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Interactions within the mammalian DNA methyltransferase family

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Abstract

Background: In mammals, epigenetic information is established and maintained via the postreplicative methylation of cytosine residues by the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. Dnmt1 is required for maintenance methylation whereas Dnmt3a and Dnmt3b are responsible for de novo methylation. Contrary to Dnmt3a or Dnmt3b, the isolated C-terminal region of Dnmt1 is catalytically inactive, despite the presence of the sequence motifs typical of active DNA methyltransferases. Deletion analysis has revealed that a large part of the N-terminal domain is required for enzymatic activity.

Results: The role played by the N-terminal domain in this regulation has been investigated using the yeast two-hybrid system. We show here the presence of an intra-molecular interaction in Dnmt1 but not in Dnmt3a or Dnmt3b. This interaction was confirmed by immunoprecipitation and was localized by deletion mapping. Furthermore, a systematic analysis of interactions among the Dnmt family members has revealed that DNMT3L interacts with the C-terminal domain of Dnmt3a and Dnmt3b.

Conclusions: The lack of methylating ability of the isolated C-terminal domain of Dnmt1 could be explained in part by a physical interaction between N- and C-terminal domains that apparently is required for activation of the catalytic domain. Our deletion analysis suggests that the tertiary structure of Dnmt1 is important in this process rather than a particular sequence motif. Furthermore, the interaction between DNMT3L and the C-terminal domains of Dnmt3a and Dnmt3b suggests a mechanism whereby the enzymatically inactive DNMT3L brings about the methylation of its substrate by recruiting an active methylase.

Background

The mechanism of how chromatin architecture is established, maintained and modified is crucial for a thorough understanding of gene regulation and DNA replication. In higher eukaryotes DNA methylation and histone modifications appear to be the main agents responsible for the formation of active or inactive chromatin. However, what has become clear in the last few years is that the enzymes responsible for these epigenetic modifications do not act independently, but interact with one another.

DNA methylation or the transfer of a methyl group to the cytosine of CpG dinucleotides is carried out by DNA methyltransferases (MTases). In eukaryotes, these

enzymes are grouped into one family consisting of five members: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3L [1,2]. Dnmt1 is responsible for the maintenance of DNA methylation after each round of replication [3,4]. Dnmt3a and Dnmt3b are the main players involved in *de novo* methylation, a type of methylation occurring mainly during early development [5–7]. Dnmt3L shares some homology with Dnmt3a and Dnmt3b and plays a central role in the establishment of maternal genomic imprinting even though it does not have *in vitro* catalytic activity [7–10]. Finally, no particular function has yet been found for Dnmt2, a protein similar to the prokaryotic MTases. It is unable to methylate DNA *in vitro* and was shown not to be essential for *de novo* or maintenance methylation in ES cells even though it carries the key sequence motifs of DNA methyltransferases [11].

The typical eukaryotic (cytosine-5) DNA methyltransferase is a protein about three times larger than its prokaryotic counterpart. Based on functional and structural data it was proposed to result from the fusion between three genes, one of them being an ancestral prokaryotic MTase [12]. By analogy with the prokaryotic enzymes, the C-terminal region has been referred to as the catalytic domain and the N-terminal region as the regulatory domain. Both domains are linked by a short stretch of repeated GlyLys dipeptides. The enzyme exhibits a preference for hemi-methylated substrates [13–15], a property suggested to reside in the C-terminal domain [16]. The N-terminal domain can interact with numerous proteins like DMAP1, PCNA, and Rb [17–19]. It is also multifunctional in that it contains a DNA binding region, a cysteine-rich region, several Zn-binding domains and two regions responsible for the localization to replication foci [20–23]. Surprisingly, the isolated C-terminal domain of Dnmt1 is catalytically inactive despite the presence of the highly conserved sequence motifs typical of active MTases [12,24,25]. Only deletion mutants that retain a substantial part of the N-terminal domain, in addition to the C-terminal domain, are active. Thus, contrary to prokaryotic MTases, the catalytic activity of Dnmt1 has become dependent upon the regulatory domain through evolution. *De novo* MTases are also organized into a large regulatory N-terminal domain and a smaller catalytic C-terminal domain but, in contrast to Dnmt1, their isolated C-terminus is capable of methylating DNA [26]. The lack of extensive homology between the N-terminal domains of maintenance and *de novo* MTases points towards a possible functional difference of this domain. In the first part of this work, we show that the N- and C-terminal domains of Dnmt1 interact with each other. This interaction, initially detected by a yeast two-hybrid assay, was confirmed by immunoprecipitation and mapped by deletion analysis.

