The *Drosophila* MBD2/3 protein mediates interactions between the MI-2 chromatin complex and CpT/A-methylated DNA

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Summary

Methyl-DNA binding proteins play an important role in epigenetic gene regulation. The *Drosophila* genome encodes a single protein (MBD2/3) with extended homologies to the vertebrate methyl-DNA binding proteins MBD2 and MBD3. However, very little is known about its functional properties. We have now characterized an MBD2/3 null mutant allele that is viable and fertile. This mutation caused a strong dominant suppression of position-effect variegation and also resulted in a high rate of chromosome segregation defects during early embryogenesis. Confocal analysis of mutant embryos showed local displacement of MI-2 from DNA and indicated that MBD2/3 is associated with only a subset of MI-2 complexes. In addition, band shift experiments demonstrated a specific binding of MBD2/3 to CpT/A-methylated DNA, which reflects the endogenous DNA methylation pattern of *Drosophila*. Consistently, the localization of MBD2/3 was disrupted in embryos with reduced levels of DNA methylation. Our data provide novel insights into the function of MBD2/3 proteins and strongly suggest the existence of methylation-dependent chromatin structures in *Drosophila*.

Key words: DNA methylation, *Drosophila*, MBD2/3, MI-2

Introduction

Epigenetic regulation is mediated by DNA methylation and by covalent histone modifications (Bird, 2002; Li, 2002). Both mechanisms are intricately linked at the molecular level. DNA methylation has been shown to be dependent on defined histone modification patterns in several organisms (Jackson et al., 2002; Tamaru and Selker, 2001). However, it has also been demonstrated that histone modification patterns can depend on DNA methylation (Soppe et al., 2002; Tariq et al., 2003). In particular, we have recently shown a mutual relationship between DNA methylation and histone methylation in *Drosophila* (Kunert et al., 2003; Weissmann et al., 2003). These results strongly suggested a cooperative action of distinct epigenetic mechanisms.

Methyl-DNA binding proteins provide an attractive mechanistic link between DNA methylation and covalent histone modifications (Bird and Wolffe, 1999). These proteins specifically bind to methylated DNA and recruit histone-modifying enzymes to their target sites. The mechanistic details of this process are best understood for the vertebrate methyl-DNA binding proteins MeCP2 and MBD2. MeCP2 has been shown to be associated with the transcriptional co-repressor Sin3a and with histone deacetylase activity (Jones et al., 1998; Nan et al., 1998). More recent results have also demonstrated an interaction between MeCP2 and histone methyltransferase activity (Fuks et al., 2003). Together, these data indicate a close physical interaction between methyl-DNA binding proteins and histone-modifying enzymes. Similar interactions have also been demonstrated for the vertebrate MBD2 protein. MBD2 has been co-purified with the MI-2 complex that contains the nucleosome remodelling enzyme MI-2 and the histone deacetylases HDAC1 and HDAC2 (Ng et al., 1999; Wade et al., 1999; Zhang et al., 1999). In addition, the complex also contains the histone-binding proteins RbAp46 and RbAp48, the metastasis-associated protein 2 (MTA2), and the methyl-binding domain containing protein MBD3. The latter protein is closely related to MBD2 but it has no detectable methyl-DNA binding activity (Wade et al., 1999; Zhang et al., 1999).

The vertebrate *MBD2* and *MBD3* genes are probably the result of a gene duplication from a common *MBD2/3* ancestor (Hendrich and Tweedie, 2003). *MBD2/3* genes are widely conserved during evolution and homologues have been described in numerous organisms (Hendrich and Tweedie, 2003). The *Drosophila* genome also encodes a single MBD2/3 homologue, with more than 70% amino acid similarity to vertebrate MBD2 and MBD3 (Tweedie et al., 1999). MBD2/3 is expressed specifically in embryos and two developmentally regulated isoforms, resulting from alternative splicing, have been described (Ballestar et al., 2001; Marhold et al., 2002; Tweedie et al., 1999): the long isoform contains all functional domains, while the short isoform (MBD2/3Δ) lacks part of the putative methyl-CpG binding domain and an adjacent *Drosophila*-specific domain that is not found in the vertebrate homologues. Consistent with a conserved function of MBD2/3, the protein has been shown to be associated with some fly homologues of the vertebrate MI-2 complex (Ballestar et al., 2001; Tweedie et al., 1999). Intriguingly, the putative methyl-

CpG binding domain of MBD2/3 contains a number of deviations from the consensus MBD that seemed to be incompatible with a standard methyl-CpG binding activity (Ballestar et al., 2001; Tweedie et al., 1999). A very weak association with a CpG-methylated DNA fragment could be demonstrated in other experiments, but this interaction was observed with the short isoform and therefore seemed to be independent of the full-length methyl-CpG binding domain (Roder et al., 2000). Together, these results suggested that MBD2/3 might represent a functional homologue of mammalian MBD3, rather than MBD2 (Ballestar et al., 2001; Tweedie et al., 1999).

