Human Molecular Genetics, 2008, Vol. 17, No. 17 ***2654–2664***
doi:10.1093/hmg/ddn165
Advance Access published on June 10, 2008

Regulation of DNA methylation activity through *Dnmt3L* promoter methylation by Dnmt3 enzymes in embryonic development

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Received February 1, 2008; Revised and Accepted May 28, 2008

The genomic DNA is methylated by *de novo* methyltransferases Dnmt3a and Dnmt3b during early embryonic development. The establishment of appropriate methylation patterns depends on a fine regulation of the methyltransferase activity. The activity of both enzymes increases in the presence of Dnmt3L, a Dnmt3a/3b-like protein. However, it is unclear how the function of Dnmt3L is regulated. We found here that the expression of Dnmt3L is controlled via its promoter methylation during embryonic development. Genetic studies showed that Dnmt3a, Dnmt3b and Dnmt3L are all involved in the methylation of the *Dnmt3L* promoter. Disruption of both *Dnmt3a* and *Dnmt3b* genes in mouse rendered the *Dnmt3L* promoter devoid of methylation, causing incomplete repression of the *Dnmt3L* transcription in embryonic stem cells and embryos. Disruption of either *Dnmt3a* or *Dnmt3b* led to reduced methylation and increased transcription of *Dnmt3L*, but severe hypomethylation occurred only when Dnmt3b was deficient. Consistent with the major contribution of Dnmt3b in the *Dnmt3L* promoter methylation, methylation of *Dnmt3L* was significantly reduced in mouse models of the human ICF syndrome carrying point mutations in *Dnmt3b*. Interestingly, Dnmt3L also contributes to the methylation of its own promoter in embryonic development. We thus propose an auto-regulatory mechanism for the control of DNA methylation activity whereby the activity of the *Dnmt3L* promoter is epigenetically modulated by the methylation machinery including Dnmt3L itself. Insufficient methylation of the *DNMT3L* promoter during embryonic development due to deficiency in *DNMT3B* might be implicated in the pathogenesis of the ICF syndrome.

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INTRODUCTION

Methylation of cytosines is the only known genome modification in vertebrates. Formation of cell-type-specific genomic methylation patterns is thought to be one of the underlying mechanisms for the differential programming of cell lineages. Cytosine methylation is required in various biological processes such as X-chromosome inactivation, genomic imprinting, transposon silencing and the regulation of tissue-specific gene expression. Aberrant DNA methylation contributes to many human diseases, including cancer (1,2).

DNA methylation patterns are established mainly during embryonic development, through highly regulated processes of *de novo* methylation and demethylation (3). Afterwards, they are stably maintained by the action of the maintenance methyltransferase Dnmt1 (4). The *de novo* methylation is carried out by three members of the Dnmt3 family. Dnmt3a and Dnmt3b are the two functional *de novo* methyltransferases with various isoforms. They are highly expressed in embryonic stem (ES) cells, early embryos and developing germ cells, but in differentiated cells only the longer isoform of Dnmt3a is expressed (5–8). In human, mutations in *DNMT3B* cause the ICF syndrome characterized by immunodeficiency, centromere instability and facial anomalies (6,9,10). Defective methylation at the classical satellites is a genomic hallmark of ICF patients. Dnmt3L is the third member of the Dnmt3 family. It shares homology with Dnmt3a and Dnmt3b but lacks the crucial catalytic motifs of the cytosine methyltransferases and is enzymatically inactive *per se* (11). Like Dnmt3a and Dnmt3b, it is highly expressed in ES cells, early embryos and the developing germ cells which undergo dramatic reprogramming of DNA methylation (12–14). Although inactive as an enzyme, knockout experiments have revealed that Dnmt3L is essential for the establishment of maternal methylation imprints during oogenesis, and for the methylation of retrotransposons, satellites repeats and some imprinted genes during spermatogenesis (12,15–19). Biochemical studies demonstrated that Dnmt3L can greatly stimulate the methylation activity of both Dnmt3a and Dnmt3b (20–23). Impaired interaction between DNMT3B and DNMT3L might contribute to the ICF syndrome (14).

Given the role of Dnmt3L in the regulation of the methyltransferase activity, investigation of the regulation of Dnmt3L itself is of potential importance for the understanding of the mechanism of DNA methylation in development and disease. The promoter of *Dnmt3L* has been reported to be unmethylated in ES cells but methylated in somatic tissues, and *in vitro* methylation of *Dnmt3L* with a bacterial methyltransferase inactivated its expression in transfected mammalian cells (24). Despite the inverse correlation of promoter methylation and expression, the mechanistic aspects of *Dnmt3L* methylation and functional significance in development have not been addressed. In this study, we explore these points by genetic and biochemical studies using knockout ES cells and mouse embryos deficient in Dnmt3a, Dnmt3b and Dnmt3L. We show that Dnmt3b is the major methyltransferase responsible for the methylation of the *Dnmt3L* promoter. Dnmt3a and Dnmt3L itself also participate in the methylation process. Hypomethylation due to deficiency in methyltransferase activity causes up-regulation of *Dnmt3L* during the differentiation of mouse ES cells and embryonic development. We therefore conclude that the methylation activity in embryonic cells might be subjected to autorepression at the *Dnmt3L* promoter by the Dnmt3 proteins. Since methylation of the *Dnmt3L* promoter is also impaired in the mouse models for the human ICF syndrome, the deregulation of *DNMT3L* expression could be implicated in the ICF syndrome.

RESULTS

Down-regulation of Dnmt3L in the epiblast and up-regulation in the ExE lineages during the early post-implantation mouse embryonic development

The mouse embryo development of early post-implantation period is characterized by epigenetic reprogramming of cellular fates. Although DNA methyltransferases Dnmt3a and Dnmt3b have been shown to be required for genome-wide *de novo* methylation at the early post-implantation stage (6), mechanistic study of the reprogramming of genomic methylation patterns during this stage is still lacking. To obtain clues about the *de novo* DNA methylation, we prepared specific anti-Dnmt3 antibodies with their performance validated using different knockout ES cells as negative controls (Fig. S1) and examined the expression patterns of Dnmt3a, Dnmt3b and Dnmt3L in the embryonic day (E) 5.5–E7.5 embryos by indirect fluorescence immunostaining. Dnmt3a is found in the epiblast (E5.5–E6.5) and ectoderm (E7.5), and in the visceral and parietal endoderm and trophoblast giant cells (Fig. 1A, E and I). Dnmt3b exists at a high level in the epiblast (E5.5–E6.5) and ectoderm (E7.5), and at a lower but substantial level in the extraembryonic epiblasts (ExEs), the visceral endoderm and the E7.5 mesoderm cells (Fig. 1C, G and K). Interestingly, the expression of Dnmt3L undergoes an obvious transition from the epiblast to ExE over this period. The signal intensity of Dnmt3L decreased gradually in the epiblast, whereas increased in the ExE lineages from E5.5 to E7.5 (Fig. 1B, F and J).