Dnmt3L is expressed during gametogenesis and was shown to be essential for maternal genomic imprinting [7]. This gene was placed in the Dnmt3 sub-family due to its homology to the PHD-like domain of Dnmt3a/3b. So far no catalytic activity has been reported for the enzyme, which is in accordance with its lack of key methyltransferase motifs [10]. We have looked at the possibility that DNMT3L, through its interaction with another MTase, could bring about the methylation of a suitable substrate. A prime candidate is DNMT2, a catalytically inactive protein that nevertheless carries the conserved sequence motifs of active MTases. The potential binding partners of DNMT3L within the MTase family, Dnmt2 and Dnmt1, were tested using the yeast two-hybrid system.

Results

Dnmt1 interactions

A physical interaction between N- and C-terminal domains of Dnmt1 was investigated using the yeast two-hybrid system. When the entire N-terminus was tested with the C-terminal domain, a strong interaction was detected as measured both by the *lacZ* and the *HIS3* reporter assay (Fig. 1B, line 1). This interaction occurred independently of whether the N-terminal domain was used as bait or prey but was lost when N- or C-domains were tested individually, which indicates that the interaction is vector independent and not due to autoactivation. Several 5'- and 3'-end deletions of the N- and C-terminal domains were generated to localize more precisely the region responsible for the interaction. For the N-terminal domain, the results indicated that a surprisingly large region is necessary since none of the 3'-end deletions showed activation of the reporter genes and only the 5'-end deletions that removed a comparatively small area (the first 227 amino acids) were positive (Fig. 1B, lines 2–8). For the C-terminal domain, a deletion of most of a region corresponding to the small domain described in prokaryotic MTases was also unable to interact with the full-length N-terminal region (Fig. 1B, line 9). Taken together, these results suggest that the interaction between the N and C-domains depends on their intact globular folding.

The validity of the two-hybrid interaction was checked by immunoprecipitation. The N- and C-terminal domains, engineered with different tags, were expressed either individually or co-expressed in COS-7 cells (Fig. 2, lanes 1–3). As expected from the yeast two-hybrid data, upon immunoprecipitation with α-Flag AB, the Flag-tagged C-terminal domain was pulled down in this assay (Fig. 2, lanes 4, 5). When probed with the anti-Flu AB, the blot revealed the presence of the Flu-tagged N-terminal domain but only when N- and C-domains were co-expressed. This finding clearly shows that the N-terminal domain

