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# RET finger protein enhances MBD2- and MBD4-dependent transcriptional repression

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**Abstract**

We recently demonstrated that MBD4 possesses the ability to repress transcription through methyl-CpG and is associated with methylated promoters in the *CDKN2A* and *MLH1* genes. In order to further investigate the role of MBD4 in methylation-based transcriptional repression, a yeast two-hybrid screening was performed, and the RET finger protein (RFP) was found to be one of the major proteins that interact with the transcriptional repression domain in MBD4. The effect of the MBD4-mediated transcriptional repression in methylated *CDKN2A* and *MLH1* promoters was extremely enhanced by the overexpression of RFP. Furthermore, RFP forms a protein complex not only with MBD4 but also with MBD2 or MBD3 and was shown to enhance transcriptional repression through MBD2. These results suggest that RFP is a mediator connecting several MBD proteins and allowing the formation of a more potent transcriptional repressor complex. Because RFP has been detected at high levels in a variety of tumor cell lines as well as testis, and embryos, RFP may have an important role in the enhancement of transcriptional repression through MBD proteins in tumorigenesis, spermatogenesis, and embryogenesis.
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*Keywords:* RFP; MBD2; MBD4; Transcriptional repression

Methylation at CpG dinucleotides is the most common epigenetic modification of vertebrate genomes. In mammals, 70% of all CpG dinucleotides are methylated, with the exception of CpG islands, which are GC-rich regions that mostly overlap the promoter regions of RNA polymerase II-transcribed genes [1]. DNA methylation maintains stable gene silencing at imprinted genes and on the inactive X chromosome and has been shown to be essential for normal development in both mice and frogs [2,3]. Aberrant gene silencing in tumors *via* methylation of CpG islands in the promoter of many cancer-related genes is also well documented [4].

DNA methylation prevents some transcription factors from binding to their cognate DNA recognition sequences by modification of cytosine residues at the CpG sites. In addition, DNA methylation affects chromatin structure because of the recruitment of corepressors and the chromatin remodeling activities of proteins that bind specifically to methylated DNA [5,6]. In this context, methyl-CpG binding proteins appear to be central players in the process of DNA methylation-dependent gene silencing. Methyl-CpG binding protein 2 (MECP2), MBD1, MBD2, MBD3, and MBD4 constitute a family of vertebrate proteins that share the methyl-CpG binding domain (MBD). The MBD, consisting of about 70 residues, possesses a unique α/β-sandwich structure with characteristic loops and is able to bind single methylated CpG pairs as a monomer [7]. All the MBDs except MBD4, a DNA glycosylase that forms a protein complex with the DNA mismatch repair protein MLH1 [8], have been shown to be involved in transcriptional repression. It has been established that MECP2 and MBD2 are involved in histone deacetylase-dependent repression [5,6], and it is likely that this is also the case for MBD3 [9]. Unlike MECP2 and MBD2, MBD1 does not appear to interact with histone deacetylase 1 (HDAC1)

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or HDAC2 [10]. We recently demonstrated that MBD4 also has the ability to repress transcription through methyl-CpG sequences [11]. MBD4 is involved in HDAC-dependent repression and directly binds to HDAC1 and corepressor SIN3A. Furthermore MBD4 specifically binds to highly methylated promoters of *CDKN2A* and *MLH1* genes. A recent publication has reported that MBD2 and MBD4 specifically associates with the methylated metallothionein-1 (MT-1) promoter in cancer cells and inhibits its activity [12]. These results suggest that the MBD4 is one of the essential components in transcriptional silencing in cancer.

The RET finger protein (RFP), which belongs to the B box zinc finger protein family, has a tripartite motif consisting of a Ring finger, a B box finger, and a coiled–coil domain. RFP becomes oncogenic when its tripartite motif is fused with the tyrosine kinase domain of the RET protein [13]. RFP has been detected in the nuclei of various cells, including peripheral and central neurons, hepatocytes, adrenal chromaffin cells, and male germ cells. Among these, RFP is expressed at high levels in pachytene spermatocytes and round spermatids during spermatogenesis, suggesting that it participates in male germ cell differentiation. RFP mRNA is also highly expressed in a variety of human and rodent tumor cell lines [13]. Moreover, RFP binds and co-localizes with Enhancer of Polycomb 1 (EPC1) [14] and CHD4 [15] in the nucleus, and is involved in transcriptional repression. The repressive activity of RFP resides mainly in its coiled–coil domain, which represents the binding sites for EPC1 and CHD4.

In the present study, we demonstrated the importance of a unique mediator, RFP, which binds the transcriptional repression domain (TRD) of MBD4 to form the repressive complex. RFP mediates enhanced transcriptional repression in MBD4 as well as MBD2. Our findings suggest that MBD proteins co-operate with each other to repress transcription through RFP.