The staining patterns of Dnmt3 proteins at E7.5 match very well with previous reports (6,18). By a more detailed study to distinguish the two Dnmt3a isoforms, we demonstrated that Dnmt3a2 is the predominant form of Dnmt3a in the epiblast (E5.5 and E6.5) and ectoderm (E7.5) (Fig. S2).

Promoter methylation of *Dnmt3L* during early post-implantation development

The temporally and spatially regulated distribution of Dnmt3L in the course of post-implantation development suggests that it may play a role in the control of methylation activity and its expression is subjected to strict control in embryonic development. We next asked whether the promoter of *Dnmt3L* acquires DNA methylation when its expression undergoes repression during this period. To address this question, genomic DNA from epiblasts and ExEs of E5.5, E6.5 and E7.5 embryos was isolated for bisulfite-based methylation analyses. The overall methylation levels were examined by combined bisulfite treatment and restriction analysis

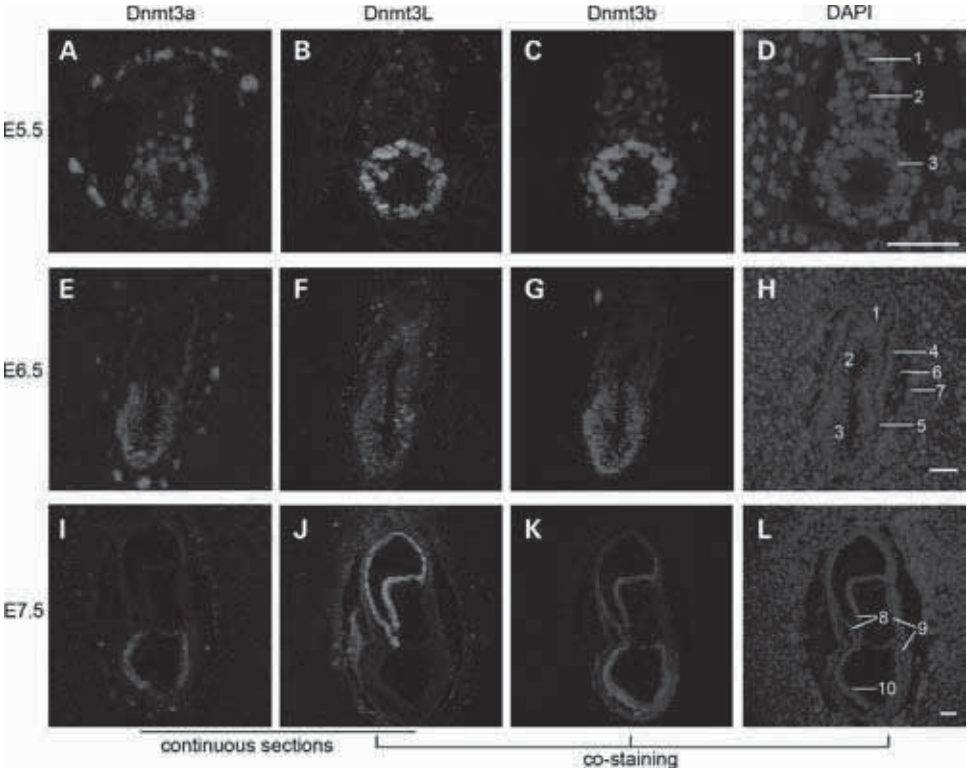

Figure 1. Expression of Dnmt3a, Dnm3b and Dnmt3L in the early post-implantation mouse embryo. Serial sections of mouse embryos at E5.5, E6.5 and E7.5 were immunostained with anti-Dnmt3a full-length (**A, E** and **I**; red), and co-immunostained with monoclonal anti-Dnmt3L (**B, F** and **J**; green) and anti-Dnmt3b full-length (**C, J** and **K**; red) antibodies. Sections were counterstained with the DNA dye DAPI (**D, H** and **L**; blue). 1, ectoplacental cone; 2, extraembryonic epiblast; 3, epiblast; 4, extraembryonic visceral endoderm; 5, embryonic visceral endoderm; 6, parietal endoderm cells; 7, trophoblast giant cells; 8, amniotic folds; 9, extraembryonic and intraembryonic mesoderm; 10, embryonic ectoderm. Scale bar, 50 mm.

(COBRA) on the PCR products derived from bisulfite-treated genomic DNA, and the methylation status of CpG dinucleotides on individual strands was examined by sequencing of clones of the PCR products. The *Dnmt3L* promoter region analyzed contains 16 CpG dinucleotides, and 3 of them are located in the perspective *Taq*I restriction site CCGA (Fig. 2A). The CCGA sequence will be converted to TTGA in the bisulfite-based assay if the second C is unmethylated, but will become TCGA (*Taq*I site) if the second C is methylated. Thus, the cleavage of the PCR products by *Taq*I digestion indicates the presence of a methylated C**CG**A site and resistance to *Taq*I digestion reveals the absence of methylation. *Taq*I-based COBRA showed that the *Dnmt3L* promoter is free of methylation at the three analyzed CpG sites in the epiblast at E5.5, but becomes gradually methylated at E6.5 and E7.5 (Fig. 2B). In the ExE, only a small fraction of DNA strands became methylated at E7.5 (Fig. 2B). These observations were confirmed and extended by bisulfite sequencing analysis (Fig. 2C). The overall methylation level rose from 1.8% at E5.5 to 75% at E7.5 in the epiblast. In the ExE, however, methylation was scarce at all time points. The gradual increase in the methylation of *Dnmt3L* promoter in the epiblast coincides well with the gradual decrease in Dnmt3L protein in the early gastrulation stage, whereas the largely unmethylated state in the ExE is conductive to the up-regulation of Dnmt3L in the extraembryonic lineages (Fig. 1B, F and J).