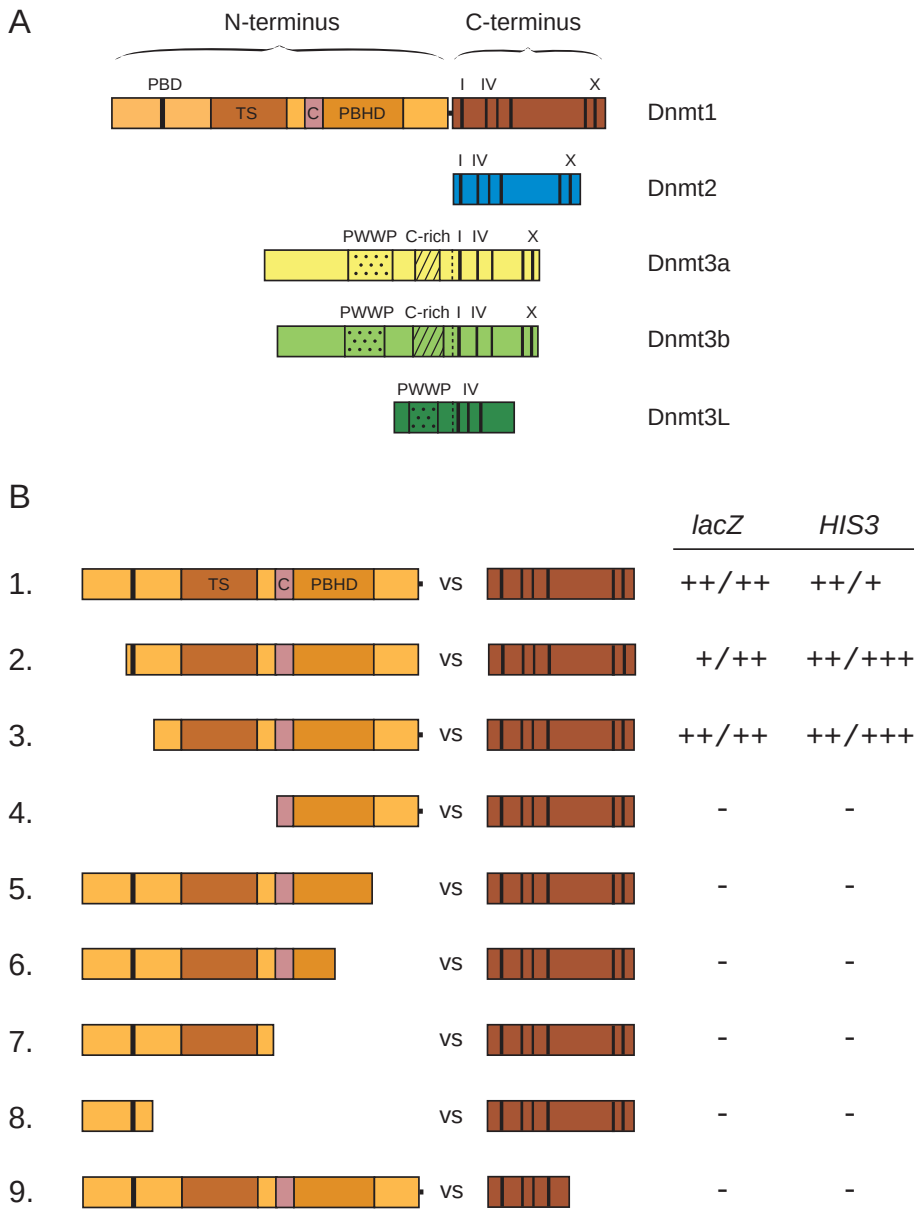

Figure 1

Dnmt1 interactions between N- and C-terminal domains. A. Overview of the general organization of the five DNA methyltransferase family members. Some of the functional domains or structural motifs recognized in the N-terminus of Dnmt1 and Dnmt3 include a PCNA binding domain (PBD), a targeting sequence (TS), a cysteine-rich region (C), a polybromo homology domain (PBHD), a tryptophan-rich region (PWWP) and another cysteine-rich region (C-rich). The vertical bars inside the C-terminus correspond to the highly conserved motifs found in most DNA methyltransferases. For simplicity, only motifs I, IV and X are labeled. The linker region – a $(GlyLys)_6$ repeat – between the N- and C-terminus of Dnmt1 is represented by a short thick horizontal line. **B.** A series of N- and C-terminal deletions of Dnmt1 were tested for interaction with their corresponding full-length terminal domain. The full-length N-terminus and the first two deletion constructs were generated by PCR amplification and inserted into pBTM117c.1 (lines 1–3, see Methods). The full-length construct was used to derive several deletions using the restriction enzymes NheI (line 5), NcoI (line 6), and BspEI (line 8). The full-length N-terminus was also cloned into pACT2 from which two deletions were derived using the enzyme BglII (lines 4, 7). The N-constructs 1, 2, 3, 5, 6 and 8 were tested as baits for interaction with the C-terminus whereas the N-constructs 1, 4 and 7 were tested as prey with the C-terminus. The C-terminus construct was obtained by digestion with EcoRI (line 9). The interaction was scored using two reporters, *lacZ* and *HIS3* (see Methods). Each member of a pair showing interaction was tested singly to rule out autoactivation. Duplicate results are separated by a slash (/).

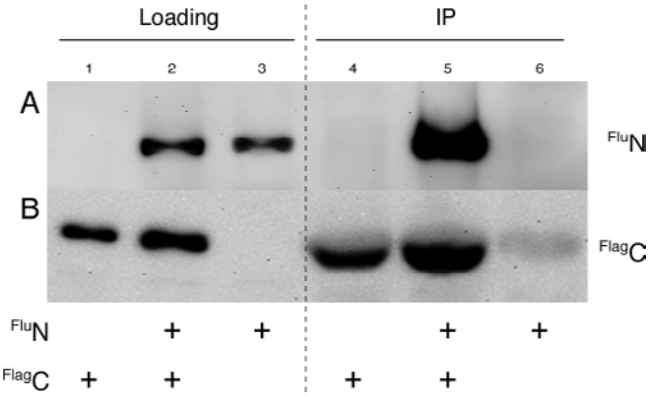

Figure 2
Co-immunoprecipitation of the N-terminal domain of Dnmt1. COS-7 cells were transfected with Flag-tagged C-terminus (lanes 1, 4), Flag-tagged C-terminus + Flu-tagged N-terminus (lanes 2, 5) or with Flu-tagged N-terminus (lanes 3, 6). The cells were lysed in EBC buffer and the supernatants immunoprecipitated with α-Flag AB. The complexes were bound to agarose beads, washed and separated by electrophoresis. After transfer to PVDF membranes, the Western blots were probed with α-Flu AB, stripped and reprobed with α-Flag AB. Lanes 1–3 show a ~2% aliquot of the initial extract used in the immunoprecipitation (IP, lanes 4–6). The recovery rate of the immunoprecipitation was about 10 %.

interacted with the C-terminal domain in order to be co-immunoprecipitated (Fig. 2, lane 5).