## Materials and methods

*Strains and plasmids*. *Escherichia coli* strain DH5αF′ was used to propagate all the plasmids. The cDNA for human MBD4 was isolated previously [11] and was subcloned into a yeast two-hybrid vector, pDBTrp (Invitrogen). The reporter strain of *Saccharomyces cerevisiae*, MaV203 (Invitrogen), was used for the yeast two-hybrid screening. The cDNA for human RFP (GenBank Accession No. NM006510) was generated by PCR amplification of the amplified DNA of a fetal brain cDNA library using KOD DNA polymerase (Toyobo) and was cloned into pcDNA3.1/V5-His (Invitrogen), pFLAG-CMV-2 (Scientific Imaging Systems, Eastman Kodak), and pGEX-2TK (Amersham Pharmacia Biotech) vectors. The fetal brain cDNA library was a generous gift from Dr. K. Yamakawa (RIKEN, Wako, Japan) [16]. The yeast two-hybrid vectors, pBTM116 and pVP16, and the reporter strain of *S. cerevisiae*, L40, were kindly provided by Dr. S.M. Hollenberg (Oregon Health Sciences University, Portland, OR) [17]. Deletion mutants of *MBD4* and *RFP* cDNAs were constructed by DNA amplifications using KOD DNA polymerase. The cDNAs for human MBD2 and MBD3 were isolated previously [11]. *MBD2*, *MBD3*, and *MBD4* cDNAs were subcloned into pcDNA3.1/V5-His, pFLAG-CMV-2, and pGEX-2TK vectors. Reporter plasmids pSF100-*CDKN2A* pro and pSF100-*MLH1* pro, which contain the β-galactosidase gene under the control of *CDKN2A* promoter or *MLH1* promoter, were described previously [11]. Plasmid pGV-C2 (Toyo Ink) was also used as an internal control reporter. Nucleotide sequences of the PCR primers used are available upon request to the authors.

*Yeast two-hybrid screening*. Yeast strain MaV203 carrying pDBTrp-MBD4 was transformed by the mouse embryo day 9 cDNA library constructed in pVP16. The cDNA library was kindly provided by Dr. T. Sato (Columbia University, New York, NY) [18]. Plasmids harboring cDNA were recovered from the histidine-, uracil-, and β-galactosidase-positive colonies. The β-galactosidase assay in liquid culture using *O*-nitrophenyl-1-thio-β-D-galactopyranoside (ONPG) was described in our earlier report [19].

*Cell culture*. HEK293T and CHO-K1 cells (obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University) were cultured in Dulbecco's modified Eagle's medium (Sigma) and Ham's F12 nutrient medium (Sigma) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen) according to recommendations from the supplier.

*Immunoprecipitation*. Immunoprecipitation was performed as described previously [20]. To detect MBD4–RFP interaction, the HEK293T cells grown in 10-cm dishes were co-transfected with a combination of 4 μg pFLAG-CMV-2 or pFLAG-RFP together with 4 μg pcDNA3.1/V5-His or pcDNA-MBD4-V5. At 48 h post-transfection, cells were lysed, immunoprecipitated, and analyzed by Western blotting using anti-FLAG monoclonal antibody M2 (Sigma) and anti-V5 monoclonal antibody (Invitrogen). To detect RFP–MBD2 and RFP–MBD3 associations, the HEK293T cells were also co-transfected with a combination of 4 μg pFLAG-CMV-2 or pFLAG-MBD2 or pFLAG-MBD3 together with 4 μg pcDNA3.1/V5-His or pcDNA-RFP-V5. The transfected cell extracts of HEK293T were immunoprecipitated and analyzed by Western blotting, using the same antibodies as used for MBD4–RFP interaction.

*In vitro binding assay*. The glutathione *S*-transferase (GST)-*in vitro* transcription and translation (IVTT) assay was performed as described previously [20]. The GST fusion protein of MBD4 or RFP was made using the pGEX-2TK vector.

*Yeast two-hybrid assay*. The yeast transformation and the β-galactosidase assay were performed as described previously [19]. Values were means and standard deviations of β-galactosidase activities obtained from three independent transformants.

*Transfection and reporter assays*. For reporter assays, the CHO-K1 cells were seeded on 6-well tissue culture dishes and were transfected with the reporter (0.5 μg; β-galactosidase gene under the control of *CDKN2A* or *MLH1* promoter), the effector (total 1 μg; MBD proteins or RFP protein), and the internal control reporter (0.15 μg; SV40 promoter/enhancer driving luciferase). The pSF100 reporter plasmid with *CDKN2A* or *MLH1* promoter was methylated by the CpG methylase, *M. Sss*I (New England Biolabs). Complete methylation was checked by restriction digestion with *Bst*UI (New England Biolabs). Transfection was performed using the Lipofectamine reagents (Invitrogen) according to supplier's recommendations. The cells were harvested 48 h after transfection. Then the β-galactosidase and luciferase assays were performed as described previously [11]. Values are means and standard deviations of the results from three independent experiments.