We have previously shown that MBD2/3 dynamically associates with *Drosophila* chromosomes during embryogenesis and with the Y-chromosome during spermatogenesis (Marhold et al., 2002). This observation has been interpreted to reflect a recruitment of MBD2/3 to epigenetically silenced loci during large-scale genome activation processes (Marhold et al., 2002). We have now characterized a loss-of-function allele for MBD2/3. Homozygous mutant flies were viable and fertile, but they showed a high incidence of chromosome segregation defects and a strong suppression of position-effect variegation. Mutant analysis also revealed a functional interaction with MI-2 and with CpT/A-methylated DNA. In conclusion, our combined data support the notion that MBD2/3 represents a functional homologue of mammalian MBD2. In addition, they reveal novel functions of MBD2/3 in the regulation of pericentric heterochromatin stability.

Materials and methods

Fly stocks and characterization of the *MBD[1]* allele

The *P{EPgy2}MBD-like[EY04582]* and *P{EP}EP(3)1112* strains were obtained from the Bloomington Stock Center. The precise position of the EP-element insertions were determined from sequencing data provided by the *Drosophila* genome project (Accession Numbers CC060346.1 and AQ025180.1, respectively). For subsequent experiments, we renamed the *P{Epgy2}MBD-like[EY04582]* allele *MBD[1]*. The *Mi-2[4]* allele has been described previously (Kehle et al., 1998).

Antibodies

The following antibodies have been described previously: rabbit anti-MI-2 (Brehm et al., 2000), rabbit anti-MBD2/3 (Ballestar et al., 2001), human anti-DNA (NatuTec) (Kunert et al., 2003) and rabbit anti-NAP1 (Lankenau et al., 2003). For the generation of the monoclonal antibodies against MBD2/3, Lou/C rats were immunized with a KLH-coupled peptide (NALKRKFARSQGGNAAGAAC) that is specific for the long isoform of the protein. After an 8-week interval, a final boost was given 3 days before fusion of the rat spleen cells with the murine myeloma cell line P3X63-Ag8.653. Hybridoma supernatants were tested in an ELISA using the same peptide coupled to ovalbumin. Antibodies reacting specifically with the peptides were confirmed by western blotting with 1:1000 diluted hybridoma supernatants. Based on the western blot results the hybridoma supernatant MBD 8E7 (rat IgG1) was used for further experiments.

Immunostaining of *Drosophila* embryos

Embryo immunostaining was performed as described previously (Marhold et al., 2002). Briefly, *MBD[1]* or wild-type Oregon R embryos were collected from a population cage. Embryos were then washed, dechorionated, fixed and permeabilized. After mounting, embryos were analyzed by confocal microscopy.

Band shift assays

GST-MBD2/3 and GST-MBD2/3Δ fusion proteins were obtained by cloning the coding region of the two isoforms in pGEX4T1 (Amersham) and expression in BL-21 bacteria according to the manufacturer's protocol. GST-MBD2a was obtained from Hidetoshi Fujita (Fujita et al., 2003). Gel mobility shifts were performed with the DIG Gel Shift Kit (Roche) according to the manufacturer's protocol, except that the oligonucleotides were labelled radioactively. To analyze interactions with CpG methylation we used the double-stranded GAM12 oligonucleotide and its unmethylated counterpart GAC12 (Lewis et al., 1992). To analyze interactions with CpT/A methylation, the following oligonucleotides were synthesized (MWG-Biotech, Germany): MATF, GAT AGC TGM AGM TGC AGC TGM AGC TGC AGC TGC AGM TGC ATC; and MATR, CTA TCG ACG TMG ACG TMG ACG TCG ACG TCG AMG TCG AMG TAG (M represents 5-methylcytosine). For controls, we also synthesized the corresponding unmethylated oligonucleotides. Prior to the binding assay, oligonucleotides were boiled for 10 minutes at 95°C in TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0) and slowly cooled down to 22°C. Annealing was verified by standard agarose gel electrophoresis. Probes were then labelled with T4-polynucleotide kinase (New England Biolabs) and gamma-[^{32}P]ATP (Amersham), and column-purified using the Qiaquick nucleotide removal kit (Qiagen). One picomol of radiolabelled probe was mixed with 100 ng of GST-purified recombinant proteins (GST-MBD2a, GST-MBD2/3 or GST-MBD2/3Δ), incubated for 15 minutes at room temperature and then electrophoresed on a native 8% polyacrylamide gel in 0.5×TBE buffer. As competitor we used the unlabelled probe in 400-fold concentration.

