Mutations in DNA methyltransferase DNMT3B in ICF syndrome affect its regulation by DNMT3L

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Deficiency in DNA methyltransferase DNMT3B causes a recessive human disorder characterized by immunodeficiency, centromeric instability and facial anomalies (ICF) in association with defects in genomic methylation. The majority of ICF mutations are single amino acid substitutions in the conserved catalytic domain of DNMT3B, which are believed to impair its enzymatic activity directly. The establishment of intact genomic methylation patterns in development requires a fine regulation of the de novo methylation activity of the two related methyltransferases DNMT3A and DNMT3B by regulatory factors including DNMT3L which has a stimulatory effect. Here, we show that two DNMT3B mutant proteins with ICF-causing substitution (A766P and R840Q) displayed a methylation activity similar to the wild-type enzyme both in vitro and in vivo. However, their stimulation by DNMT3L was severely compromised due to deficient protein interaction. Our findings suggest that methylation defects in ICF syndrome may also result from impaired stimulation of DNMT3B activity by DNMT3L or other unknown regulatory factors as well as from a weakened basal catalytic activity of the mutant DNMT3B protein per se.

INTRODUCTION

Cytosine methylation in DNA serves as an epigenetic mark indispensable for the regulation of genome integrity and functions involved in a variety of cellular processes including genomic imprinting, inactivation of the X chromosome, repression of transposons and control of tissue-specific gene expression (1,2). Aberrant DNA methylation is an underlying mechanism for diseases such as cancer and several congenital disorders with mental retardation (3,4).

Methylycytosines account for 5% of the total cytosines in mammalian genomes and display stable patterns in any given type of cell. The DNA methylation patterns are perpetuated through somatic divisions by the maintenance methyltransferase Dnmt1 that acts in concert with DNA replication. Reprogramming of DNA methylation patterns is required for the progression of gametogenesis and early embryonic development. The generation of new methylation patterns depends on the de novo methylation processes driven by methyltransferases, Dnmt3a and 3b (5,6). Dnmt3a and Dnmt3b have a C-terminal catalytic domain fused to a long N-terminal region with regulatory functions (6). Their N-terminal regions contain a PWWP domain with chromatin-targeting functions (7,8) and a cysteine-rich PHD zinc finger domain that may mediate methylation-independent transcriptional repression through interaction with the transcriptional repressor RP58, heterochromatin protein HP1B, histone deacetylases and histone methyltransferase SUV39H1 (9–12). The C-terminal domains of Dnmt3a and 3b are catalytically active on their own in vitro (13). Disruption of either or
both genes in mice led to failure in the establishment of genomic methylation patterns in embryonic development and caused lethal phenotypes (14).

In humans, mutations in the DNMT3B gene are responsible for the recessive syndrome characterized by a variable phenotype consisting of immunodeficiency, centromeric heterochromatin instability and facial anomalies (ICF) (reviewed in 15,16). The cytogenetic instability is virtually confined to the pericentromeric region of, in particular, chromosomes 1, 9 and 16. At the molecular level, sequences such as the pericentromeric classic satellite repeats of pericentromeric regions are hypomethylated in patient DNA (17). The methylation defects have been ascribed to reduced enzymatic activity of the mutant proteins (13,18). Accordingly, most mutations identified to date fall in the catalytic domain (19,20).

Dnmt3L, the third member of Dnmt3 family methyltransferases, is related to Dnmt3a and Dnmt3b but lacks the conserved catalytic motifs characteristic of the cytosine methyltransferases. Like Dnmt3a and Dnmt3b, Dnmt3L is highly expressed in germ and early embryonic cells that undergo dramatic DNA methylation reprogramming. Mouse gene targeting experiments established the role of Dnmt3L as a positive regulator required for the establishment of DNA methylation marks at imprinted and repeated sequences in germ cell development (21–23). The methylation activity of Dnmt3a and Dnmt3b in vivo is significantly stimulated in cultured human cells by co-expression of Dnmt3L which is inactive itself (24,25). The stimulation by Dnmt3L has also been demonstrated for both Dnmt3a and Dnmt3b with purified recombinant proteins (26,27). The binding of Dnmt3L presumably induces a conformational change of Dnmt3a, which increases the affinity for DNA and the methyl group donor S-adenosylmethionine (27). Although the interaction with Dnmt3L involves the catalytic domain of the de novo methyltransferases (25–27), the effect of the mutations found in ICF patients on the regulation of methylation activity has not been investigated.

