in primordial germ cells (PGCs), prospermatogonia and neonatal SSCs, but declines significantly in differentiated spermatogonia (Bourc'his and Bestor, 2004; La Salle et al., 2007). Transplanting DNMT3L overexpressing germline stem cells (D3L-TG GSC) into recipient testes results in spermatogenic arrest at the spermatocyte stage (Takashima et al., 2009). However, there are no differences in the number of the re-populated SSCs between wild-type and D3L-TG GSC-transplanted testes, suggesting excessive DNMT3L does not affect the function of GSCs. In contrast, targeted disruption of *Dnmt3l* causes gradual germ cell loss in adult mice, implying that DNMT3L may have a function in SSC maintenance (Bourc'his et al., 2001; Hata et al., 2002). Our recent study indicated that DNMT3L is required for maintaining the quiescent status of spermatogonial progenitor cells, which is crucial for maintaining the germline stem cell pool. *Dnmt3l*-KO germ cells also failed to re-populate busulfan-treated recipient testes, indicating the importance of DNMT3L in germline stem cell function (Liao and Lin et al., unpublished data). Our results suggest that DNMT3L is involved in more than just retrotransposon silencing and establishment of imprinting marks in male germ cells. DNMT3L may affect the epigenetic signature (La Salle et al., 2007) and thus the expression patterns of genes involved in germline stem cell maintenance and function. Recent genome-wide DNA methylation analysis by Smallwood et al. (2011) supported the notion that at least in oocytes, DNMT3L-dependent methylation is significantly more widespread than in ICRs and retrotransposons.

The potential roles of DNMT3L in maintaining the DNA methylation landscape in pluripotent cells

High levels of DNMT3L expression can be observed in cell lines such as ESCs, embryonal carcinoma (EC) cells, embryonic germ cells and male germline stem cells, which can easily be reprogrammed to the pluripotent status when cultured in ES cell media (Guan et al., 2006; Takashima et al., 2009). The expression level of DNMT3L drops dramatically when

pluripotent cells start to differentiate (Hata et al., 2002; Hu et al., 2008). DNMT3L is one of the proteins, along with other pluripotent factors and chromatin modifiers, that are up-regulated during the transition stage between partially and fully reprogrammed iPSCs (Mikkelsen et al., 2008). DNMT3L has also been categorised as a marker of pluripotent cells in the literature (Mikkelsen et al., 2008; Samavarchi-Tehrani et al., 2010). However, it is not clear whether DNMT3L is functionally significant for either maintaining the properties of pluripotent cells or their full differentiation potential.

Pawlak and Jaenisch (2011) demonstrated that *Dnmt3a*- and *Dnmt3b*-deficient fibroblasts can be reprogrammed to iPSCs via the activation of endogenous pluripotent factors. These reprogrammed cells maintain self-renewal properties and display similar gene expression patterns to regular iPSCs and ESCs. The authors therefore concluded that *de novo* methyltransfearses are dispensable for the reprogramming process. However, these *Dnmt3a* and *Dnmt3b* double-deficient iPSCs have only limited differentiation potential, as demonstrated by deficient teratoma formation. After injecting to 2n blastocysts, the cells did not contribute to developing chimeras in mid-gestation stages. The differentiation deficiency of the DNMT3A and DNMT3B double-deficient iPSCs can be rescued after re-introduction of the *Dnmt3a* and *Dnmt3b* genes. If DNMT3L is involved in assisting DNMT3A and/or DNMT3B for establishing and maintaining the methylation landscape in pluripotent stem cells, one would expect to see similar differentiation defects in *Dnmt3l*-KO ESCs or iPSCs. The fact that *Dnmt3l*-KO mice can survive without serious pathological symptoms in somatic lineages argues against the previous statement (Bourc'his et al., 2001). However, it is important to note that these *Dnmt3l*-KO embryos are generated from inter-crossing $Dnmt3l^{+/-}$ males and females. Oocytes generated from the $Dnmt3l^{+/-}$ females have maternally derived *Dnmt3l* transcripts, which can still be detected in the cytoplasm of early pre-implantation embryos (Mo and Lin et al., unpublished data). Since DNMT3L expression drops sharply after implantation in wild-type embryos (Hu et al., 2008), the differences in DNMT3L expression between *Dnmt3l*-KO embryos and wild-type littermates is therefore only restricted to a very brief period from morula to blastocyst and potentially epiblast. The long-term effect for DNMT3L deficiency in pluripotent stem cells would therefore need to be examined separately.