Analysis of MBD2/3 localization in demethylated embryos

Oregon R embryo collections (0-30 min) were dechorionated and demethylated with 5-azacytidine, as described previously (Kunert et al., 2003).

Results

To address the function of MBD2/3 in vivo, we identified and characterized an insertion mutant that contains an *EP* element in the 5′-coding sequence of the *MBD2/3* gene, 54 bp downstream of the initiation codon (Fig. 1A). This mutant allele was designated *MBD[1]*. In order to characterize this mutation, we isolated mRNA from homozygous *MBD[1]* embryos and analyzed it for the presence of *MBD2/3* transcripts by northern blotting. This revealed that *MBD2/3* expression was reduced to background levels (Fig. 1B). Consistently, western blotting failed to detect any MBD2/3 protein in extracts from homozygous mutant embryos (Fig. 1C). Last, we also immunostained homozygous *MBD[1]* embryos with a polyclonal MBD2/3-specific antibody and did not observe any signals above the background level (Fig. 1D). These results strongly suggest that the *MBD[1]* mutation represents a null allele.

Homozygous mutant flies were viable and fertile (data not shown). This indicated that MBD2/3 is not essential for *Drosophila* development. However, a more detailed analysis of mutant embryos by immunofluorescence microscopy revealed that a significant fraction (~20%) appeared smaller and more rounded than matched controls (Fig. 2A). However, this phenotype did not seem to have a significant effect on embryonic viability (data not shown). We also analyzed the effect of the *MBD[1]* mutation on the organization of embryonic DNA. To this end, we collected homozygous mutant and control embryos, immunostained them with an antibody

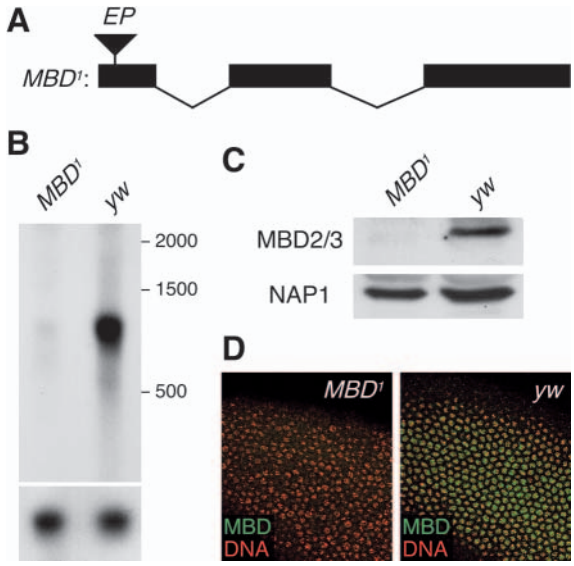

Fig. 1. Characterization of the *MBD¹* allele. (A) Overview indicating the exon-intron structure of the *MBD2/3* locus and the position of the *P* element insertion. (B) Northern analysis of embryonic poly(A)$^+$ RNA from 0- to 6-hour-old embryos. The upper panel shows significant levels of MBD2/3 mRNA in control (*yw*) embryos, while no signal was detectable in *MBD¹* mutant embryos. A probe against the elongation factor *EF1α48D* was used as a loading control (lower panel). (C) Western analysis of 0-6 hours-old embryonic protein extracts. Nucleosome assembly protein 1 (NAP1) was used as loading control. (D) Confocal sections from embryos double immunostained with antibodies against MBD2/3 (green) and DNA (red). Mutant embryos showed only background levels for MBD2/3, while the DNA signal showed no quantitative differences between mutant and control.