*RNA interference*. The cDNA for the Chinese hamster *Rfp* was sequenced, and the 19-base sequence corresponding to 1470–1488th nucleotides relative to the start codon was chosen as targeting sequence by short interference RNA (siRNA) with two protruding deoxythymine residues (5′-CCCUAUGA GUGGGAUUGAUdTdT-3′, sense strand sequence), and a double-stranded siRNA was purchased from Japan Bioservice (Asaka, Saitama, Japan). The siRNA for the firefly luciferase gene, *GL2*, was used as the negative control [21]. The CHO-K1 cells were seeded on 6-well tissue culture dishes and were transfected with mock (without siRNA), *RFP* siRNA (final concentration; 20 nM), or *GL2* siRNA (final concentration; 20 nM). Transfection was performed using the Oligofectamine reagents (Invitrogen) according to the supplier's recommendations. At 24 h post-siRNA transfection, cells were again transfected with reporter, effector, and internal control reporter DNAs as described above and harvested 48 h after DNA transfection. Then the β-galactosidase and luciferase activities were measured, and Western blotting was performed using anti-RFP antibody (Immuno-Biological Laboratories) and anti-β-actin monoclonal antibody clone AC-15 (Sigma).

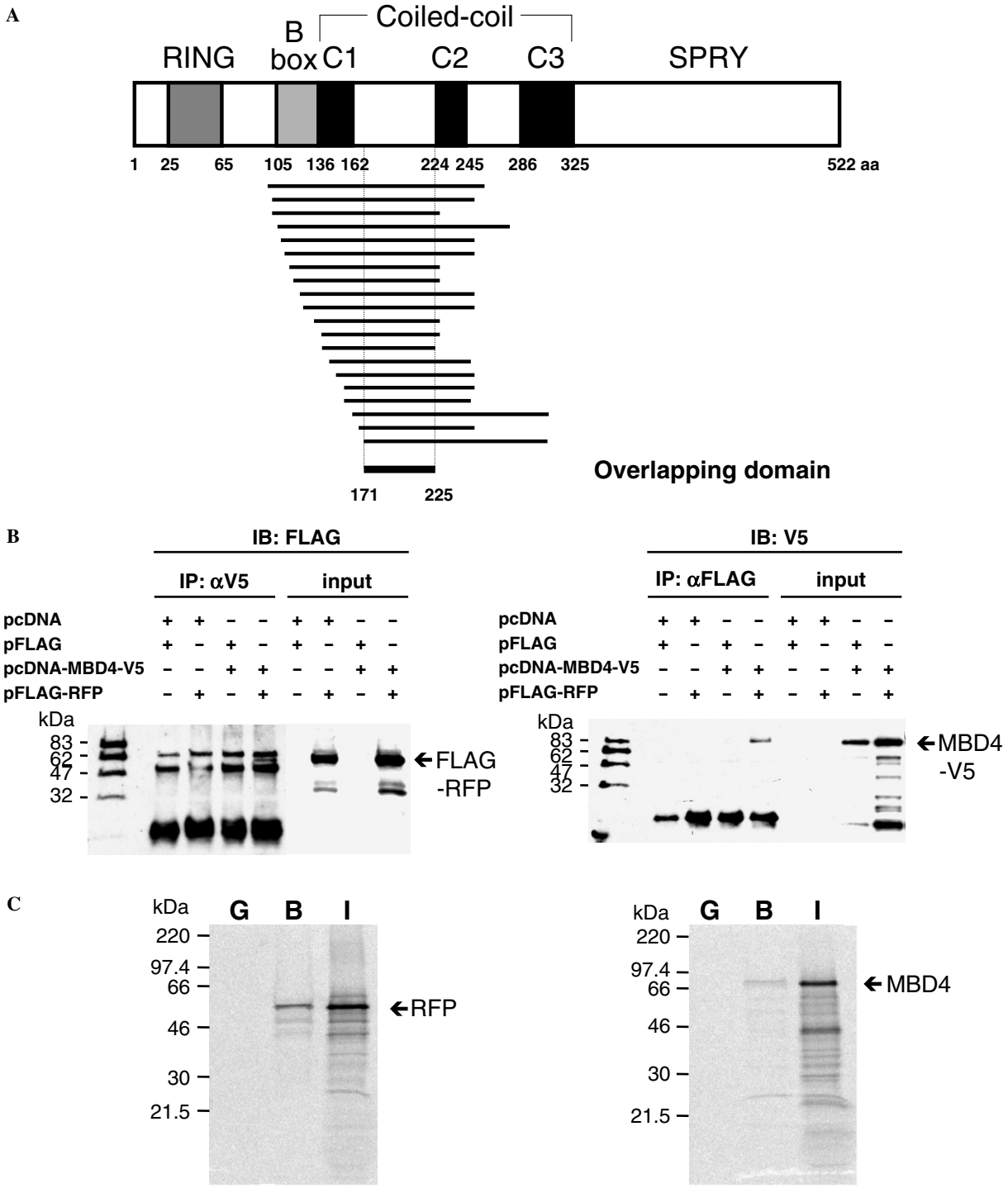