Discussion

In vitro proteolytic cleavage between the N- and C-domains has been shown to result in a large increase in the velocity of methylation of unmethylated DNA relative to hemi-methylated DNA [22]. This finding has been interpreted as the release of the inhibition by the N-terminal domain of the intrinsic *de novo* activity of the C-terminal domain [22]. This interpretation implies that the C-terminal domain by itself has enzymatic activity. However, this is in disagreement with the lack of catalytic activity reported by several groups for the C-terminal domain alone [12,24,25]. The interaction between N- and C-domains described here can partly explain this discrepancy as these domains were not physically separated in the proteolytic cleavage experiment. Therefore, the N- and C-domains most likely remained associated accommodating a role of the N-terminal domain in the catalytic process. Lines of evidence for the complex role of the N-terminal region of Dnmt1 in the methylation process include a) deletion mapping of Dnmt1, which indicates that a large part of the N-terminus is required for activity [12,24]; b) fusion of a eukaryotic N-terminus to various prokaryotic (whole) MTases, which changes the preference of the prokaryotic enzyme towards hemi-methylation [27]; and c) the first ~500 amino acids of the N-terminus repress enzymatic activity [16]. What does this interaction mean? Since the C-terminal domain carries the conserved sequence motifs found in active MTases, this suggests that, at the primary structure level, it is potentially capable of catalysis, but needs somehow to be activated by the N-terminus. The nature of this activation is likely due to a change in the tertiary structure of the protein. Our suggestion of a conformational change is in agreement with the allosteric activation model of Dnmt1, which proposes a double DNA binding site – for unmethylated DNA within the C-terminal and for methylated DNA within the N-terminal domain [16,25,28]. On the other hand, *in vitro* binding studies using smaller fragments of Dnmt1 revealed that a rather small fragment of about 135 amino acid centered around the cysteine-rich region interacts with the catalytic domain [25]. These results are hard to reconcile with the mapping results described here, which points towards a requirement for rather intact domains. However, since most fragments appear to interact with each other in those *in vitro* binding assays, it is possible that *in vivo* the folding of the N-terminal domain prevents some of these interactions. So far, attempts at recovering catalytic activity by mixing *in vitro* the C-terminal domain with these small N-terminal fragments have been unsuccessful [25], which supports the reported requirement for a mostly intact N-terminal domain in addition to the C-terminal domain for achieving methylation activity [12,24]. Our deletion mapping results point to an intact tertiary structure of the N-terminal domain rather than to a sequence motif as the essential element binding to the C-terminal domain and suggest that the active conformation of the C-terminal domain requires the presence of the correctly folded N-terminal domain for activity.

Interactions within the Dnmt family

The similar organization of Dnmt1 and Dnmt3a/3b led us to examine whether the N-and C-domain interaction of Dnmt1 also occurs in Dnmt3a or Dnmt3b. However, contrary to Dnmt1, the N-terminal domain of Dnmt3a (N-Dnmt3a) failed to interact with the corresponding C-domain – independently of whether the N-terminus was used as bait or prey (Fig. 3A). The N-terminal domain of Dnmt3b (N-Dnmt3b) also failed to interact with C-Dnmt3b suggesting an absence of intra-molecular interactions in Dnmt3a or Dnmt3b.

Although DNA methyltransferases are known to be found in complexes with methyl-CpG-binding proteins and histone modifying enzymes, little is known about interacting partners within the methyltransferase family itself. For instance, it is conceivable that Dnmt2 and Dnmt3L,