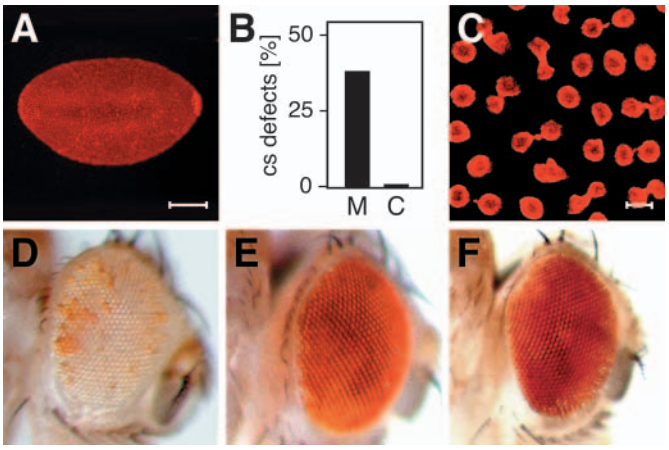

Fig. 2. Phenotype of *MBD¹* mutants. (A) Embryo stained with anti-DNA antibody (red). A significant number of mutant embryos appeared rounder and shorter than control embryos. Scale bar: 50 μm. (B) Percentage of embryos with detectable chromosome segregation defects. The data are derived from the microscopical analysis of 200-300 *MBD¹* mutant (M) and control (C) embryos, respectively. (C) Chromosome segregation defects in *MBD¹* mutant embryos. Embryos were collected, stained with an antibody against DNA and screened for abnormal mitotic figures. This revealed multiple chromosomal bridges in *MBD¹* mutants. Scale bar: 5 μm. (D) Eye phenotype of flies carrying a *pUAST-lacZ* (control) transgene in the w^{m4h} background. (E) The *MBD¹* mutation results in a strong dominant suppression of the w^{m4h} phenotype. (F) A similar effect was also observed with an independent *MBD2/3* mutant allele (see Materials and methods for details).

against DNA and analyzed them by confocal microscopy. This revealed chromosome segregation defects in 37% of *MBD¹* embryos, but only in 1% of control embryos (Fig. 2B). More specifically, we observed a high number of chromosome bridges and lagging anaphase chromosomes (Fig. 2C), which indicated a potential role of MBD2/3 in the stability of pericentric heterochromatin. This prompted us to investigate the effect of the mutation on the expression of a variegating pericentric *white* gene. To this end, we introduced the *MBD¹*mutation into the w^{m4h} background. Our experiments revealed a strong dominant suppression of *white* variegation (Fig. 2E, compare to 2D), which can be seen by an uniform red eye colour and the loss of variegating spots in the eyes of the adult progeny. The same effect was also observed with an independent mutant *MBD2/3* allele (Fig. 2F) that contains a *P*-element insertion 300 bp upstream from the transcriptional start site (see Materials and methods for details). This confirmed the specificity of our observation and is consistent with a role of MBD2/3 in chromatin regulation.

We then sought to confirm the association between MBD2/3 and the MI-2 complex at the functional level. It has been shown previously that MBD2/3 and MI-2 interact in vitro (Tweedie et al., 1999). Similarly, both proteins have been co-fractionated in protein extracts from *Drosophila* SL-2 cells (Ballestar et al., 2001). In order to look for a genetic interaction between *MBD2/3* and *Mi-2*, we crossed homozygous *MBD¹* flies with flies carrying a heterozygous mutant allele for *Mi-2* ($Mi\text{-}2^4$). Compound heterozygotes for both mutations had significantly rougher and smaller eyes in about 25% of the progeny, while both homozygous *MBD¹* flies and heterozygous $Mi\text{-}2^4$ flies had completely normal eyes (Fig. 3A). This result strongly suggested a functional interaction between MBD2/3 and MI-2. We also analyzed the interaction between MBD2/3 and MI-2 by determining the subcellular distribution of MI-2 protein in *MBD¹* mutants. We immunostained wild-type and mutant embryos with a specific antiserum against MI-2 and analyzed the subnuclear distribution of the protein by confocal microscopy. This revealed a homogeneous distribution of MI-2 in wild type embryos (Fig. 3B). However, the protein appeared to be absent from about 10-15 nuclear foci in the mutant (Fig. 3C). These results are consistent with a functional interaction between MBD2/3 and MI-2, and suggest that the MI-2 complex might be absent from a subset of target loci in MBD2/3 mutants.

In order to analyze the relationship between MBD2/3 and MI-2 in higher detail, we performed double immunostaining. We have shown previously that MBD2/3 forms nuclear foci at the cellular blastoderm stage that remain detectable until after gastrulation (Marhold et al., 2002). However, the precise nature of these foci could not be determined further because of the lack of suitable antibodies. As a prerequisite to double immunostaining experiments we raised a monoclonal MBD2/3-specific antibody (MBD 8E7) by immunizing rats with an MBD2/3 peptide. The peptide was selected from the exon 2 region of MBD2/3 that is not present in the short MBD2/3 isoform, and the antibody recognized a single band in western blots from embryonic nuclear extracts that