In this paper, we characterize the physical and functional interactions between Dnmt3b and Dnmt3L proteins both in vitro and in vivo. Two ICF mutations in the Dnmt3b catalytic domain do not show any reduction in enzymatic activity, but show much weakened stimulation by Dnmt3L. This observation suggests that the ICF syndrome is not necessarily caused by a change of basal enzymatic activity of the mutant methyltransferase itself. Disruption of the interaction with regulatory factors can also affect the methylation function. Our finding therefore provides a new molecular etiology for ICF syndrome and underscores the importance of the regulation of DNA methylation activity in development and pathogenesis.

RESULTS

Dnmt3b associates physically and functionally with Dnmt3L via the catalytic domain

There is growing evidence that Dnmt3L co-operates with Dnmt3a and Dnmt3b in de novo methylation (23,24,28). Dnmt3b displays similar expression patterns at several germ cell and early embryonal developmental stages as Dnmt3a and Dnmt3L (14,23). A plausible physical and functional interaction between Dnmt3b and Dnmt3L was investigated here.

We first performed co-immunoprecipitation with extracts from murine ES cells which express Dnmt3L and Dnmt3b. Dnmt3b was detected in the ES immunoprecipitate obtained from murine ES cells which express Dnmt3L and Dnmt3b. We first performed co-immunoprecipitation with extracts from murine ES cells which express Dnmt3L and Dnmt3b. Dnmt3b was detected in the ES immunoprecipitate obtained from murine ES cells which express Dnmt3L and Dnmt3b.
with non-specific rabbit IgG (Fig. 1B, lane 2) validates the specificity of the assay. A splicing variant of Dnmt3b present in ES cells, Dnmt3b6 (29), was missing in the immunocomplexes (Fig. 1B, compare lanes 1 and 3). Dnmt3b6 skips a region of 64 amino acids from the catalytic domain at the C-terminus and thus may be enzymatically inactive (29).

In agreement with previous results (25–27), the absence of Dnmt3b6 isoform in the anti-Dnmt3L immunoprecipitate implied that the C-terminal catalytic domain might be necessary for the interaction with Dnmt3L. We then performed a glutathione S-transferase (GST) pull-down assay to identify the interaction region in Dnmt3b. Purified GST–Dnmt3L fusion protein was incubated with in vitro translated (IVT) Dnmt3b fragments. The full-length and C-terminal (D3b-C) fragments of Dnmt3b but not the N-terminal fragment (D3b-N, amino acids 1–550) could be pulled down by GST–D3L (Fig. 1A and C, lane 3). GST alone could not pull down any protein (lane 2). Our data confirm that the C-terminal MTase domain of Dnmt3b is responsible for the interaction with Dnmt3L.

We then performed an in vivo analysis of methylation activity in cultured human cells for functional characterization of the Dnmt3b–Dnmt3L interaction. The assay plasmid p220.2 was transfected into 293 c18 cells, together with a series of Dnmt3b and Dnmt3L expression constructs. We used a probe complementary to the EBNA1 region of p220.2 in Southern hybridization to assess the methylation level of the recovered assay plasmid. Three HhaI fragments of 1707, 876 and 738 bp would arise from the complete digestion when no methylation occurred; fragments of increased sizes would reflect de novo methylation which blocked HhaI cleavage (Fig. 1D). The fraction of HhaI fragments of larger size increased when the full-length or catalytic domain