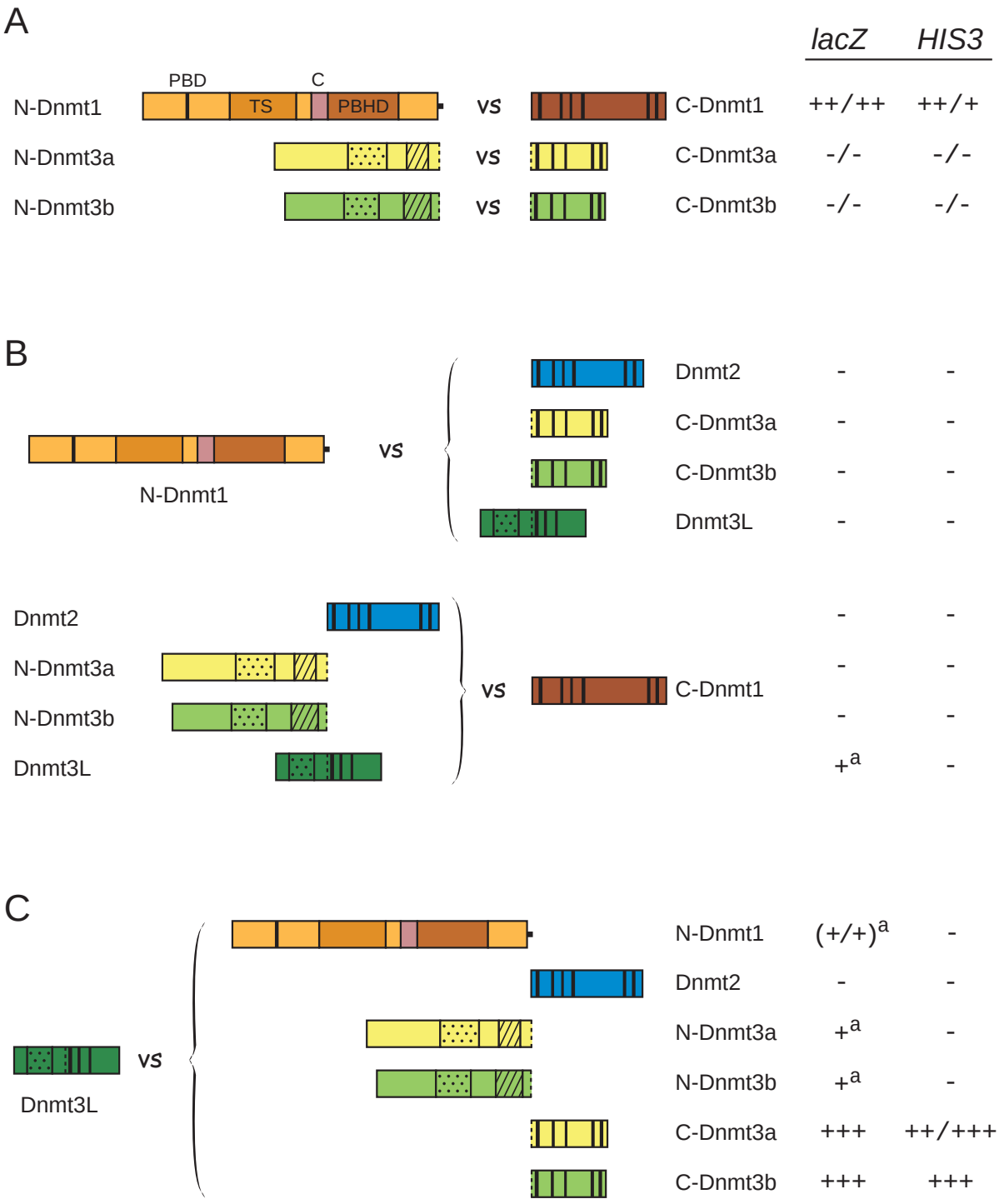

Figure 3
Interactions within the Dnmt family. Several combinations of N- and C-terminal domains of Dnmt1, Dnmt3a and Dnmt3b as well as whole Dnmt2 and DNMT3L were tested for interaction. The results are compiled in Table 1 and a subset is diagrammed here. **A.** N-terminus of Dnmt1, Dnmt3a and Dnmt3b versus their corresponding C-terminus; **B.** (top) N-Dnmt1 versus Dnmt2, DNMT3L, C-Dnmt3a and C-Dnmt3b; **B.** (bottom) C-Dnmt1 versus Dnmt2, DNMT3L, N-Dnmt3a and N-Dnmt3b; **C.** DNMT3L versus the N-Dnmt1, Dnmt2, N- and C-Dnmt3a, N- and C-Dnmt3b. The interactions were scored as in Fig. 1 and each member of a pair showing interaction was tested singly to rule out autoactivation. DNMT3L cloned in pACT2 shows weak autoactivation (marked a; see also Table 1).