Role of the Dnmt3 members in the promoter methylation and transcriptional repression of *Dnmt3L* during *in vitro* differentiation of ES cells

We then wanted to investigate the enzymes responsible for the methylation of the *Dnmt3L* promoter and the role of DNA methylation in its transcriptional regulation. We employed an *in vitro* stem cell differentiation system which mimics the mouse early embryonic development. The molecular events of cell differentiation in an embryoid body, an induced aggregate of stem cells cultured *in vitro*, are thought to recapitulate early embryonic development to a certain extent (25,26). We first demonstrated that the promoter of *Dnmt3L* is subject to cytosine methylation upon *in vitro* differentiation (Fig. 3A and B). To determine the contribution of Dnmt3a, Dnmt3b and Dnmt3L in the regulation of the *Dnmt3L* promoter, wild-type (WT) and Dnmt3-deficient ES cells were induced to form embryoid bodies (EBs) for differentiation. EBs were collected at different days and subjected to DNA and RNA extraction. Both COBRA and bisulfite sequencing analysis were performed to examine the *Dnmt3L* promoter methylation. As shown in Fig. 3A and B, the *Dnmt3L* promoter underwent gradual methylation in the WT, *Dnmt3a*$^{-/-}$ and *Dnmt3b*$^{-/-}$ ES cells upon differentiation, whereas in the *Dnmt3a*$^{-/-}$/*3b*$^{-/-}$ cells, no methylation was detected. Therefore, both Dnmt3a and Dnmt3b participate in the methylation of the *Dnmt3L* promoter. Interestingly, Dnmt3b has a much larger contribution as the promoter

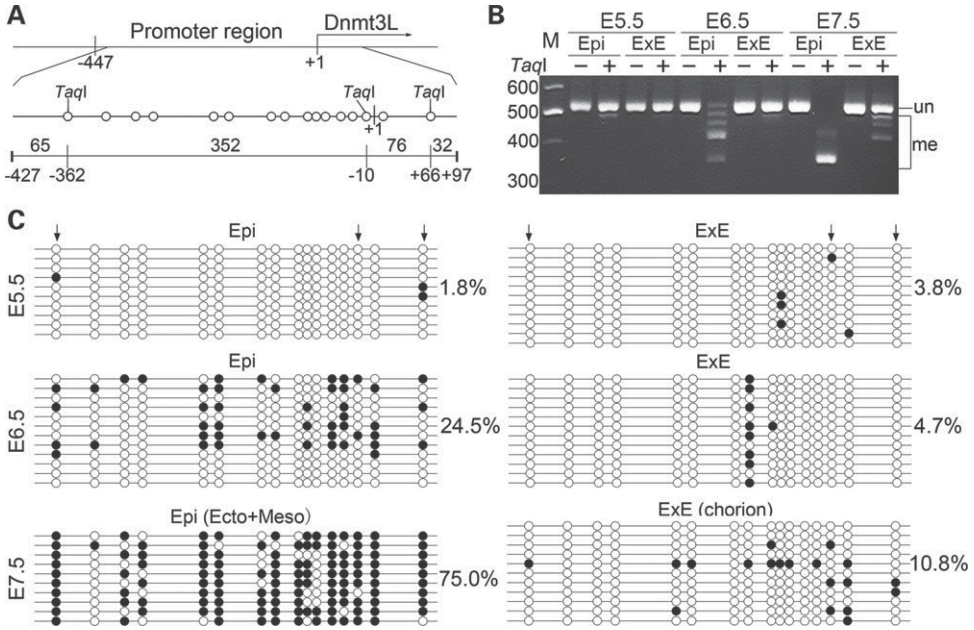

Figure 2. Analysis of DNA methylation of the *Dnmt3L* promoter in the Epi and ExE of early post-implantation mouse embryos. (**A**) Schematic diagram of the *Dnmt3L* promoter (24). The translational starting site is indicated as +1. The relative position of the CpG dinucleotides (open circles) and the *Taq*I sites in the region PCR-amplified after bisulfite treatment are shown below. The lengths of the *Taq*I restriction fragments of the PCR fragment are indicated above the bottom line. (**B**) COBRA methylation analysis of the *Dnmt3L* promoter. For each sample, genomic DNA was obtained from 5–16 embryos. The top band of 524 bp (un) is *Taq*I resistant and the fragments were thus derived from embryonic DNA unmethylated at the CpG dinucleotides within the three *Taq*I sites. The smaller *Taq*I-digested fragments (me) were derived from DNA with full or partial methylation at the analyzed CpG dinucleotides. (**C**) Methylation analysis by bisulfite sequencing. Each line corresponds to a single strand of DNA, and each circle represents an individual CpG dinucleotide. Open circles, unmethylated CpG sties; filled circles, methylated CpG sites. Arrows indicate the CpG dinucleotides within the *Taq*I recognition sequence. Epi, epiblast; ExE, extraembryonic epiblast; Ecto, ectoderm; Meso, mesoderm.

methylation was more severely affected in Dnmt3b-deficient ES cells (Fig. 3A and B). The effect of Dnmt3a deficiency on the methylation rate appeared minimal. Another interesting observation is that Dnmt3L contributes to the methylation of its own promoter. In the absence of Dnmt3L, its promoter methylation was impaired, reaching only 29.4% at day 2 and 48.8% at day 4, significantly lower than the level of WT ES cells at the corresponding time points (Fig. 3A and B). The impairment was not caused by targeted deletion of exons 3–8 of *Dnmt3L* (18), as the promoter region could be methylated by ectopic expression of Dnmt3L in *Dnmt3L*$^{-/-}$ ES cells (Fig. S3).

To analyze the relationship between the altered *Dnmt3L* promoter methylation and expression, we performed real-time RT–PCR to measure the mRNA level at various stages. *Dnmt3L* was first up-regulated upon aggregation (day 1), and then took a down turn in the following days of differentiation in all four ES cell lines (Fig. 3C). Although the overall patterns of change in the mRNA level are similar among different cell lines, the levels of *Dnmt3L* mRNA vary greatly with their genotypes, especially after day 3 when significant promoter methylation of *Dnmt3L* was underway in the WT ES cells. While repression of *Dnmt3L* occurred efficiently in WT ES cells, a significant level of expression persisted in both *Dnmt3b*$^{-/-}$ and double knockout ES cells. In contrast, *Dnmt3L* was efficiently repressed in *Dnmt3a*$^{-/-}$ cells with only a minor elevation of expression at later time points compared with WT ES cells (Fig. 3C). Therefore, comparison of different cell lines indicates that the transcription of *Dnmt3L* during EB differentiation is inversely related to its promoter methylation levels. The high degree of negative correlation strongly suggests that silencing of *Dnmt3L* transcription is closely associated with the occurrence of DNA methylation. On the other hand, the overall down-regulation of *Dnmt3L* transcription regardless of DNA methylation may be accounted for by the contribution of other epigenetic modifications such as histone methylation and/or decrease of transcriptional factors during ES cell differentiation.