Figure 2. Subcellular colocalization of Dnmt3b and Dnmt3L. (A) Nuclear targeting of FLAG-Dnmt3L by HA-Dnmt3b in NIH 3T3 cells. Fluorescence immunostaining of cells transfected with various constructs (indicated on the left) was carried out using anti-HA (red) and anti-FLAG (green) antibodies. Nuclear territory was defined by DAPI staining (blue) with heterochromatin domains stained more intensely. Scale bar: 5 μm. (B) Co-localization of endogenous Dnmt3b and Dnmt3L on chromosomes in ES cells. Chromosome spreads prepared from ES cells were co-immunostained with antibodies against Dnmt3b (red) and Dnmt3L (green). The murine chromosomes were also counterstained by DAPI (blue) with prominent staining at the centromeres. Scale bar: 2 μm. (C) Chromosome targeting of Dnmt3L by Dnmt3b. Chromosome spreads of human HEK 293T cells transfected with HA-Dnmt3b and FLAG-Dnmt3L expression constructs indicated on the left were subjected to indirect immunofluorescence staining using antibodies against HA (red) and FLAG (green). Scale bar: 2 μm. See Supplementary Material for a colour version of this figure.
of Dnmt3b was expressed together with Dnmt3L or its C-terminal domain (Fig. 1D, lanes 2, 4, 6 and 8). This result shows the dependence on their respective C-terminal domains for the stimulation of methylation activity in vivo. The methylation activity of the Dnmt3b catalytic domain was much lower than the full-length Dnmt3b (Fig. 1D, compare lane 1 with 5) but readily detectable, especially when co-expressed with Dnmt3L (lane 6). The stimulation effect of Dnmt3L was reduced but readily detectable when only the C-terminal domains from the respective proteins were used (compare lanes 6 and 8). These observations are consistent with the notion that the N-terminal half of both Dnmt3b and Dnmt3L might play a role for the in vivo functions of these proteins; for example, for targeting the enzyme to the DNA substrate existing in chromatin (7,8).

Dnmt3b determines the subcellular localization of Dnmt3b–Dnmt3L complex

To further explore the functional relationship between Dnmt3b and Dnmt3L proteins, we examined the formation and subcellular distribution of the complex in cultured cells expressing either transfected or endogenous proteins by indirect fluorescence immunostaining. In transfected NIH 3T3 cells, the exogenous hemagglutinin (HA)-tagged Dnmt3b was localized in the nucleus, whereas FLAG-tagged Dnmt3L was distributed in the whole cell (Fig. 2A). Co-expression of the two proteins led to the nuclear localization of Dnmt3L. The cytoplasmic distribution of Dnmt3L did not change when truncated fragments of either protein containing only an N-terminal part were expressed. On the contrary, Dnmt3L nuclear targeting could happen when both proteins had only the C-terminal domain (Fig. 2A, bottom row). This domain in the respective proteins is therefore both necessary and sufficient for the Dnmt3L nuclear localization.

Dnmt3b displays a specific staining pattern of speckles on metaphase chromosomes in cells expressing the transfected or endogenous protein (7). We found that Dnmt3L in murine ES cells is localized on metaphase chromosomes as well (Fig. 2B). An identical pattern of enhanced staining at particular loci was revealed in the double staining for the two proteins. To ascertain the role of Dnmt3b in this targeting event, we performed immunostaining of chromosome spreads prepared from 293T cells transfected with either individual or both proteins. Chromosome association of Dnmt3L was detected when it was expressed together with Dnmt3b (Fig. 2C). Dnmt3L alone failed to bind to the metaphase chromosomes. This observation indicates that Dnmt3L can be recruited to chromosomes by Dnmt3b.

ICF mutations in the Dnmt3b catalytic domain affect the capacity to be stimulated by Dnmt3L in vivo and in vitro

The above results showed that Dnmt3L can interact with and stimulate the catalytic activity of Dnmt3b. We surmised that ICF mutations in the Dnmt3b catalytic domain might affect the functional interaction with Dnmt3L. To test this possibility, we introduced eight ICF-causing mutations identified in the catalytic domain of the human DNMT3B gene into the mouse Dnmt3b expression construct and analyzed their methyltransferase activities in vivo in the presence and absence of Dnmt3L. The Dnmt3b variants with or without Dnmt3L were co-transfected into 293 c18 cells with the assay plasmid p220.2. The methylation survey showed that six of the eight ICF mutations examined reduced the methylation activity and the enzymatic activity was elevated in the presence of Dnmt3L, albeit to different degrees (Fig. 3B). To our surprise, two mutations, A772P and R846Q (equivalent of A766P and R840Q in the human DNMT3B), showed catalytic activities close to the wild-type enzyme, but the rate of stimulation by Dnmt3L was much reduced. Western detection of the proteins expressed in the transfected 293 c18 cells allowed us to rule out the possibility that the major difference in methylation activity was caused by variations in protein expression levels (Fig. 3B, lower panels).