which are both enzymatically inactive, could complement each other perhaps in a process analogous to the N- and C-domain interaction of Dnmt1. Therefore, several possible interactions within the Dnmt family were investigated using the yeast two-hybrid assay. Each member of the Dnmt family as well as two members of the MBD family were cloned in the appropriate yeast vectors and tested with one another. A summary of all protein pairs tested is presented in Table 1, while the results for the interacting partners of Dnmt1 and DNMT3L are diagrammed in Fig. 3. In the first case N-Dnmt1 (the N-terminal domain of Dnmt1) was tested with Dnmt2, DNMT3L, MBD2, MBD3, C-Dnmt3a (C-terminal domain of Dnmt3a) and C-Dnmt3b. MBD2 and MBD3 were chosen as their homologues play a role in mouse development [29]. These closely related proteins carry a methyl-CpG binding domain (MBD) but only MBD2 binds to methylated DNA. Both proteins are involved in transcriptional repression through deacetylation and chromosome remodeling albeit through different protein complexes [30–32]. The results of the two-hybrid assay show that none of the pairs tested were able to activate the reporter genes (Fig. 3B). C-Dnmt1 also failed to interact with Dnmt2, DNMT3L, MBD2, MBD3, N-Dnmt3a or N-Dnmt3b (Fig. 3B). Therefore, although none of the proteins tested were able to interact with the N- or C-terminal domains of Dnmt1, it is possible that the full-length protein could behave differently. Further, our survey of Dnmt1 interactions was not exhaustive and one of the pairs we did not test (N-Dnmt1 vs N-Dnmt3a/3b) has recently been shown to interact [33].

Dnmt2 did not interact with Dnmt1, N-Dnmt3a, N-Dnmt3b or DNMT3L and therefore failed to provide new clues as to its biological function (Fig. 3C and Table 1). Finally, DNMT3L was tested with N-Dnmt1, Dnmt2, N-Dnmt3a, N-Dnmt3b, C-Dnmt3a, C-Dnmt3b, MBD2 or MBD3 (Fig. 3C). A strong interaction was detected with C-Dnmt3a and C-Dnmt3b. It must be noted that, for practical reasons, human cDNA clones for DNMT3L, MBD2 and MBD3 were used against mouse proteins in all these assays. Our results therefore mean that the interspecies interaction DNMT3L with DNMT3A/B has been maintained through evolution even though mouse and human Dnmt3L share only ~74 % homology at the nucleotide level [9]. Thus it is unlikely that the lack of interaction between human MBD2/3 and mouse MTase proteins results from species differences since mouse and human MBD2/3 share ~90 % homology [30].

Discussion

Dnmt3L is expressed during gametogenesis. It is essential for the establishment of maternal genomic imprints as its targeted disruption leads to defective methylation of normally maternally methylated sequences [7]. Our finding that DNMT3L interacts with *de novo* MTases suggests a mechanism whereby the catalytically inactive Dnmt3L recruits an active MTase. While this work was in progress, Dnmt3L was reported to interact *in vitro* with Dnmt3a/3b [6]. These authors also showed that Dnmt3L was recruited to heterochromatin foci when co-expressed with Dnmt3a/3b whereas it is mainly cytoplasmic when singly expressed in NIH 3T3. They concluded that Dnmt3L might cooperate with Dnmt3a/b to carry out *de novo* methylation of maternally imprinted genes in oocytes.

In spite of well-conserved sequence motifs in their C-terminal domain, Dnmt1 and Dnmt3a/3b share little similarity. For instance, contrary to Dnmt1, the C-terminal domain of Dnmt3a/b is enzymatically active [26]. Also, the cysteine-rich region, which is found in both enzyme sub-families, has different functions. In Dnmt1 this region binds Zn, which is required for allosteric activation [22,25], whereas it acts as transcriptional repressor in Dnmt3a/3b [34]. Phylogenetic analysis also suggests that both enzyme sub-families may have evolved from different prokaryotic ancestors [35,36]. The lack of interaction between N- and C-terminal domains of Dnmt3a/3b is a good indication that the mechanism that regulates methylation for these enzymes is significantly different from that of Dnmt1. This difference is likely due to the preference for hemi-methylated substrates that Dnmt1 has acquired through evolution – a property absent in prokaryotic MTases. A corollary of our results is that a hybrid of N-Dnmt3 with C-Dnmt1 would be inactive, since Dnmt3a/3b are not allosterically regulated [37]. The lack of *in vivo* interaction between Dnmt1 and MBD2 or MBD3 is in agreement with the *in vitro* data indicating that human Dnmt1 can be immunoprecipitated only when both MBD2 and MBD3 are present [38].