Fig. 1. Interaction between MBD4 and RFP. (A) *Rfp* cDNA clones obtained from yeast two-hybrid screening. The amino-terminal is on the left, and a RING finger, a B box finger, three coiled–coil domains (C1, C2, and C3), and a SPRY domain are designated. Numbers correspond to amino acid positions in the protein. (B) Western blots of immunoprecipitates. Immunoprecipitaion was performed as described in Materials and methods. (C) *In vitro* binding assay was performed as described in Materials and methods and Results. G, protein bound by GST alone; B, protein bound by the MBD4 or RFP; I, input protein (10% of the total). (D) Domain of MBD4 interacting with RFP. The two-hybrid assay was performed as described in Materials and methods and Results. The filter β-galactosidase assays are shown in the middle. Parentheses indicate the mean β-galactosidase activities in the yeast cells containing only the corresponding BD plasmid. Region representing transcriptional repression activity (TRDs) is also indicated by the thick black line at the bottom [11]. MBD, methyl-CpG binding domain; MID, hMLH1 interacting domain; Glycosylase, glycosylase domain. (E) Domain of RFP interacting with MBD4. The mean β-galactosidase activity in yeast cells containing only MBD4 BD plasmid was 0.1 U.

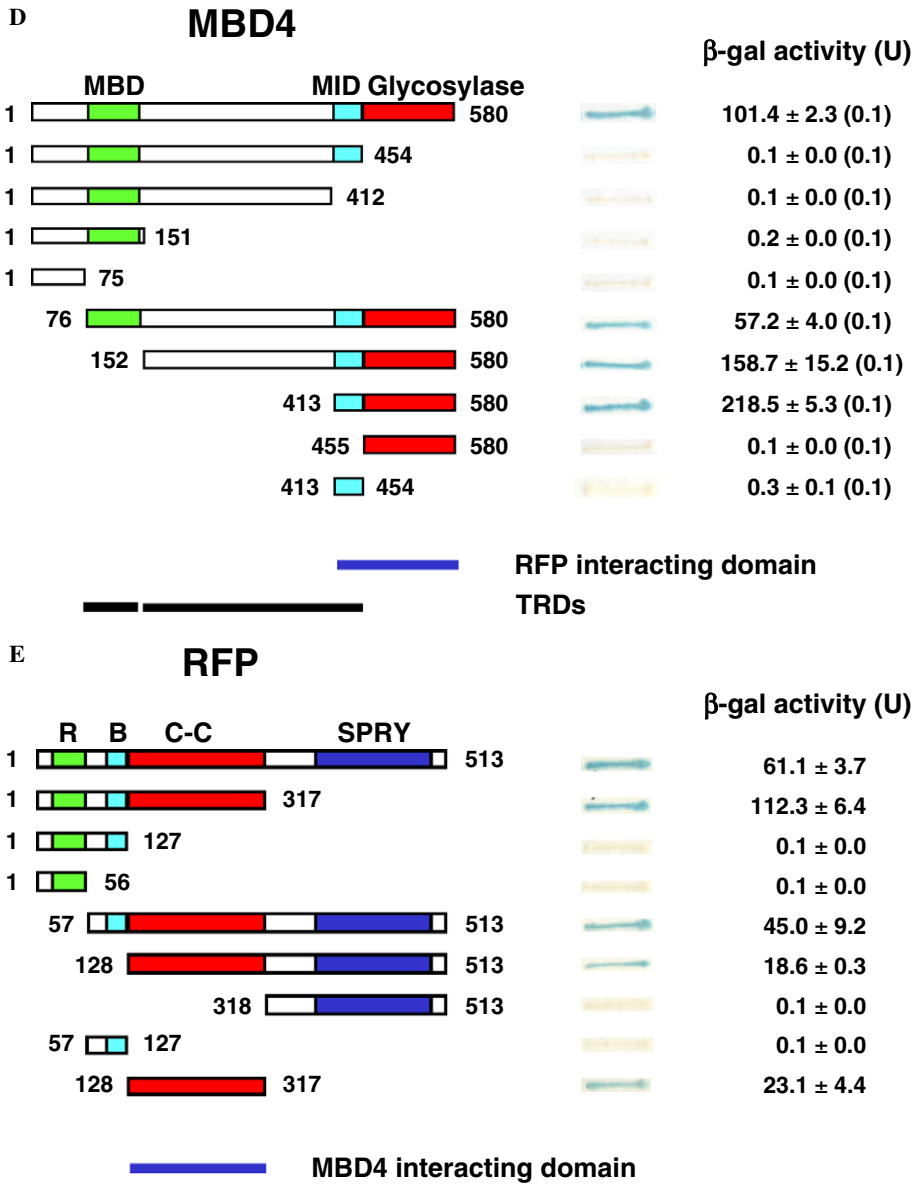


Fig. 1 (*continued*)