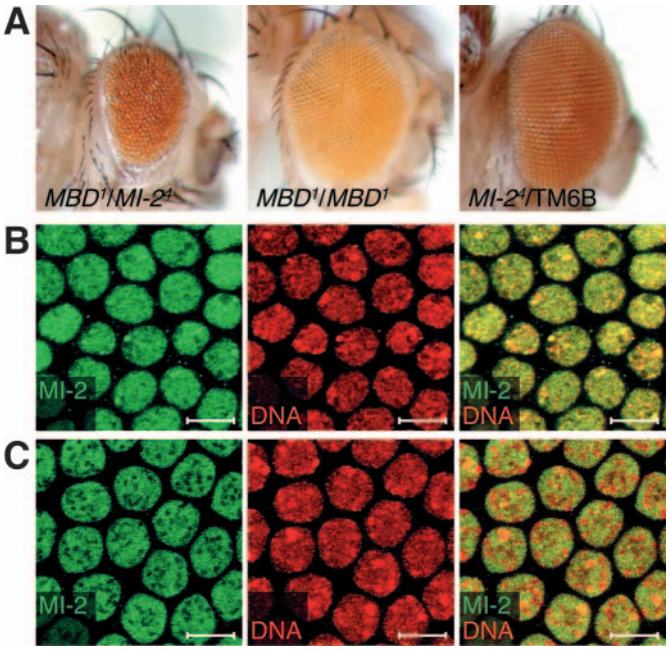

Fig. 3. Functional interaction between MBD2/3 and MI-2. (A) *MBD*^{1}*/MI-2*^{4} compound heterozygotes (left panel) showed significantly smaller and rougher eyes than either parent strain (middle and right panels). (B,C) Analysis of MI-2 protein distribution in control (B) and mutant (C) embryos by double immunostaining with antibodies against MBD2/3 (green) and DNA (red) and subsequent confocal microscopy. Scale bars: 5 μm. (B) MI-2 was ubiquitously distributed in wild type nuclei. (C) MI-2 was displaced from a subset of chromosomal sites in *MBD*^{1} embryos.

corresponds to the long isoform of MBD2/3 (Fig. 4A). The specificity of the antibody was confirmed by the absence of detectable signals in western blots of protein extracts from homozygous *MBD*^{1} embryos (Fig. 4A). Similarly, immunostaining of homozygous *MBD*^{1} embryos with our 8E7 antibody failed to detect any signals above the background level (Fig. 4A). Double immunostaining of wild-type embryos with the 8E7 antibody and an MI-2-specific antiserum revealed a speckled nuclear pattern for MBD2/3 (Fig. 4B), which was in agreement with our previous observations (Marhold et al., 2002). By contrast, MI-2 was found in a rather ubiquitous distribution in embryonic nuclei (Fig. 4B). This result argued against MBD2/3 being an integral component of all MI-2 complexes and suggested a more peripheral association with only a subset of MI-2 complexes.

To look for a potential interaction between MBD2/3 and methylated DNA we first tried to double immunostain embryos with antibodies against MBD2/3 and 5-methylcytosine. However, 5-methylcytosine staining requires an extensive sample denaturation with 2 M hydrochloric acid, which affected the distribution of MBD2/3 epitopes (data not shown). We therefore used band shift assays to determine the affinity of MBD2/3 for methylated oligonucleotides. Two previous studies have failed to detect an interaction between MBD2/3 and CpG-methylated probes (Ballestar et al., 2001; Tweedie et al., 1999). A third study described a preferential binding of the short MBD2/3 isoform to a human DNA probe containing a single methylated CpG (Roder et al., 2000). However, the short isoform lacks parts of the methyl-DNA binding domain, which rendered the significance of this finding unclear. In addition, CpG methylation is virtually absent from *Drosophila* genomic DNA and most of the 5-methylcytosine is present in the context of CpT and CpA dinucleotides (Lyko et al., 2000). This prompted us to test a CpT- and CpA-methylated oligonucleotide probe in band shift assays. In a first set of experiments we determined the affinity of both MBD2/3 isoforms to an oligonucleotide probe that was densely methylated at CpG dinucleotides on both strands. Consistent with the results obtained by others (Ballestar et al., 2001; Tweedie et al., 1999), we did not observe any interactions between MBD2/3 or MBD2/3Δ and CpG-methylated DNA (Fig. 5A). Under the same conditions, human MBD2 revealed a readily detectable interaction with the probe (Fig. 5A). To analyze the interaction between MBD2/3 and non-CpG methylated DNA, we then used a double-stranded oligonucleotide that contained eight 5-methylcytosine residues in an asymmetrical CpT and CpA context (see Materials and methods for details). This revealed a protein-DNA complex for the long isoform of *Drosophila* MBD2/3, but not for the short isoform, that lacks part of the methyl-DNA binding domain (Fig. 5B). Most of the shifted signal was detected in the high-molecular weight range, which may indicate that MBD2/3 had formed dimers or oligomers during the binding reaction. The specificity of the interaction between MBD2/3 and the CpT/A-methylated oligonucleotide was confirmed by the addition of unlabelled competitor probe, which strongly reduced the band shift signal (Fig. 5B). Furthermore, human MBD2 showed no detectable affinity for the CpT/A-methylated probe (Fig. 5B).