Role of the Dnmt3 members in the promoter methylation and transcriptional repression of *Dnmt3L* in embryonic development

To extend our study in the *in vitro* ES cell differentiation system, we next investigated promoter methylation and transcriptional regulation of *Dnmt3L* in mouse embryos. Both the COBRA and bisulfite sequencing were performed on genomic DNA isolated from E9.5 WT and mutant mouse embryos deficient in Dnmt3 proteins (Fig. 4A and B). We found that the *Dnmt3L* promoter was methylated to a similar level of ~58% in the WT and *Dnmt3a*$^{-/-}$ embryos, but the methylation level reached only 12.5 and 42.2% in the absence of *Dnmt3b* or *Dnmt3L*. Removal of both *Dnmt3a* and *Dnmt3b* abolished methylation completely. Defective promoter methylation also led to de-regulation of *Dnmt3L* expression in the mutant embryos (Fig. 4C). Compared with the WT embryos, the *Dnmt3L* mRNA level in the *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$ and *Dnmt3a*$^{-/-}$/*3b*$^{-/-}$ embryos was 1.5, 4.2 and 26.5-fold higher, respectively (Fig. 4C, upper panel). In contrast, high level expression of *Dnmt3L* in trophoblast tissues was detected in all samples,

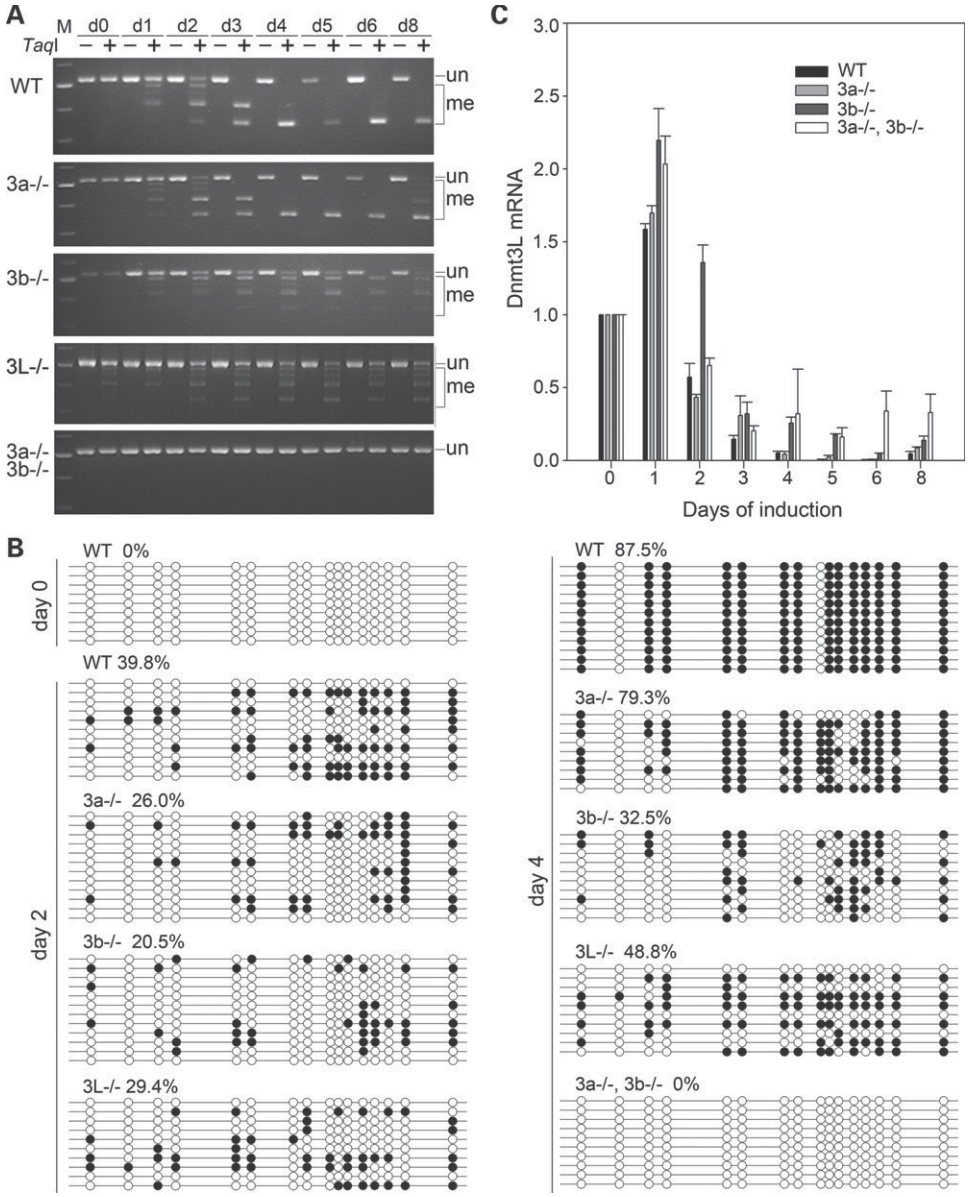

Figure 3. Promoter hypomethylation and increased mRNA levels of *Dnmt3L* in mutant ES cells during *in vitro* differentiation. (**A**) COBRA methylation analysis of the *Dnmt3L* promoter at different stages of differentiation of ES cells. The genotypes of the ES cells (wild-type or Dnmt3 knockout mutant) are indicated at the left and the time points (day 0–8) for methylation analysis indicated on the top. Labeling of the *Taq*I restriction fragment is the same as in Fig. 2B. (**B**) Methylation analysis by bisulfite sequencing. The genotypes of the ES cells (wild-type or Dnmt3 knockout mutant) and the methylation level determined are indicated on the top. The time points (days 0, 2 and 4) for methylation analysis are indicated at the left. (**C**) Analysis of *Dnmt3L* mRNA levels by real-time RT-PCR. The bars represent means ± SD which were derived from three independent experiments each performed in triplicate. The values are relative to the expression level in undifferentiated ES cells (day 0) which was set to 1.

regardless of their genotypes (Fig. 4C, lower panel). These results indicate that all three components of the *de novo* methyltransferases are involved in the promoter methylation of *Dnmt3L* and its transcriptional repression during embryonic development, and Dnmt3b has a predominant contribution.