The evaluation of the de novo methyltransferase activity of Dnmt3b mutant proteins in living cells could be complicated by multiple factors, such as the stability of proteins in 293 c18 cells over the prolonged period of culturing upon transfection, subcellular localization and substrate accessibility, chromatin structure of the co-transfected assay plasmid, site preference on the assay plasmid and potential interactions with other proteins. It is, therefore, important to confirm the effect of these mutations with purified proteins in vitro. To this end, we expressed and purified recombinant Dnmt3L and nine Dnmt3b proteins with or without ICF mutations (Fig. 4A). The in vitro methylation activity was measured by scintillation-counting of the incorporation of 3H-labeled methyl group into a biotinylated PCR fragment using the avidin microplate method. The methylation activity of six mutants (V612A, H820R, G669S, L670T, V732G and R829G) was close to the background and stimulation by Dnmt3L did not bring methylation level over 30% of the activity of the wild-type enzyme (Fig. 4B). On the contrary, mutants A772P and R846Q showed similar methylation activity as the wild-type, but the ratio of stimulation by Dnmt3L was significantly lower (1.9- and 1.5-fold for the mutants versus 3.4-fold for the WT enzyme). The reduction in the level of stimulation of these two mutants remained constant with varying concentrations of the Dnmt3L protein (Fig. 4C).

As there might be species-specific difference in protein function, we also performed the in vivo and in vitro methylation assays using the human DNMT3B and DNM3L proteins. As shown in Supplementary Material, Figure S2, the two ICF mutant proteins carrying mutations outside of the catalytic center A766P and R840Q exhibited full activity both in vivo and in vitro, whereas the other two mutants V606A and H814R had almost no activity. Further, the degree of stimulation by DNM3L was markedly reduced by A766P and R840Q mutations. These results confirm that the distinct properties of these two mutations observed in the mouse Dnmt3b protein could be extended to the specific ICF mutations in humans.

ICF mutations of Dnmt3b disturb the interactions with Dnmt3L

The observation that the catalytic domain of Dnmt3b interacts with Dnmt3L, and most of the ICF missense mutations lie in this domain, prompted us to test the effect of the mutations
on the protein–protein interaction with Dnmt3L. To this end, we assessed the interaction strength for each mutant by the mammalian two-hybrid assay which is based on the transcriptional activation of a luciferase reporter gene. The N-terminal regions of Dnmt3b and Dnmt3L were removed because the presence of the N-terminal domain invalidated the assay due to the strong transcriptional repression activity of the PHD domain (9). The C-terminal regions of Dnmt3b and Dnmt3L fused, respectively, to the GAL4-DNA binding domain and VP16 activation domain, were co-expressed in 293 c18 cells in the presence of a luciferase reporter containing five GAL4-binding sites. As shown in Figure 5A, the luciferase activity for five mutants, including the two catalytically active mutants A772P and R846Q, dropped below 37% of the wild-type value, indicating a severely compromised interaction with Dnmt3L. In contrast, mutants G669S and R829G had similar interaction strength and V612A retained 69% of the activity in comparison to the wild-type.

To further confirm the defect in protein–protein interaction seen for the two catalytically active mutants A772P and R846Q, we performed immunofluorescence co-staining of 3T3 cells co-transfected with the HA-tagged full-length Dnmt3b and FLAG-tagged Dnmt3L. As shown in Figure 5B, a significant fraction of the Dnmt3L protein was left in the cytoplasm when co-expressed with mutants A772P and R846Q, whereas the wild-type Dnmt3b recruited Dnmt3L almost exclusively into the nucleus. This observation suggests a reduced ability for A772P and R846Q to interact with Dnmt3L. These results, together with the data from the mammalian two-hybrid assays, indicate that mutations in the Dnmt3b catalytic domain can also have impact on its interaction with Dnmt3L.

**Dnmt3b and Dnmt3L proteins are co-expressed in early post-implantation mouse embryos**

Co-operation of Dnmt3b and Dnmt3L in genomic methylation in animal development requires simultaneous expression of the two proteins in early embryonic cells undergoing methylation reprogramming. To address this requirement, we analyzed the expression patterns of the two proteins in mouse post-implantation embryos by immunohistochemistry using affinity-purified antibodies specific for Dnmt3b and Dnmt3L. At E5.5, both Dnmt3b and Dnmt3L are expressed highly in epiblast. Weaker expression of these two proteins in visceral endoderm and extra-embryonic ectoderm could also be detected. No staining was found in parietal endoderm and deciduum (Fig. 6Aa, b, g and h). Lack of expression in deciduum is expected because there should be no de novo methylation activity existing in this somatic tissue of maternal origin.
This result indicates the co-expression of Dnmt3b and Dnmt3L in the pregastrulation embryo.