Conclusions

The goal of this study was to investigate the interaction between regulatory and catalytic domains within the mammalian DNA methyltransferase family. The two-hybrid assay has revealed that such an interaction occurs in Dnmt1, which immediately suggests the possibility of an intra-molecular interaction between both domains. Immunoprecipitation assays have shown that this interaction can occur *in vivo* while deletion analysis suggests that this interaction requires an intact tertiary domain structure rather than small and independent binding motifs. The fact that this interaction appears specific to the maintenance enzyme Dnmt1 could reflect the need to restrict the catalytic activity of the enzyme to hemi-methylated sites in order to faithfully maintain a given methylation pattern. Another type of interaction has been identified by the two-hybrid assay between the catalytically inactive DNMT3L and the *de novo* enzymes. Since the isolated C-terminal domains of Dnmt3a/b are catalyti-

Table 1: Overview of all tested interactions within the Dnmt family.

	cloned in pACT2	cloned in pBMT117c.1	*lacZ*	*HIS3*	Comments
	Dnmt 2	N-Dnmt 1	-	-	
	N-Dnmt 3a	C-Dnmt 1	-	-	
	C-Dnmt 3a	N-Dnmt 1	-	-	
Dnmt 1	N-Dnmt 3b	C-Dnmt 1	-	-	
	C-Dnmt 3b	N-Dnmt 1	-	-	
vs	DNMT 3L	C-Dnmt 1	-	-	
	DNMT 3L	N-Dnmt 1	(+/+)	-	weak autoactivation
	DNMT 3L	control	(+/+)	-	weak autoactivation
other Dnmt's	N-Dnmt 1	DNMT 3L	-	-	
	N-Dnmt 1s	DNMT 3L	-	-	short isoform Dnmt1
	DNMT 3L	N-Dnmt 1s	(+)	-	weak autoactivation
	Dnmt 2	C-Dnmt 1	-	-	
Dnmt 3a	control	C-Dnmt 3a	-	-	
	control	C-Dnmt 3b	-	-	
vs	C-Dnmt 3a	N-Dnmt 3a	-	-	
	C-Dnmt 3b	N-Dnmt 3b	-	-	
Dnmt 3b	N-Dnmt 3a	C-Dnmt 3a	-	-	
	N-Dnmt 3b	C-Dnmt 3b	-	-	
	DNMT 3L	control	(+/+)	-	weak autoactivation
	control	C-Dnmt 3a	-	-	
DNMT 3L	control	C-Dnmt 3b	-	-	
	DNMT 3L	N-Dnmt 3a	(+)	-	weak autoactivation
vs	DNMT 3L	N-Dnmt 3b	(+)	-	weak autoactivation
	DNMT 3L	C-Dnmt 3a	+++/+++	++/++	
other Dnmt's	C-Dnmt 3a	DNMT 3L	+++	+++	
	DNMT 3L	C-Dnmt 3b	+/+	-	
	C-Dnmt 3b	DNMT 3L	+++	+++	
	Dnmt 2	DNMT 3L	-	-	
Dnmt 2	Dnmt 2	control	-	-	
	Dnmt 2	N-Dnmt 1	-	-	
vs	Dnmt 2	C-Dnmt 1	-	-	
	Dnmt 2	N-Dnmt 3a	-	-	
other Dnmt's	Dnmt 2	N-Dnmt 3b	-	-	
	Dnmt 2	DNMT 3L	-	-	
	MBD 2	MBD 3	++	++	positive control
	MBD 2	N-Dnmt 1	-	-	
MBD2	MBD 2	C-Dnmt 1	-	-	
	MBD 2	N-Dnmt 3a	-	-	
vs	MBD 2	N-Dnmt 3b	-	-	
	MBD 2	DNMT 3L	-	-	
Dnmt's	Dnmt 2	MBD 2	++	+++	strong autoactivation
	Dnmt 2	MBD 2	++	+++	strong autoactivation
	control	MBD 2	++	+++	strong autoactivation
MBD3	MBD 3	N-Dnmt 1	-	-	
	MBD 3	C-Dnmt 1	-	-	
vs	MBD 3	N-Dnmt 3a	-	-	
	MBD 3	N-Dnmt 3b	-	-	
Dnmt's	MBD 3	DNMT 3L	-	-	

The results are grouped in six panels corresponding to the different proteins tested. To allow for completeness within each panel, some of the results are presented in two different panels. N-Dnmt1s corresponds to the N-terminal domain of the short isoform, while the other abbreviations are explained in the text. Duplicate results are separated by a (/).

cally active, this interaction suggests rather a mechanism whereby DNMT3L recruits an active protein without the need for conformational changes. This analysis of interactions within the Dnmt family adds to our growing understanding of the complex network of interactions that are likely to play an important role in the regulation of DNA methylation and epigenetic information in mammalian cells.