## Results

### *MBD4 interacts with RFP*

To identify the factors interacting with MBD4 and responsible for transcriptional repression, we performed a yeast two-hybrid screening by using the human *MBD4* full-length cDNA as a bait and the mouse embryo day 9 cDNA library. The mouse cDNA library was used to allow the detection of evolutionarily important interacting proteins. Of the approximately $1 \times 10^7$ independent Trp-/Leu- transformants examined, 96 clones were His+, Ura+, and β-galactosidase-positive. A great majority of the clones (59 of 96, 61.5%) contained the central region of RET finger protein (Rfp) covering the B box finger and the coiled–coil domain (Fig. 1A); therefore, we further analyzed RFP as a major MBD4 interacting protein. The minimal overlapping region of the *Rfp* cDNA clones located at residues 171–225 comprised the coiled–coil domain. As the next step, we cloned and sequenced the 1542-bp human *RFP* full-length cDNA that encoded a polypeptide consisting of 513 amino acid residues. To check whether MBD4 forms a protein complex with RFP in the cell, we performed an immunoprecipitation analysis (Fig. 1B). FLAG-tagged RFP was detected in the immunoprecipitates with V5-tagged MBD4. Likewise, V5-tagged MBD4 was present in the FLAG-tagged RFP immunoprecipitates. This result indicated the existence of protein complexes containing both MBD4 and RFP *in vivo*.

To confirm the direct interaction between MBD4 and RFP, we produced GST-fused MBD4, and an *in vitro* pulldown analysis was carried out (Fig. 1C). GST and

GST-fused MBD4 were immobilized on glutathione–agarose beads and incubated with $^{35}$S-labeled RFP. Only GST-fused MBD4 bound the RFP. In the reciprocal experiment, GST-fused RFP similarly bound $^{35}$S-labeled MBD4.

*Interaction domains in MBD4 and RFP*

We determined the domain of MBD4 that interacted with RFP using the yeast two-hybrid assay (Fig. 1D). *MBD4* cDNAs containing a full-length message as well as nine deletion mutants were inserted into *lexA* DNA binding domain (BD) pBTM116 plasmids. The full-length *RFP* cDNA was inserted into a VP16 transcriptional activation domain (AD) plasmid, pVP16. Various combinations of these plasmids were used for transformation of the *S. cerevisiae* L40 strain. All the yeast cells containing only the BD plasmids with various MBD4 portions showed background levels of β-galactosidase activity (0.1 U). Our experiments as summarized in Fig. 1D indicated that the minimal region conserving the interacting activity of MBD4 with RFP corresponded to a region between residues 413 and 580.

Next, we proceeded to analyze the domain of the RFP interacting with MBD4 by the yeast two-hybrid assay (Fig. 1E). The full-length *MBD4* cDNA was inserted into the BD plasmid, and the *RFP* cDNAs containing a full-length and eight deletion mutants were inserted into the AD plasmid. As presumed from the results of two-hybrid screening demonstrated in Fig. 1A, MBD4 interacted with the coiled–coil domain of RFP.

*RFP enhances MBD4-dependent transcriptional repression*

As one of the important clues for resolving the role of the MBD4–RFP interaction, RFP was found to repress transcription through EPC1 [14] and CHD4 [15]. To address a fundamental role of RFP in MBD4-mediated repression, we investigated the effect of RFP on promoter activities in CHO-K1 cells (Fig. 2). The methylated *CDKN2A* and *MLH1* promoters were previously shown to be associated with MBD4 and were repressed transcriptionally by MBD4. In the absence of MBD4, RFP increased β-galactosidase activities to some extent for both methylated *CDKN2A* and *MLH1* promoters. However, RFP enhanced MBD4-dependent transcriptional repression in both methylated *CDKN2A* and *MLH1* promoters. These results suggest that MBD4 enhances transcriptional repression in association with RFP.