Hypomethylation of the *Dnmt3L* promoter in mouse ICF models

Deficiency in *DNMT3B* in human ICF patients cause hypomethylation on a number of target sequences including the classic satellite 2 repeats (27). Based on our findings, *DNMT3B* mutations might also affect the methylation of the *DNMT3L* promoter. To address this possibility, we analyzed the methylation status of *Dnmt3L* in mouse ICF models which recapitulate clinical phenotypes (28). Methylation analysis showed that in both brain and tail of ICF mice carrying missense mutations A609T (T/T) and D823G (G/G) in the catalytic domain of Dnmt3b (Fig. 5A), the methylation level of the *Dnmt3L* promoter was severely decreased compared with that of the WT mice (Fig. 5B and C). Quantification by bisulfite sequencing revealed that in the brain of mutant

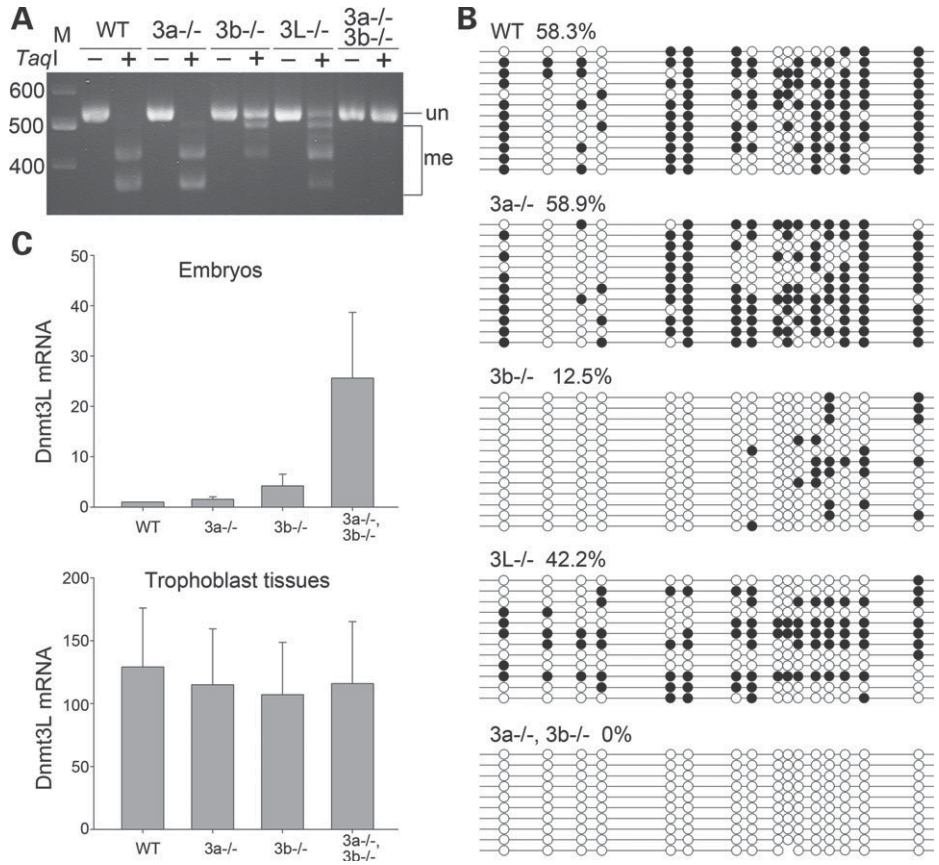

Figure 4. Promoter hypomethylation and increased mRNA levels of *Dnmt3L* in E9.5 mutant mouse embryos. (**A**) COBRA methylation analysis of the *Dnmt3*L promoter. The genotypes of the embryos are indicated on the top. Each DNA sample was obtained from two E9.5 embryos. (**B**) Bisulfite sequencing analysis. (**C**) Analysis of *Dnmt3L* mRNA by real-time RT–PCR. RNA samples of both embryonic and trophoblast tissues were isolated from wild-type ($n = 6$), Dnmt3a$^{-/-}$ ($n = 8$), Dnmt3b$^{-/-}$ ($n = 10$) and Dnmt3a$^{-/-}$/3b$^{-/-}$ ($n = 6$) embryos. The *Dnmt3L* mRNA levels were normalized against the amount of β-actin mRNA in each sample. The bars represent the means ± SD and results are shown as fold up-regulation relative to the level in the wild-type, which was set to 1.

mice, the promoter was only methylated to 34.7 and 50.0%, much lower than 73.6% in the WT. These results further ascertain the critical role of Dnmt3b in the methylation of the *Dnmt3L* promoter during mouse development, and suggest that the functional deficiency of Dnmt3b can not be fully compensated by Dnmt3a and Dnmt3L.

To examine the importance of DNMT3B in the regulation of *DNMT3L* in humans, we first mapped the human *DNMT3L* promoter (Fig. S4). A 250-bp promoter region was confirmed to be methylated upon differentiation of human ES cells *in vitro* (Fig. S5). The region was then analyzed by bisulfite sequencing in the DNA samples from three ICF patients. We found that the methylation levels of the *DNMT3L* promoter in patient leukocytes were modestly lower than in normal controls (Fig. S6).

Recruitment of Dnmt3b to the *Dnmt3L* promoter during ES cell differentiation

Having demonstrated the functional importance of Dnmt3b in the methylation of the *Dnmt3L* promoter in development, we next asked whether Dnmt3b becomes physically associated with the promoter in ES cells upon differentiation. Chromatin immunoprecipitation (ChIP) assay was thus performed in both proliferating (day 0) and differentiating ES cells (day 3). The *Dnmt3b*$^{-/-}$ ES cells aggregate and develop normally during EB differentiation and were used as a negative control in this experiment. Quantification of ChIP data revealed an 11-fold enrichment of Dnmt3b at the *Dnmt3L* promoter upon differentiation of WT ES cells, whereas no significant difference was detected in the *Dnmt3b*$^{-/-}$ ES cells (Fig. 6A). The specificity of the Dnmt3b antibody was validated because control ChIP with the equal amount of mouse IgG showed the background binding in all four samples.