At the advanced egg cylinder stage of E6.5 embryos, Dnmt3b retains strong expression in the epiblast and weaker expression in visceral endoderm and extra-embryonic ectoderm, with no expression in parietal endoderm (Fig. 6Ac and d). Dnmt3L expression seems rapidly down-regulated with the onset of gastrulation but weak expression is still apparent in extra-embryonic ectoderm and proximal epiblast. Unlike Dnmt3b, Dnmt3L does not seem to be expressed in visceral endoderm and parietal endoderm (Fig. 6Ai and j).

DISCUSSION

In this study, we have investigated the interaction between Dnmt3b and Dnmt3L and how this is affected by ICF mutations (A772P and R846Q) in the Dnmt3b catalytic domain reduce its capacity to be stimulated by Dnmt3L in vitro.
mutations in relation to the regulation of DNA methyltransferase activity using in vivo and in vitro assays. It is a general view that mutations in the catalytic domain of DNMT3B impair its methyltransfer ability, thereby causing ICF syndrome. However, here we found that two ICF-causing mutations in Dnmt3b do not impair its catalytic activity but rather impair the stimulation of this activity by Dnmt3L. Taken together, our results suggest an important role for Dnmt3b–Dnmt3L interactions in the regulation of DNA methylation during mammalian development. Disruption of the interaction could have a pathological consequence due to dysregulated DNA methylation activity.

Structural and functional roles of amino acids of DNMT3B altered in ICF patients

For better understanding the molecular basis of the etiology of the ICF syndrome, we examined the possible functional significance of the mutated residues in the homology model of the catalytic domain of human DNMT3B (Supplementary Material, Fig. S1), which was built based on the bacterial M. Hhal methyltransferase (30). Although human DNMT3B and M. Hhal methyltransferase are distantly related, they share high sequence similarity in the 10 conserved motifs which form the binding sites for the S-adenosyl-L-methionine (AdoMet) cofactor and target cytosine. In particular, most of the catalytically important amino acids involved in AdoMet binding and catalysis occupy the corresponding spatial positions. Biochemical data have suggested that Dnmt3b adopts two conformations regarding the state of the DNA-binding cleft (27). A closed conformation is favored by the free enzyme, but upon binding of Dnmt3L, the free enzyme undergoes a substantial conformational change so that the cleft becomes open, allowing the binding of DNA and AdoMet. Dnmt3L promotes the conformational transition from a closed state to an open state compatible for the rapid formation of a ternary Dnmt3b–AdoMet–DNA complex to initiate the enzymatic process.

Inspection of the two catalytically competent mutations (A766P and R840Q) in the DNMT3B model revealed that both of them would have no effect on the conformational transition nor might they interfere with AdoMet binding and catalysis (Supplementary Material, Fig. S1). A766 is situated in the TRD loop of the smaller subdomain, which is far...
from the catalytic center. Although mutation of alanine to proline may cause local structural variation with increased rigidity, it is unlikely to have impact on the overall conformation owing to the intrinsic flexibility of this loop. Thus, this residue is unlikely to be involved in DNA binding or catalysis. Instead, as it is positioned on the surface of the smaller subdomain, A766 might play a role as part of the interface in the protein–protein interaction with DNMT3L. Substitution by proline would thus perturb the stimulation by DNMT3L as we observed. R840 is located in the α-helix close to the C-terminal end in the large domain. Similar to A766, this residue also lies on the surface and is located distantly from the catalytic center. Mutation of this residue to glutamine would not affect the structure of the α-helix which does not depend on the side chain of an amino acid, but would cause a change in the local electrostatic property due to the replacement of the positively charged arginine. In summary, both A766 and R840 could be part of the interface to interact with DNMT3L which would explain why their mutations impair the interaction of DNMT3B and DNMT3L, but have no effect on the catalytic activity of DNMT3B itself.