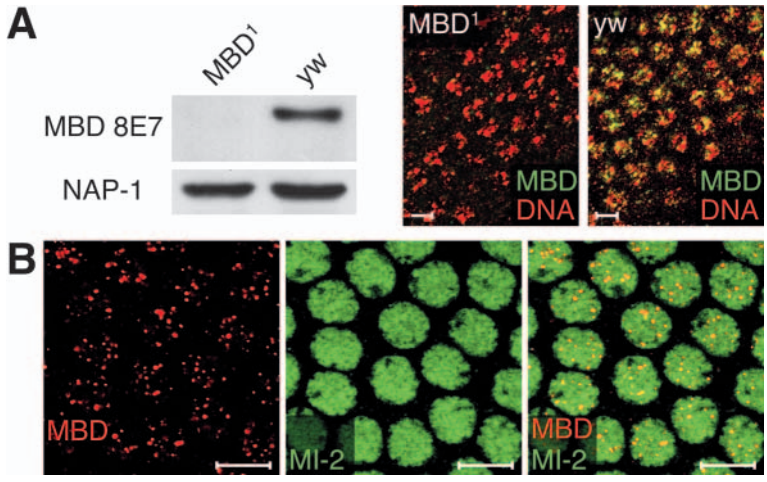

Fig. 4. MBD2/3 colocalizes with a subset of MI-2 proteins. (A) Establishment of a novel MBD2/3-specific antibody. Antibody specificity was confirmed by western analysis of protein extracts from 0- to 6-hour-old embryos (left panel) and confocal analysis of immunostained embryos (right panels). Significant signals for MBD2/3 were detected in control (*yw*), but not in *MBD*^{1} embryos. (B) Confocal sections of embryos double immunostained with antibodies against MBD2/3 (red) and MI-2 (green). MBD2/3 was found to decorate only a limited number of nuclear foci, while MI-2 was broadly distributed over the entire nucleus. Scale bars: 5 μm.

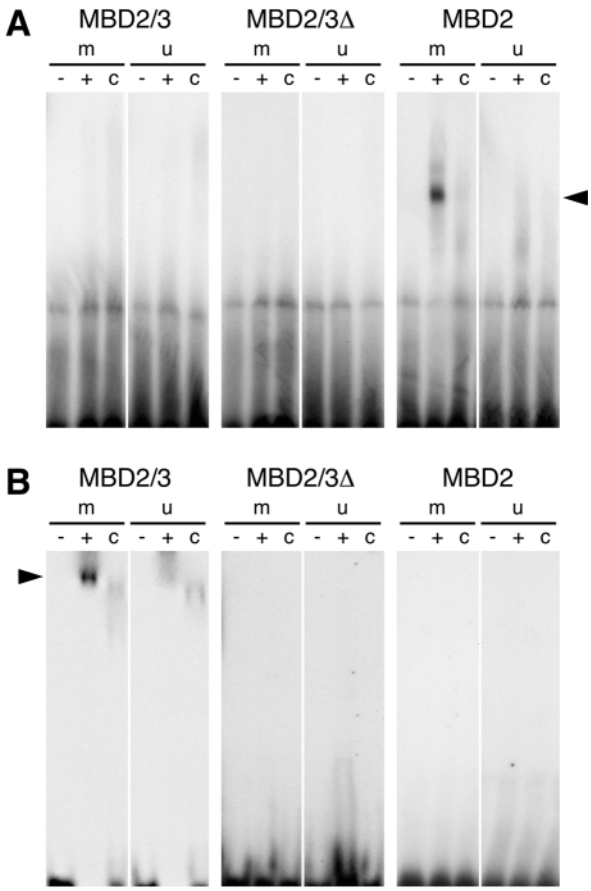

Fig. 5. MBD2/3 interacts with CpT/A-methylated oligonucleotides in band shift assays. *Drosophila* GST-MBD2/3, GST-MBD2/3Δ and human GST-MBD2 were analyzed for their ability to bind to radioactively labelled oligonucleotide probes. Binding assays were performed either in the absence (–) or presence (+) of recombinant proteins and competed with a 400-fold excess of the corresponding unlabelled probe (c). (A) Binding assays with a CpG-methylated probe (m) or with the corresponding unmethylated probe (u). (B) Binding assays with a CpT/A-methylated probe (m) or with the corresponding unmethylated probe (u). Arrowheads indicate the position of shifted oligonucleotide probes.

From these results, we concluded that the long isoform of MBD2/3 interacts directly and specifically with CpT/A-methylated DNA.