DISCUSSION

During mammalian embryo development, DNA methylation undergoes dramatic reprogramming. Genome-wide demethylation occurs actively on the paternal genome shortly after fertilization and passively on the maternal genome through the subsequent cleavage divisions. Then a wave of *de novo* methylation begins in the inner cell mass probably from the late morula stage, and a considerable level of methylation might become re-established in the blastocyst (29–33). The reprogramming of genomic methylation patterns allows for the establishment of differential gene expression profiles required for lineage specification. For example, early embryonic genes

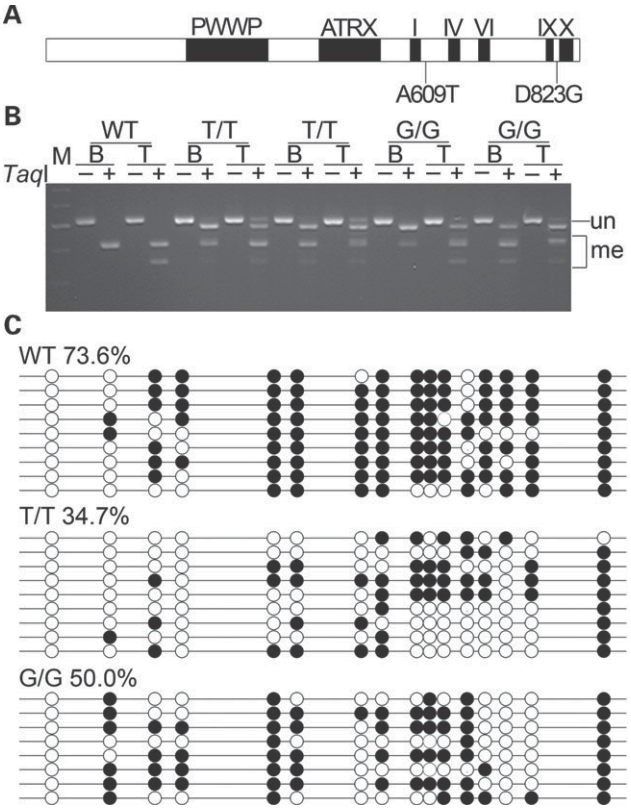

Figure 5. Hypomethylation of the *Dnmt3L* promoter in mouse ICF models. (**A**) Schematic diagram of the mouse Dnmt3b protein. The conserved PWWP and ATRX domains, the conserved motifs (I, IV, VI, IX and X) in the catalytic domain are indicated above. The two ICF mutations made into the mouse models (28) are indicated below. (**B**) COBRA methylation assay of the *Dnmt3L* promoter. Genomic DNA of brains (B) and tails (T) from one wild-type and two mutant mice for each ICF mutation was analyzed. T/T and G/G, the genotypes indicated on the top, represent homozygous A609T and D823G mutations. The DNA samples used were previously described (28). (**C**) Methylation profiles of the *Dnmt3L* promoter revealed by bisulfite sequencing analysis in brain samples. Each DNA sample was obtained from three embryos.

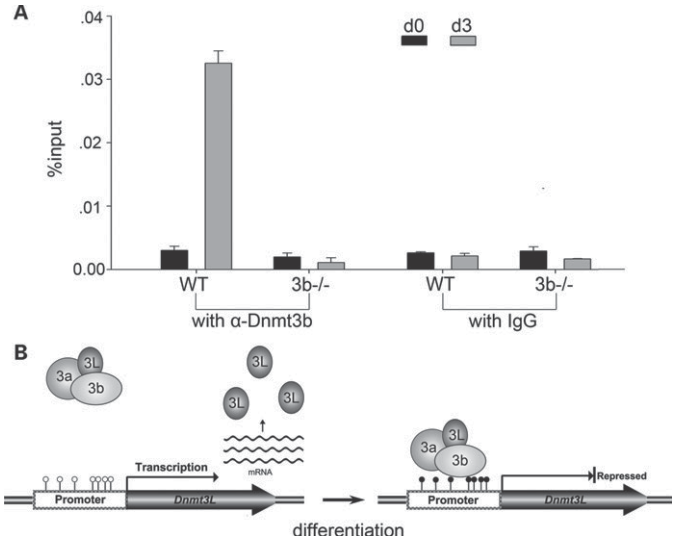

Figure 6. Recruitment of Dnmt3b to the *Dnmt3L* promoter in ES cells upon differentiation. (**A**) Chromatin immunoprecipitation assay in wild-type and *Dnmt3b*$^{-/-}$ ES cells before (day 0) and during (day 3) differentiation. The amount of DNA in immunoprecipitated chromatin was measured by real-time PCR using primers specific for the *Dnmt3L* promoter region. The percentage of signal intensity in immunoprecipitated sample over the input DNA is represented with the bar height. The bars represent means ± SD which were derived from three individual ChIP experiments. (**B**) Model of methylation-mediated auto-suppression of *Dnmt3L* expression. Mechanism controlling the access of Dnmt3 proteins to the promoter is unknown.

such as the pluripotency genes *Oct4* and *Nanog* undergo transcriptional silencing in differentiating cells (34). In the present study, we showed that *Dnmt3L*, a gene controlling DNA methylation, is subjected to epigenetic regulation too. Remarkably, *Dnmt3L* is highly expressed in the early epiblast, and then down-regulated in the embryo proper from mid-gastrulation onwards. The silencing process of *Dnmt3L* is closely correlated with and to a certain extent depends on *de novo* methylation of its promoter. DNA methylation at *Dnmt3L* starts at the onset of gastrulation in the epiblast (E6.5) and reaches a high level by mid-gastrulation (E7.5), a stage when the Dnmt3L protein is missing. All three known components (Dnmt3a, Dnmt3b and Dnmt3L) of the methylation machinery are involved in the process. Deficiency of either component compromises (but does not fully abolish) the methylation activity, resulting in incomplete silencing of *Dnmt3L* in the embryonic cells. Taken together, Dnmt3L, a regulator of *de novo* DNA methylation, is regulated at the transcriptional level by DNA methylation during the early post-implantation period. The mechanistic aspects and biological significance of methylation-mediated *Dnmt3L* silencing are discussed in more detail below.