**Differential catalytic activities of DNMT3B mutants and their response to DNMT3L stimulation may account for the phenotype variability in ICF syndrome**

The stimulation of Dnmt3b methyltransferase activity by Dnmt3L dropped from 3.4 to 1.5–1.9-fold due to the two ICF mutations. Such an in vitro biochemical difference may reflect differential enzyme properties that can determine the manifestation of the disease in ICF patients because the difference was only measured in vitro with recombinant proteins using a DNA fragment as a substrate. Methylation in vivo is expected to involve additional factors such as post-translational modification of Dnmt3b (e.g. sumoylation), interaction with other proteins including HP1 and chromatin remodeling factors and the regulation of subcellular localization of the Dnmt3b complex. For example, the mutational effect of A766P and R840Q could be aggravated through the reduction of DNMT3L concentration in the nucleus. Indeed, weakened interaction of mutant Dnmt3b with Dnmt3l also impairs the recruitment of Dnmt3l into the nucleus (Fig. 5B). The amplification of a mutational effect in vivo is evident in transfected cultured cells: A772P (human A766P) and R846Q (human R840Q) mutants showed no stimulation of methylation by Dnmt3l (DNMT3L) (Fig. 3 and Supplementary Material, Fig. S2) despite the 1.5–1.9-fold stimulation observed for these two mutants in vitro.

Although defective genomic methylation, facial dysmorphism and immunodeficiency are common features for the ICF syndrome, there are large molecular and clinical variations. For example, genes on the inactive X chromosome are demethylated only in certain patients (31) and mental retardation affects most but not all patients (32,33). A group of patients lacking mutations in DNMT3B have recently been categorized as a subtype of the ICF syndrome (type 2) characterized by demethylation of centromeric alpha-satellite repeats (34), partially explaining the phenotypic heterogeneity of the disease. Our biochemical characterization of mutations from type 1 patients revealed a wide spectrum of Dnmt3b mutants associated with various levels of residual enzymatic activity and their differing response to Dnmt3l stimulation. At one end of the spectrum are mutations A772P and R846Q that are fully active but are deficient in their stimulation by Dnmt3l. At the other end are mutations G669S, L670T and H820R with negligible enzymatic activity irrespective of Dnmt3l stimulation. The mutation V732G retained considerable activity but was not fully stimulated by Dnmt3l. These observations lead us to suggest that differences in biochemical features of DNMT3B mutants may contribute to variability in the pathogenesis of ICF syndrome, thus providing an explanation for the phenotypic heterogeneity among the subtype 1 patients. In support of this suggestion, patients with mutations A766P, R840Q and S270P had no apparent mental retardation (20,33). S270P is the only missense mutation found outside of the catalytic domain of DNMT3B. The fact that all these three variants are fully active (this work and data not shown) implies that the severity of the condition is inversely related to the level of residual methylation activity of DNMT3B. A refinement of genotype–phenotype correlation awaits more patients to be diagnosed and characterized both at the clinical and molecular levels.

**Potential role of DNMT3B–DNMT3L interaction in embryo development**

Biological requirements for a fine control of de novo methylation activity can be understood in light of (i) only 5% of cytosines in the mammalian genome are methylated and the generation of specific differential methylation patterns is important for cell lineage diversification, (ii) de novo methylation occurs in specific cell lineages at particular developmental stages, (iii) massive global methylation of the undermethylated genome is accomplished by the de novo enzymes with weak enzymatic activity compared with the maintenance methyltransferase Dnmt1.

Control of methylation activity can be realized by virtue of co-ordinated expression of methyltransferases and their regulators in development (35). There is an absolute requirement for the positive regulator Dnmt3l for de novo methylation of imprinted genes and repeat sequences in the male and female germ cells (21–23,28,36). Our observation of co-expression of the Dnmt3b and Dnmt3l proteins in post-implantation embryos is consistent with the idea that Dnmt3l may function as a stimulatory factor in the de novo methylation of the somatic embryonic genome as well. Given the interaction properties and co-existence of the two Dnmt3 proteins in mouse embryo cells, Dnmt3l might contribute to this somatic de novo methylation but its function may not be essential, as no apparent DNA methylation abnormality was detected in mice homozygous for Dnmt3l disruption (22,23). In particular, disruption of Dnmt3l did not appear to affect the methylation on minor satellite repeats (Supplementary Material, Fig. S3) which were found to be demethylated in Dnmt3b deficient mice (14). The lack of methylation defects in Dnmt3l−/− mice may be interpreted as a result of the persistent expression of a less-active Dnmt3b in gastrulation embryos (as we observed at E7.5) which may function to compensate for the missing stimulation
by Dnmt3L in the wave of global somatic de novo methylation. Thus, an interspecies difference in the dependence on Dnmt3L participation may exist for the establishment of methylation patterns. In this connection, the human DNMT3L was interestingly only detected in zygotes and blastocysts but absent in oocytes (37), where the function of the mouse counterpart in the methylation of imprinted genes was first established (22,23). The expression pattern of human DNMT3L resembles that in rhesus monkey (38), suggesting a function restricted at embryonic stages in primates.