Lack of DNMT3L has recently been shown to cause hypomethylation at retrotransposons and minor satellite sequences in *Dnmt3l*-KO ESCs (Ooi et al., 2010), indicating a surprising role for DNMT3L in maintaining DNA methylation marks in ESCs. However, hypomethylation was only observed in *Dnmt3l*-KO ESCs after around 30 passages. This may be caused by an accumulative effect of reduced processive catalytic activity from DNMT3A in the absence of DNMT3L (Jurkowska et al., 2011).

Another characteristic of pluripotent stem cells is the strong epigenetic silencing of newly infected retroviruses. With the role of DNMT3L in retrotransposon repression in germlines, it is logical to predict that DNMT3L is involved in *de novo* methylation of newly infected retroviruses. Indeed, DNMT3L has recently been proven to facilitate *de novo* methylation by DNMT3A and DNMT3B on newly infected Moloney murine leukaemia virus (Mo-MLV) in ESCs, as demonstrated by the significantly reduced DNA methylation level on the newly integrated Mo-MLV sequences in *Dnmt3l*-KO ES cells compared with wild-type ESCs (Kao and Lin et al., unpublished data; Ooi et al., 2010). These observations suggest that DNMT3L may contribute to certain properties demonstrated in pluripotent stem cells.

The potential roles of DNMT3L in cancer cells

Aberrant DNA methylation patterns have been consistently found in many different types of cancer cells (Robertson, 2005; Sharma et al., 2010). These patterns include global hypomethylation associated with the over-expression of proto-oncogenes, growth factors and retrotransposons that result in aberrant overgrowth and genomic instability. On the contrary, many tumour suppressor genes are found to be hypermethylated in various tumours, a direct association with their phenotypes (review by Szyf et al., 2004; Esteller, 2007; Montoya-Durango and Ramos, 2010). De-regulation of certain DNMT isoforms has been associated with specific cancer cell types (review by Robertson, 2005; Ellis et al., 2009). DNMT3L is highly expressed in germ cells and undifferentiated pluripotent stem cells under normal

developmental processes. Therefore, it is not surprising that DNMT3L is also found in several germ cell lineages derived from cancers that share characteristics with pluripotent stem cells. These include seminomatous and non-seminomatous testicular germ cell tumours (TGCTs). DNMT3L has been demonstrated to be essential for the growth of human EC, which is highly associated with the metastasis of non-seminomatous TGCTs (Minami et al., 2010).