Contribution of Dnmt3a, Dnmt3b and Dnmt3l in the transcriptional regulation of *Dnmt3L*

We have evaluated the contribution of Dnmt3 proteins in the DNA methylation and transcriptional regulation of *Dnmt3L* in differentiating mutant mouse ES cells *in vitro* and in post-implantation embryos null for *Dnmt3a*, *3b* and *3L*. We found that all the three Dnmt3 members participate in the methylation of the *Dnmt3L* promoter in both the ES cells and embryos. Great variations in the methylation and transcription levels of Dnmt3L were detected among the different single and double knockouts. This has allowed us to assess the contribution of each individual gene in the epigenetic regulation of *Dnmt3L*. We found that Dnmt3b and Dnmt3L have a much larger contribution than Dnmt3a. The level of promoter methylation levels show a nice correlation to the degree of transcriptional silencing in different knockout ES cells and embryos. The sensitive response of transcription to the change of DNA methylation implies that other epigenetic modifications such as histone methylation alone cannot replace the regulatory function of DNA methylation. A seemingly contradictory exception is found with the Dnmt3a-deficient cells and embryos. While having a marginal effect on the establishment of promoter methylation, disruption of *Dnmt3a* causes, respectively, 2.5 (day 5) and 1.5-fold up-regulation of *Dnmt3L* in the differentiating mutant ES cells and the E9.5 knockout embryos (Figs 3C and 4C). Therefore, Dnmt3a has a potential role in transcriptional repression of *Dnmt3L*, independent of its methylation function. This idea fits well with the previous observation that Dnmt3 members are able to mediate transcriptional repression through their

N-terminal PHD domains, which recruit histone deacetylase activities (35–37). Since all Dnmt3 members are present in the early post-implantation embryo (Fig. 1) and they form complexes by direct interaction in embryonic cells (34,38), we postulate that Dnmt3a might cooperate with Dnmt3b and Dnmt3L mainly as a transcription repressor at the *Dnmt3L* locus during development. This may account for a strong synergistic effect between the two methyltransferases, mostly obviously seen in experiments with knockout embryos: the up-regulation of *Dnmt3L* in the double knockout E9.5 embryos exceeds by 4.6-fold over the sum of that in the *Dnmt3a* and *Dnmt3b* single knockout embryo (Fig. 4C). The cooperation in the transcriptional repression of *Dnmt3L* in a methylation-independent fashion appears to be different from the situation of the epigenetic regulation of the *Oct4* and *Nanog* genes we reported previously (34). Dnmt3a and Dnmt3b show a clear synergistic effect both in the methylation of the *Oct4* and *Nanog* promoters and in their transcriptional silencing. Another previous report showed that *Dnmt3a* and *Dnmt3b* are required to maintain the methylation at a regulatory region of the oocyte-specific form of the *Dnmt1* gene in ES cells (7). It remains unresolved whether the maintenance methylation at the *Dnmt3L*, *Oct4* and *Nanog* promoters upon cell differentiation would also involve the two Dnmt3 enzymes.

Auto-repression of the methyltransferase activity by the promoter methylation of *Dnmt3L*

Though Dnmt3L lacks intrinsic methyltransferase activity, it is well established that it serves as a key regulator of DNA methylation. Besides its function in stimulating the activity of both Dnmt3a and Dnmt3b (20–22), more recent biochemical studies have shown that Dnmt3L has a potential targeting function in the determination of specific genomic loci to be methylated. Complex of Dnmt3L with Dnmt3a C-terminal domains might contribute to the recognition of the differentially methylated region of maternal imprinted genes (39). Dnmt3L can also bind via its N-terminal PHD domain specifically to unmethylated lysine 4 of H3, potentially mediating the recruitment of Dnmt3a enzymes to inactive chromatin regions (38). The dual ability of Dnmt3L in enzymatic stimulation and target-specific recruitment of Dnmt3a and 3b makes its expression a good regulatory point. Interestingly, different promoters have been reported to control the expression of sex-specific *Dnmt3L* isoforms in mouse germ cells (40).

Based on the regulatory function of the Dnmt3 members in the expression of *Dnmt3L* in mouse ES cells and embryos, we postulate an auto-repression mechanism for the control of the cellular methylation activity (Fig. 6B). If the *de novo* methyltransferase activity is excessive, the methylation machinery might feed back negatively to the Dnmt3L activator by promoter methylation. Conversely, if the methyltransferase activity is too low, for example, due to a point mutation in a methyltransferase, the negative feed-back system may allow overexpression or delayed silencing of *Dnmt3L*, thus resulting in a compensation of the methylation activity. The self-adjustment of the total methyltransferase activity might help establish precise genomic methylation patterns in development. This model does not exclude the importance of other epigenetic mechanisms in the transcriptional regulation of *Dnmt3L*. The high expression level of all Dnmt3 members in ES and epiblast cells indicates that unknown factor(s) protecting the *Dnmt3L* promoter from methylation and silencing remain yet to be identified.

The developmental significance of the regulation of Dnmt3L

At the commencement of gastrulation, most target sequences in the genome has been methylated in the wave of the genome-wide methylation beginning from the late morula (29,30,33). The subsequent development of embryo involves rapid cellular migration and differentiation by which the progenitors of different cell lineages are produced. This process might involve the methylation or demethylation of tissue-specific genes to generate various lineages. The dynamic expression of Dnmt3L in the early embryo may suggest a role of Dnmt3L in the *de novo* methylation of the genome. This view is apparently at odd with a lack of any somatic phenotype in the *Dnmt3L* knockout mice (15,18). However, the *Dnmt3L* knockout mice which were phenotypically normal were invariably derived from a heterozygous mother and a heterozygous father. The heterozygous mother could have deposited Dnmt3L in the oocytes and this deposit could support the early development of the homozygous embryo. Indeed, *Dnmt3L* is actively transcribed in the growing oocyte (18). Therefore, the characterization of a Dnmt3L embryonic function requires a careful phenotype analysis and genome-wide methylation profiling in the mutant embryos obtained with a new cross (*Dnmt3L* homozygous mother × *Dnmt3L* heterozygous father).

Dnmt3L, as a general activator of *de novo* methyltransferases, might have to be down-regulated after the early genome-wide *de novo* methylation has completed. If the expression of *Dnmt3L* would persist, further differentiation of the epiblast and germ layers might be disturbed due to excessive methylation. In fact, in our study of the *Dnmt3L* transgenic mice, we found that the expression of an extremely low level of *Dnmt3L* might be incompatible with normal development (unpublished data). Excessive methylation activity in the case of forced expression of transgenic Dnmt3b in post-natal mouse led to abnormal methylation and neoplasia (41). A recent survey for cervix cancer suggests that up-regulation of *DNMT3L* due to promoter hypomethylation might contribute to tumorigenesis in human (42).