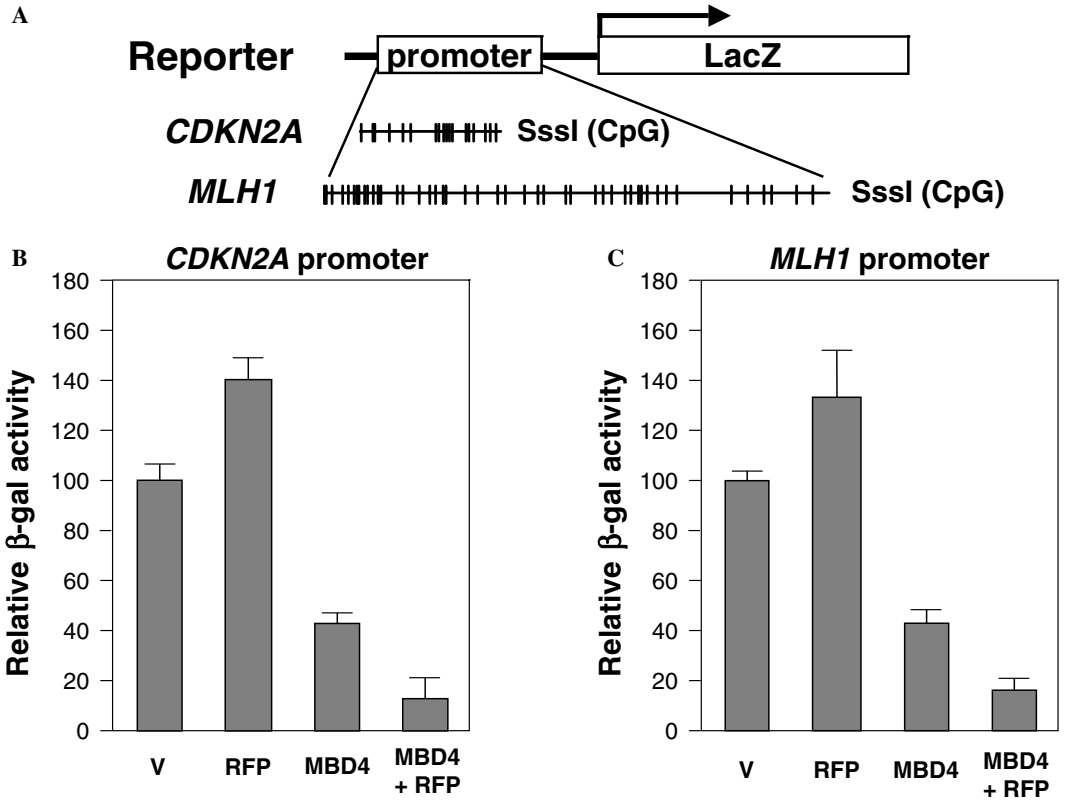


Fig. 2. Enhancement of MBD4-dependent transcriptional repression by RFP. (A) PCR-amplified DNA fragments from *CDKN2A* and *MLH1* genes were subcloned upstream of a *lacZ* gene in a pSF100 vector. The methyl-CpG sites modified by *Sss*I methyltransferase are shown by vertical bars. *Sss*I-treated pSF100 reporter with *CDKN2A* (B) or *MLH1* (C) promoter was co-transfected with pGV-C2 and RFP expressing plasmid (pcDNA-RFP), MBD4 expressing plasmid (pcDNA-MBD4), or both of these plasmids (MBD4 + RFP). The relative β-galactosidase activity of methylated pSF100 in combination with pGV-C2 and the empty pcDNA3.1/V5-His vector (mock) was set to 100 as the normalization, and the relative β-galactosidase activities (mean with standard deviation) were determined after correction by the transfection efficiency of pGV-C2.

*RFP associates with MBD2 and MBD3*

Recently, RFP was found to interact with CHD4 [15], which is involved in transcriptional repression, in co-operation with its associating proteins such as MBD2 and MBD3, HDAC1 and HDAC2, MTA1, MTA2, and MTA3, and RBBP4 and RBBP7 [6,9]. Therefore, we tested the association of RFP with MBD2 or MBD3 by immunoprecipitation experiments (Fig. 3A and B). FLAG-tagged MBD2 and MBD3 (FLAG-MBD2 and FLAG-MBD3, respectively) were detected in the immunoprecipitates with V5-tagged RFP (RFP-V5). Likewise, V5-tagged RFP was