Another plausible explanation for the lack of major methylation defects in Dnmt3L−/− mice is the possible leaky expression of the intact C-terminal region of Dnmt3L in mice in which only some exons encoding the N-terminal domain was targeted (22,23,36). However, this does not seem the case as northern blotting using a full-length cDNA probe did not detect transcripts potentially arising from leaky expression from the IRES-βgeo allele used for producing homozygous mice, though it detected a weak transcript of smaller size from the hygromycin allele in targeted ES cells (23). The expressed C-terminal domain could well stimulate Dnmt3b enzymatic activity in post-implantation embryos, as the N-terminal domain is not required for this effect. Therefore, a potential role of Dnmt3L in DNA methylation reprogramming in other developmental processes besides gametogenesis might need to be re-examined.

Our inference that the co-operation of DNMT3L might be required for DNA methylation in human embryos is based on the observation that ICF patients with catalytically competent mutant forms of DNMT3B lacking DNMT3L-interacting ability nevertheless display similar genomic methylation defects to other patients. One way to test the regulatory role of DNMT3L during embryonic development would be to carry out a detailed examination of genomic methylation in mouse embryos confirmed to be deficient in Dnmt3L. Delayed completion of embryonic methylation by Dnmt3b and/or defective somatic methylation may be expected due to a lack of Dnmt3L stimulation.

MATERIALS AND METHODS

Plasmids

Various regions of Dnmt3b were cloned into a mammalian expression vector, pcDNA3, to generate HA-tagged fusion proteins. Mutations in mouse Dnmt3b and human DNMT3B were introduced by PCR. The Myc-tagged DNMT3B mammalian expression construct was described elsewhere (25). Mutant DNMT3B were cloned into a mammalian expression vector, pcDNA3/Myc, to generate Myc-tagged fusion proteins. For expression and purification of 6 × His-tagged proteins, the Dnmt3b and DNMT3B coding sequences were cloned into pET28a (Novagen). Regions of Dnmt3L were cloned into expression vector, pCMV-FLAG-2 (Sigma), to generate FLAG-tagged proteins. For expression of GST fusion, mouse Dnmt3L cDNA was cloned into pET41b (Novagen) and human DNMT3L was cloned into pGEX-5X-1 (Amerham Biosciences). We verified all PCR-cloned constructs by DNA sequencing.

Mice, cell lines and transfections

Dnmt3L-deficient mice and ES cell lines were described elsewhere (14,23). Cultures of HEK 293T, 293 c18 (ATCC no. CRL-10852), NIH 3T3 and mouse ES cells (gift of Victor Lin, Columbia University) were maintained in DMEM with standard supplements. Plasmid constructs were transfected into HEK 293T and NIH 3T3 cells using LipofectAMINE (Invitrogen). 293 c18 cells were transfected using the calcium phosphate co-precipitation method.

Antibodies

Polyclonal antibodies specific for Dnmt3L were raised by immunizing a rabbit with a GST–Dnmt3L fusion (amino acids 1–78). The antibodies were affinity purified with an antigen-coupled glutathione–Sepharose column. Monoclonal anti-Dnmt3b (IMG-184) was from Imgenex Corporation. Other antibodies used have been described previously (7). Monoclonal anti-Dnmt3L was provided by the Antibody Research Center (Shanghai).

Immunoprecipitation

For co-immunoprecipitation of Dnmt3b and Dnmt3L, 4 × 10^6 of ES cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1.7 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Protein A–Sepharose beads (Amersham Biosciences) were added and the mixture was rotated for 1 h at 4°C. Precleared supernatants were incubated with the IP antibody overnight at 4°C. Immunoprecipitates were collected and washed four times in RIPA buffer. Proteins in immunoprecipitates were analyzed by standard western blotting.

In vitro translation and GST pull-down assay

GST–Dnmt3L protein was purified from Escherichia coli BL21 (DE3) carrying an expression construct. In vitro transcription/translation was performed using the TNT-coupled reticulocyte lysate system (Promega). Five micrograms of purified GST fusion protein were incubated with GST–Sepharose 4B beads (Amersham Biosciences) in NET buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 50 μg/ml bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin and 1 mM PMSF] in a total volume of 500 μl at 4°C for 2 h. Twenty microliters of in vitro translation products were then added to the slurry and incubated at 4°C for another 2 h. The Sepharose beads were vigorously washed five times with NET buffer, and bound proteins were resolved on an SDS–PAGE gel followed by autoradiography.