It has been shown before that the mouse MBD2 protein loses its defined localization pattern in DNA methyltransferase-mutant cell lines (Hendrich and Bird, 1998). In order to confirm the methyl-DNA binding activity of MBD2/3 in vivo we analyzed the localization of MBD2/3 in embryos with decreased levels of DNA methylation. We incubated dechorionated pre-blastoderm embryos with the DNA methyltransferase inhibitor 5-azacytidine under conditions that had previously been shown to cause efficient and specific demethylation of genomic DNA (Kunert et al., 2003). Drug- and mock-treated embryos were then double-immunostained with MBD2/3- and DNA-specific antibodies. This revealed a clear mislocalization of MBD2/3 in demethylated embryos (Fig. 6A, compare with 6B). The protein lost its defined focal pattern and showed a homogeneous cellular distribution (Fig. 6A). Similar results were also obtained after an RNAi-mediated knockdown of the Dnmt2 DNA methyltransferase protein (data not shown). Our data suggests that the localization of MBD2/3 requires wild-type levels of DNA methylation and thus confirms the interaction between MBD2/3 and methylated DNA in vivo.

Discussion

The presence of a functional DNA methylation system in *Drosophila* has been questioned for a long time. The fly homologues of central vertebrate DNA methylation factors have initially been interpreted to be evolutionary remnants with little or no functional significance (Tweedie et al., 1999). This view has been challenged by the recent demonstration of catalytic activity for the *Drosophila* DNA methyltransferase homologue Dnmt2 (Kunert et al., 2003). We have now used a mutant allele for the putative *Drosophila* methyl-DNA binding protein MBD2/3 to

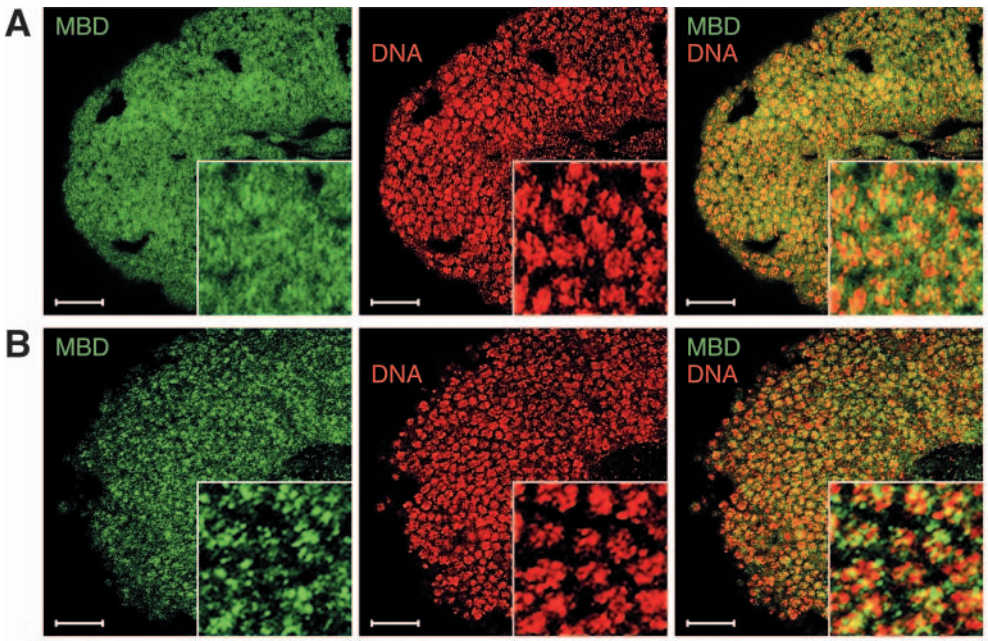

Fig. 6. The localization of MBD2/3 depends on DNA methylation. (A) Embryos were demethylated with the DNA methyltransferase inhibitor 5-azacytidine and double immunostained with antibodies against MBD2/3 (green) and DNA (red). This caused a readily detectable displacement of MBD2/3 from DNA. (B) For comparison, pictures from mock-treated embryos are also shown. Scale bars: 25 μm, insets show fourfold magnified details.