In addition, hypomethylation of the *Dnmt3l* promoter has been observed in several other cancer cells and neoplasias, including cervical cancer, ocular surface squamous and gastric cancer cell line (Gokul et al., 2007; Kim et al., 2010; Manderwad et al., 2010). Over-expression of DNMT3L in HeLa cells was also shown to mimic the carcinogenesis process (Gokul et al., 2009). However, little is known about the functional significance of DNMT3L's expression in these cancer cells. It has recently been demonstrated that DNMT3L is responsible for the promoter methylation of thymine DNA glycosylase in gastric cancer cell lines, and may be therefore linked to fine-tuning the DNA methylation landscape (Kim et al., 2010). Various types of functional non-coding RNAs have been associated with carcinogenesis and cancer metastasis. These include the aforementioned piRNAs (reviewed in Siddiqi and Matushansky, 2011) and large intervening non-coding RNAs (lincRNAs; Gupta et al., 2010). LincRNAs affect the chromatin landscape of the host cells. For example, the breast cancer metastasis-associated lincRNA HOTAIR binds polycomb repressive complex 2 (PRC2) on its 5′ domain and LSD1/CoREST/REST complex on its 3′ domain (Gupta et al., 2010; Tsai et al., 2010). This feature would allow the co-ordinated addition of H3K27me3 by PRC2 and demethylation of H3K4me3 or H3K4me2 by the LSD1 complex in the target sequences of the lincRNAs. The resulting repressive chromatin signature, especially the demethylated H3K4, may be favourable for the DNMT3A–DNMT3L complex to add *de novo* methylation mark in cancer cell type. The presence of piRNAs and their associated proteins Hiwi and Hili in various human cancers (review by Siddiqi and Matushansky, 2011) also suggests that the potential piRNA-mediated *de novo* methylation machinery proposed in germ cells may be hijacked by cancer cells and trigger similar DNA methylation pattern amongst germ/stem/cancer cells, in addition to cancer-specific DNA methylation patterns.

Conclusion

Epigenetic factors undoubtedly play important roles in cell fate decision/commitment. Here, we reviewed the epigenetic roles of DNMT3L, which contributes to the establishment of genomic methylation patterns and the production of fully functional gametes. Protein crystal structures and *in vitro* biochemical analyses of the DNMT3 family advanced the understanding of DNMT3L's biological functions. Recent cell line studies further suggested that DNMT3L fine-tunes DNMT3A's and DNMT3B's activities and/or their target selection mechanisms, reducing DNMT3A homo-oligomerisation and relocating DNMT3A to heterochromatic foci; increasing the DNMT3A–DNMT3L complex sensitivity to H3K4 methylation; guiding DNMT3A to the well-chromatinised regions; increasing the processive catalytic activity of DNMT3A and the duration of DNMT3s/DNA association. To further investigate DNMT3L-mediated mechanisms in the establishment of most parental origin-specific imprinted marks and the repressive epigenetic marks on many retrotransposon sequences in germ cells, we need to combine both biochemical and systems biology approach in developing germ cells when the *de novo* methylation takes place.

Although genetic evidence has confirmed the involvement of the piRNA pathway in the establishment of DNA methylation marks on retrotransposons and one imprinted locus, *Rasgrf1*, the exact mechanisms are not fully understood. Nevertheless, a plausible theory states that piRNA and some Pip-body components may mediate the predisposition of specific chromatin context in the target sequence that subsequently favour the *de novo* DNA methylation by DNMT3 proteins. Time course RNA immunoprecipitation (RIP with antibodies against EZH2, KDM1B) and chromatin immunoprecipitation sequencing (ChIP-seq for H3K4 and H3K27 methylation profiles) studies throughout the duration of epigenetic reprogramming in the wild-type and piRNA-deficient male mouse germ cells may determine whether the nascent retrotransposon or Rasgrf transcripts are indeed recruiting specific histone

modifiers in a piRNA-dependent manner. In addition, the application of DNA–RNA FISH with specific probes against the piRNA-mediated DNA methylation region in Rasgrf1 DMR and the immunostaining experiment with antibodies against specific histone modifiers may provide further visual evidence. Time course RNA immunoprecipitation plus ChIP analysis in germ cells throughout the epigenetic reprogramming period can provide implication of the roles of long non-coding RNA in mediating the establishment of DNA methylation landmark. The findings will be particularly relevant to clarify whether the transcripts overlapped with both maternally and paternally methylated ICRs can recruit histone modifiers and induce chromatin modifications at the adjacent DNA sequences (ICRs).

In the near future, unmasking the roles of DNMT3L-targeted genes in developing germ cells and germline stem cells will provide significant insight into the signalling and epigenetic mechanisms that are important to germ cell homeostasis and function. This knowledge will also facilitate the understanding of DNMT3L's role in pathological conditions, including male infertility and certain types of cancers.

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Conflict of interest statement

The authors have declared no conflict of interest.

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