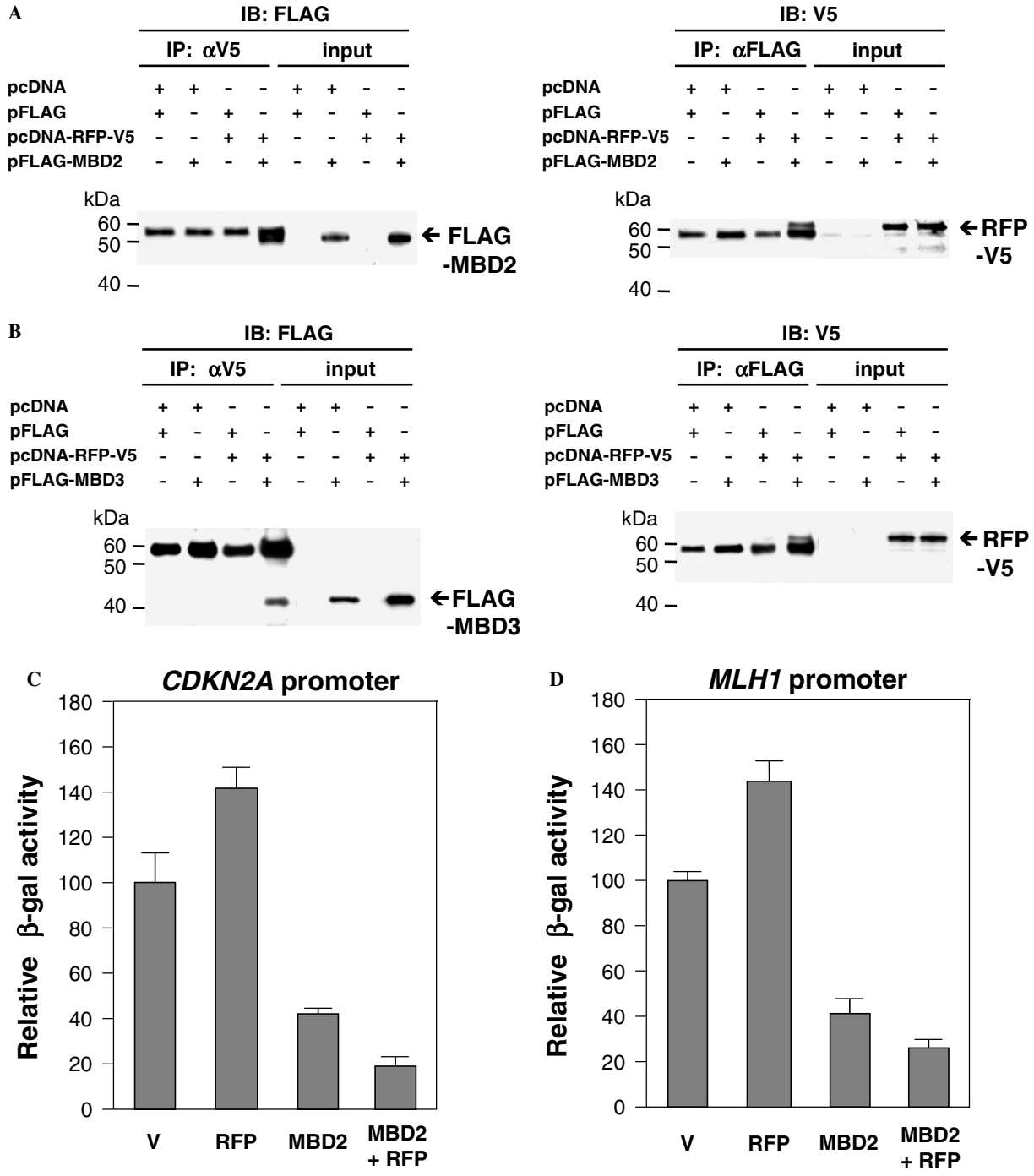


Fig. 3. Enhancement of MBD2-dependent transcriptional repression by RFP. (A,B) Western blots of immunoprecipitates. Immunoprecipitaion was performed as described in Materials and methods. *Sss*I-methylated pSF100 reporter with *CDKN2A* (C) or *MLH1* (D) promoter was co-transfected with a pGV-C2 and RFP expressing plasmid (pcDNA-RFP), MBD2 expressing plasmid (pcDNA-MBD2), or both of these plasmids (MBD2 + RFP). The relative β-galactosidase activities were determined as described in Fig. 2.

present in the FLAG-tagged MBD2 or MBD3 immunoprecipitates. These results indicated the existence of protein complexes containing MBD2 and RFP or MBD3 and RFP *in vivo*.

### *RFP also enhances MBD2-dependent transcriptional repression*

Given the fact that both MBD2 and MBD3 associate with RFP and are components of the methyl-CpG binding protein 1 (MeCP1) complex [6], it is logical to think that RFP may enhance MBD2-dependent transcriptional repression. To address this question, we investigated the transcription from the methylated *CDKN2A* and *MLH1* promoters when RFP was expressed together with MBD2 (Fig. 3C and D). Similar to the pattern seen with MBD4 and described above, RFP slightly enhanced MBD2-dependent transcriptional repression in both methylated *CDKN2A* and *MLH1* promoters. These results suggest that MBD2 enhances transcriptional repression in association with RFP.

### *Knockdown of RFP does not affect MBD2- and MBD4-dependent transcriptional repression*

The knockdown of RFP was performed by siRNA technology to see whether RFP could be an essential player in transcriptional repression from the hypermethylated *CDKN2A* and *MLH1* promoters in mammalian cells (Fig. 4). The RFP siRNA could reduce the protein level up to 80% in CHO-K1 cells. However, the transcriptional repression from both methylated *CDKN2A* and *MLH1* promoters in these siRNA treated cells did not change in comparison with untreated cells. These results suggest that RFP is not likely to be an indispensable component in MBD2- and MBD4-dependent transcriptional repression.