The ICF syndrome is the only human genetic disease known to arise from germline mutations within a DNA methyltransferase gene—*DNMT3B*. Like most other *DNMT3B* mutations, the two mutations (A609T and D823G) used in the mouse models affect the catalytic domain and thus attenuate the enzymatic activity (27,28). Both mutant proteins also display an altered nuclear subcellular localization, with less accumulation at the pericentric heterochromatin (28). Since the *Dnmt3L* promoter is hypomethylated in the mouse models of the human ICF syndrome, we speculate that dysregulation of *DNMT3L* might also occur during ICF fetal development and thus influence the phenotype formation. A modest decrease in the methylation of a *DNMT3L* promoter region was indeed detected in the leukocytes from three ICF patients (Fig. S6).

The less-severe hypomethylation in the human ICF might be explained by the following aspects. First, the human ICF samples used were leukocytes, while tail and brain DNA were analyzed for the mouse model. Second, ICF probands show great phenotypic variation. ICF mothers have a lot of spontaneous abortion. The born ICF fetuses and long-surviving patients have less-severe phenotypes in general. The patients we analyzed were born and are probably the ones that were 'more' viable. Therefore, samples from aborted ICF fetuses would be more appropriate for examining hypomethylation at the *DNMT3L* promoter.

Up-regulation of DNMT3L due to hypomethylation during embryonic development could partially compensate the deficiency in methylation activity caused by *DNMT3B* mutations, ensuring proper methylation at most genomic loci. This would mitigate the adverse effect of the DNMT3B deficiency. This idea is consistent with the finding that hypomethylation in ICF patients is restricted to a small portion of the genome (27,43). On the other hand, over-activation of DNMT3A or 3B by the up-regulated DNMT3L could lead to abnormal methylation of otherwise unmethylated sequences as most ICF mutant proteins retain a considerable amount of catalytic activity (14). Assessment of this possibility would require thorough genome-wide methylation profiling of ICF cells.

MATERIALS AND METHODS

Antibodies and immunofluorescence

The polyclonal anti-Dnmt3a (full-length) and anti-Dnmt3b (full-length) antibodies and monoclonal anti-Dnmt3L have been described previously (14,44). Immunostaining of embryo cross-sections was performed as previously described (12), and the images were captured with a confocal microscope (Leica TCS SP2 AOBS).

Mice and embryos

CD-1 mice were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences). Dnmt3 knockout mice were described previously (6,18). Mice of ICF models were described previously (28). Noon of the day on which the vaginal plug was seen was designated as E0.5. The mouse epiblasts and ExEss were isolated under a Nikon SMZ-1500 stereomicroscope, washed three times in M2 medium, and stored at -70°C until use.

Bisulfite methylation analysis

Bisulfite treatment of genomic DNA was carried out as previously described (45). Nested PCR was performed to amplify the *Dnmt3L* promoter region with the following primers: outside forward, ATT TTA ATG TGT GAG GTT TAG AGT TTT T; outside reverse, ACC TAA AAA TCT CAC AAA ATT TCA AC; inside forward, GTT TTG AGT TTT ATA GAA TTT TAT AAT TTT T; inside reverse, AAA AAC TAT CAA CAT CAA AAC TAA AAC. The annealing temperature was 57°C (with 1.5 mM Mg^{2+}) for the first round of PCR and 59°C (with 3 mM Mg^{2+}) for the second round. For COBRA analysis, 5 μl of the PCR products were restricted with *Taq*I (Takara) followed by electrophoresis in 3% agarose gel. For sequencing analysis, the PCR products were cloned into T-vectors (Takara Inc.), and individual clones were sequenced by Invitrogen Ltd, Shanghai.

ES cell culture and *in vitro* differentiation

WT and mutant ES cells were maintained as preciously described (46). To induce differentiation, ES cells were cultured for two days by the hanging drop method (700 ES cells per 20 μl in each drop) (47). EBs in hanging drops were then transferred to 100 mm Petri dishes and cultured in suspension for various days.

Quantitative RT–PCR

Total RNA was isolated from ES cells or tissues with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse-transcription was performed using the reverse transcription system (Promega Ltd) with oligonucleotide dT primer. The quantification of transcripts was performed by real-time PCR on a Realplex 2S cycler (Eppendorf Ltd) using Eva Green fluorescent dye (Biotium Inc.) according to the standard curve method (48). Expression values were normalized to that of β-actin. The PCR primers for *Dnmt3L* were as described (13) and the mouse β-actin primers were as follows: forward, TTC CTT CTT GGG TAT GGA AT; reverse, GAG CAA TGA TCT TGA TCT TC. Annealing temperatures for both reactions were 60°C.

Chromatin immunoprecipitation

The ChIP assay was carried out largely as described (49). Briefly, trypsin dispersed cells were treated with 1.42% formaldehyde for 15 min at room temperature, followed by incubation with a 0.125 M glycine solution for 5 min. Nuclei were then extracted and incubated in nuclear lysis buffer for 20 min. The genomic DNA was sheared to an average size of 500 bp with a Diagenode BioRuptor™ sonicator [16 times for ES cells and 14 times for EB cells (30 s/pulse with 1 min rest interval)]. The sheared extracts were diluted 10-fold by ChIP dilution buffer, precleared with salmon sperm DNA, BSA and protein A/G agarose beads and incubated with 3 μg antibody (monoclonal anti-Dnmt3b or mouse IgG) at 4°C overnight. Blocked protein A/G agarose beads (50 μl, 50% slurry) were then added and rotated at 4°C for 2 h followed by centrifugation. The supernatant from the control precipitation was used as input. The beads were washed and bound DNA was eluted with elution buffer (1% SDS, and 100 mM $NaHCO_3$). The eluates were then de-cross-linked and treated with proteinase K for DNA purification. The immunoprecipitated DNA was analyzed by real-time PCR with primers specific for the *Dnmt3L* promoter (forward, CCT CAT AGG CTC CAT CCA GCA T; reverse, CAG GGT CGT CAG AAC CCT AAA ACG; annealing temperature, 65°C). To process the ChIP data, the percentage of immunoprecipitated DNA over the input was calculated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Dr F. Tang for critic review of the manuscript.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from the Max- Planck-Gesellschaft of Germany and the Ministry of Science and Technology of China (2005CB522400 and 2007CB947503) and the National Science Foundation of China (30730059) to G.L.X.

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