Mammalian two-hybrid assay

For the mammalian two-hybrid assay, catalytic domains of Dnmt3b containing different mutations were cloned into the pM vector (Clontech) to generate fusions with the GAL4
DNA binding domain. The C-terminal region of Dnmt3L was cloned into the pVP16 vector (Clontech) to generate fusions with the VP16 transcriptional activation domain. pG5Luc is a reporter vector which contains the luciferase-coding region downstream of the adenovirus E1b gene minimal promoter and five GAL4-binding sites. pRL–TK is a Renilla luciferase construct to control transfection efficiency. 293 c18 cells were transfected with the four constructs as indicated for 48 h and luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer’s instruction.

**In vivo methylation assay**

In vivo methylation assays were performed essentially as described (39). Briefly, 293 c18 cells in 35 mm dishes were transfected with 0.5 μg of the episomal assay plasmid p220.2 and appropriate expression constructs. Cells were allowed to grow for 2 days before being replated into a 100 mm dish. On reaching confluence, cells were harvested for episomal DNA extraction. An aliquot of cells was also collected to confirm the expression of the transfectants by western analysis. The recovered episomal DNA was digested with an excess of HhaI enzyme followed by Southern hybridization using a 32P-radiolabeled probe derived from the EBNAI region of p220.2. Quantification of the cleavage bands was performed using the Storm phosphorimager instrument and ImageQuant software (Molecular Dynamics) to assist the assessment of methylation levels.

**Protein expression and purification**

*Escherichia coli* BL21 (DE3) expression strains were induced at a cell density of 0.7 A600 nm for 3 h at 27°C with 0.2 mM isopropyl-β-d-thiogalactopyranoside. Collected cells were resuspended in 50 mM KPi (pH 7.5), 0.5 M NaCl, 10% glycerol, 0.2 mM EDTA, 10 mM imidazole, 20 mM β-mercaptoethanol, 1 mM leupeptin and 1 mM PMSF and disrupted by sonication. The supernatants were incubated with Ni-NTA-agarose (Qiagen) for 3 h at 4°C and washed with a similar buffer containing 20 mM imidazole and 1 mM β-mercaptoethanol. The bound proteins were eluted with a buffer containing 250 mM imidazole and 10 mM β-mercaptoethanol and dialyzed overnight in 50 mM Tris–HCL (pH 7.0), 50 mM NaCl, 10% glycerol, 1 mM EDTA and 0.2 mM DTT.

**In vitro methylation assay**

For the detection of in vitro methylation activity, a biotinylated 1179 bp PCR fragment amplified from the EBNAI region of p220.2 was used as substrate. The methylation reaction contained DNA fragments (15 μM) and Dnmt3b (DNMT3B) (0.1 μM) with or without Dnmt3L (DNMT3L) (0.1–4 μM) in 20 mM Tris–HCl (pH 7.5), 30 mM NaCl, 1 mM EDTA, 50 μg/ml BSA and 1.25 μM S-[methyl-3H] AdoMet (80 Ci/mmol) (Amersham Biosciences) in a total volume of 20 μl. Incorporation of methyl-3H into the substrate DNA was determined following the previous procedure (40). Measurements of methylation activities at multiple time points established that the initial incubation of 60 min falls within the linear phase of the reaction for Dnmt3b (DNMT3B) wild-type and variants under all conditions.

**Immunohistochemistry**

Mouse embryos in decidua were collected on embryonic days 5.5, 6.5 and 7.5 from ICR females and fixed in Bouin’s solution (75% picric acid, 10% formaldehyde, 5% glacial acetic acid). Paraffin-embedded sections in transverse plane were prepared with a thickness of 5 μm. Deparaffinized and stepwise rehydrated samples were treated with a steam of 10 mM citrate buffer (pH 6.0) for antigen retrieval. After blocking with a solution containing 10% goat serum, 2% BSA, 0.1% Tween-20 and 0.1% sodium azide in PBS, overnight incubation with primary antibodies against Dnmt3b and Dnmt3L was carried out at 4°C followed by fluorescence detection using a corresponding secondary antibody.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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