## Discussion

In this study, we found that RFP interacts with MBD4. This result strongly supports our previous concept that MBD4 in fact plays a role in transcriptional repression in addition to its DNA repair function, because RFP is widely known to be involved in transcriptional repression through EPC1 [14] and CHD4 [15]. The interaction between RFP and CHD4 is an especially interesting aspect of the interactions of RFP. The MeCP1 complex represses transcription in a methylation density-dependent fashion and consists of the Mi-2/NuRD complex and MBD2 [6]. CHD4 is a major component of the Mi-2/NuRD complex, which also contains MBD3. These results suggest the existence of a network involving several MBD proteins.

What is the function of the RFP interaction with MBD4? RFP mediates the enhancement of transcriptional repression by MBD4 as well as MBD2. Among the MBD proteins, MBD2 has been reported to be involved with hypermethylated promoters in cancer-related genes such as *CDKN2A*, *BRCA1*, and *MGMT*. On the other hand, we recently identified that MBD4 has an additional role in transcriptional repression through methyl-CpG and associates with the hypermethylated promoters in the *CDKN2A* and *MLH1* genes [11]. Furthermore, a recent report showed that MBD2 and MBD4 specifically associated with the methylated MT-1 promoter and inhibited its activity [12]. Another report also showed that MBD4 as well as MBD2 among other MBD proteins co-localizes to

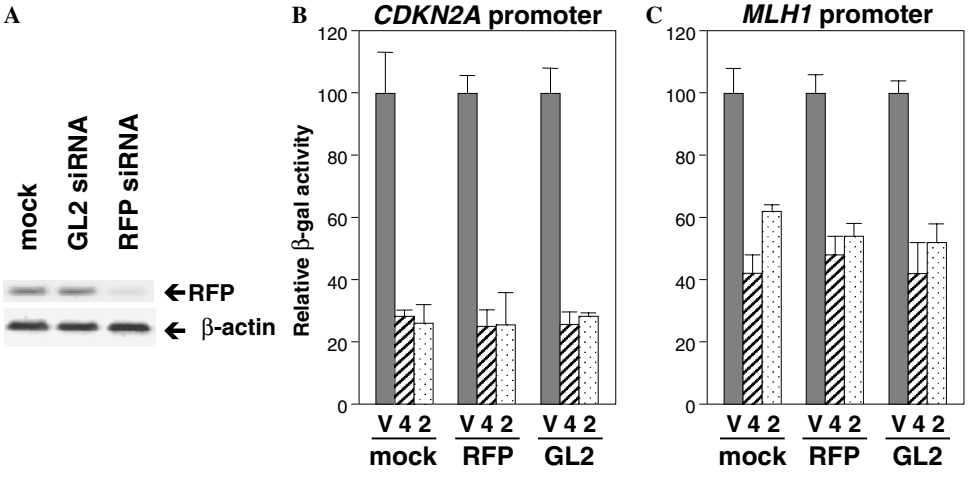


Fig. 4. Influence of siRNA targeting RFP on MBD2- and MBD4-dependent transcriptional repression. (A) Treatments with mock, siRNA targeting against RFP or GL2 were performed using CHO-K1 cells, and the immunoblot assay for RFP and β-actin (internal control) was performed 72 h after siRNA transfection. (B) At 24 h after siRNA transfection described in (A), *Sss*I-methylated pSF100 reporter with *CDKN2A* or *MLH1* promoter was co-transfected with pGV-C2 and the empty pcDNA3.1/V5-His vector, the MBD4 expressing plasmid (pcDNA-MBD4), or the MBD2 expressing plasmid (pcDNA-MBD2). The relative β-galactosidase activities were determined as described in Fig. 2. V, vector; 4, pcDNA-MBD4; 2, pcDNA-MBD2; RFP, RFP siRNA treatment; GL2, GL2 siRNA treatment.

highly methylated satellite DNA in murine cells [22]. Given all these results, the data suggest that MBD2 and MBD4 may co-operate with each other to contribute to transcriptional repression in cancer-related genes.

Transcriptional repression by MBD2 as well as by MBD4 was not affected even though RFP was reduced up to about 80% at the protein level by using siRNA technology. This fact suggests that RFP is not likely to be an indispensable component in MBD2- and MBD4-mediated transcriptional repression. In fact, we previously showed that MBD4 directly interacted with corepressor SIN3A and HDAC1 [11]. Therefore, the function of RFP should be exhibited in some specific circumstances. In this context, it is interesting to know that Northern blot analyses have demonstrated high levels of RFP expression in a variety of tumor cell lines, the testis, and embryos compared with the extremely low levels of RFP expression in most adult tissues [13]. Because global *de novo* methylation has also been well documented during germ-cell development, early embryogenesis, and early tumorigenesis, the high level expression of RFP is well matched by these timings. Taken together, it is logical to think that MBD2 and MBD4, mediated by RFP, might work together to repress transcription in cancer-related genes and developmentally regulated genes. Further analysis is needed to clarify whether RFP plays a role to enhance transcriptional repression by MBD2 and MBD4 in spermatogenesis, embryogenesis, and tumorigenesis.

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