Methods

Plasmid constructs

A diagram of the five DNA methytransferase family members used in this work is given in Fig 1A. Either whole cDNAs or cDNA fragments of mouse Dnmt1, Dnmt2, Dnmt3a, Dnmt3b1 and human DNMT3L, MBD2, and MBD3 were cloned in the yeast vectors pACT2 and/or pBTM117c.1. Inserts into pACT2 (Clontech) generate a fusion protein with the Gal4 activation domain and are referred to as preys in a yeast two-hybrid assay while baits refer to inserts into pBTM117c.1, which generate a fusion protein with the LexA DNA binding domain [39]. Each MTase or fragment thereof was amplified by the long template PCR system (Roche, Mannheim, Germany) with specific primers designed for in frame cloning. These primers carry restriction endonuclease sites immediately upstream of the initiation codon ATG. With the exception of N-Dnmt3a/3b and Dnmt2, all constructs showed one or another positive interaction, which indicates that they were correctly expressed. All fusion protein boundaries and subsequent deletions were sequenced. The amino acid boundaries of the various N-terminus constructs in Fig.1 are aa 1–1134 for #1, aa 119–1111 for #2, aa 228–1134 for #3, aa 630–1134 for #4, aa1-957 for #5, aa 1–848 for #6, aa 1–630 for # 7 and aa 1–228 for # 8. The C-terminus deletion (#9) removes aa 1388–1620. In Fig. 3, the boundaries are aa 1–1134 for N-Dnmt1, aa 1135–1620 for C-Dnmt1, aa 1–629 for N-Dnmt3a, aa 630–908 for C-Dnmt3a, aa 1–580 for N-Dnmt3b, and aa 581–859 for C-Dnmt3b. The plasmids used for the immunoprecipitation were generated by PCR amplification as described above. In this case, the specific primers allowed for the introduction of a Flag tag (DYKDDDK) or a Flu tag (YPYDVPDYA) downstream of an optimized translation initiation signal as previously described [40].

Yeast two-hybrid assays

For two-hybrid assays, the yeast strain L40 [*MAT***a***his3•200 trp1-901 leu2-3,112 ade2 LYS2:: (4lexAop-HIS3) URA3::(8lexAop-lacZ)*, Invitrogen] was transformed with the corresponding plasmids to leucine and tryptophan auxotrophy. Each two-hybrid combination was tested on at least four individual transformants. We did not observe variation between transformants. Additionally, for positive interactions, the transformation was repeated and again at least four transformants were tested. The transformants were grown at 30°C on selective medium lacking histidine to check for activation of the *HIS3* reporter (++, growth visible after 4 days; +++, growth visible after 2 days). Furthermore, activation of the *lacZ* reporter was tested in a β-galactosidase filter assay (Invitrogen). The strength of *lacZ* activation was rated according to the development of blue colonies with X-Gal. The strongest activation was obtained with the DNMT3L / C-Dnmt3a/3b interaction. It gave a dark blue color that was recorded as dark blue (+++). A light blue color that was still clearly distinguishable from a negative control was rated (+) while a medium activation, as judged by its color intensity, was rated (++). Negative controls were carried out with each construct cotransformed with the appropriate other empty vector, while MBD2/3 or the N- versus C-interaction were used as positive controls.

Immunoprecipitations

COS-7 cells were grown and transfected by the DEAE-dextran method as previously described [40]. Soluble extracts from cells overexpressing either the C-terminus or the N-terminus or both N- and C-termini were isolated after 1.5 days using EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5 % Nonidet P-40) with proteases inhibitors [40]. The pre-cleared extracts were immunoprecipitated overnight at 4°C with mouse anti-Flag (M2, Sigma, Taufkirchen, Germany). The resulting complexes were mixed with anti-Mouse IgG-agarose (Sigma, Taufkirchen, Germany) for 1 h at 4°C and centrifuged at low speed for 1 min. After 2 x 10 min washing with excess EBC buffer, the agarose beads were collected by centrifugation and mixed with 2 x Laemmli loading buffer. The mixture was boiled for 5 min and the supernatant loaded onto an 8 % SDS-PAGE. Western blotting conditions have been described previously [40].

Authors' Contributions

JBM carried out the cloning and immunoprecipitations while AEEM did the yeast work. HL was involved in the conception and coordination of this project. All authors approved the final manuscript.

Acknowledgments

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