Table. 1. Functional characteristics of mammalian MBD2/MBD3 and *Drosophila* MBD2/3

	MBD2	MBD3	MBD2/3
Methyl-DNA binding	Detectable*	Absent*	Detectable†
MI-2 complex association	Peripheral‡	Integral¶	Peripheral†
Mutant phenotype	Viable and fertile§	Embryonic lethal§	Viable and fertile†

*Hendrich and Bird, 1998.
†This study.
‡Feng and Zhang, 2001.
§Hendrich et al., 2001.
¶Ng et al., 1999; Wade et al., 1999; Zhang et al., 1999.

analyze its function. Our results showed a strong suppressor effect of MBD2/3 on pericentric position-effect variegation. This indicates a role of the protein in the organization of pericentric heterochromatin. Consistent with this finding, MBD2/3 mutants also showed a high incidence of chromosome segregation defects. A mechanistic link between the stability of pericentric heterochromatin and proper chromosome segregation has also been demonstrated in other organisms (Bernard et al., 2001; Taddei et al., 2001), and has been explained by the structural requirements of mitotic spindle attachment sites. MBD2/3 does not localize to pericentric regions in *Drosophila* embryonic nuclei (Marhold et al., 2002) and is therefore unlikely to be a structural component of pericentric heterochromatin. However, the protein might play a more indirect role and could be involved in the regulation of genes encoding heterochromatin-associated proteins. In this respect, it is also worth mentioning that MBD2/3 adds to the growing list of epigenetic mediators that play an important role in the modulation of chromosome architecture (Weissmann and Lyko, 2003).

MBD2/3 is the only gene in the *Drosophila* genome with extensive homologies to vertebrate genes encoding methyl-DNA binding proteins (Adams et al., 2000). This made the protein a primary candidate for a functional link between methylated DNA and epigenetic chromatin structures. It has been previously suggested that MBD2/3 is associated with the *Drosophila* MI-2 complex (Ballestar et al., 2001; Tweedie et al., 1999). Our data confirms this interaction on a functional level and suggests that MBD2/3 acts as a co-repressor that targets the MI-2 complex to methylated DNA. A similar function has been proposed for vertebrate MBD2 (Feng and Zhang, 2001). Other, DNA methylation-independent co-repressors are involved in recruiting the MI-2 complex to a variety of target genes (Ahringer, 2000). For example, the *Drosophila* hunchback and Tramtrack69 proteins have been implied in targeting the complex to homeotic and neuronal-specific genes, respectively (Kehle et al., 1998; Murawsky et al., 2001).

Our results also revealed a detectable interaction between MBD2/3 and methylated DNA. This interaction appeared to be specific for CpT/A methylation and could not be seen with a CpG-methylated probe that effectively interacted with the human MBD2 protein. The differential specificities of the fly and vertebrate proteins are in agreement with the methylation patterns found in the respective species. Vertebrates methylate their genome mainly at symmetrical CpG sequences and human MBD2 showed a corresponding preference for CpG-methylated DNA. Fly DNA is methylated predominantly at asymmetrical CpT/A sequences (Kunert et al., 2003; Lyko et al., 2000) and MBD2/3 showed a corresponding preference for CpT/A-methylated DNA. This difference in specificity might involve some of the sequences that are found in the N-terminal half of *Drosophila* MBD2/3, but not in the vertebrate homologues (Hendrich and Tweedie, 2003; Tweedie et al., 1999). Consistently, the CpT/A-binding activity of MBD2/3 was undetectable with the short isoform of the protein, which lacks most of these non-conserved sequences. In addition, the long isoform is expressed only during early stages of embryogenesis and it associates with methylated DNA during the cellular blastoderm stage, when DNA methylation appears to be most abundant (Kunert et al., 2003; Marhold et al., 2002). The short isoform of MBD2/3 is expressed in the mid-to late stages of embryogenesis (Marhold et al., 2002), when DNA methylation levels are much lower (Kunert et al., 2003). It is possible that the transient expression of the long isoform creates a short window of time for the establishment of DNA methylation-dependent chromatin structures during *Drosophila* embryogenesis.

Last, our results can also be used to address the functional similarities between MBD2/3 and mammalian MBD2/MBD3 (Table 1). The latter two proteins are highly similar at the sequence level but distinguished by strikingly different functional characteristics: mouse MBD2 binds methylated DNA, while MBD3 does not (Hendrich and Bird, 1998). MBD2 has been shown to be peripherally associated with the MI-2 complex (Feng and Zhang, 2001), while MBD3 is an integral component of it (Ng et al., 1999; Wade et al., 1999; Zhang et al., 1999). MBD2 knockout mice were shown to be viable and fertile, while loss of MBD3 resulted in embryonic lethality (Hendrich et al., 2001). Our results showed that MBD2/3 binds to methylated DNA, that the protein co-localized with only a subset of MI-2 proteins and that MBD2/3 mutants are viable and fertile. All these characteristics show unambiguous parallels between *Drosophila* MBD2/3 and mammalian MBD2 (Table 1) and therefore suggest that MBD2/3 is a functional homolog of mammalian MBD2, rather than MBD3.

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