

# TACC3 mediates the association of MBD2 with histone acetyltransferases and relieves transcriptional repression of methylated promoters

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## ABSTRACT

We have recently reported that a novel MBD2 interactor (MBDin) has the capacity to reactivate transcription from MBD2-repressed methylated promoters even in the absence of demethylation events. Here we show that another unrelated protein, TACC3, displays a similar activity on methylated genes. In addition the data reported here provide possible molecular mechanisms for the observed phenomenon. Immunoprecipitation experiments showed that MBD2/TACC3 form a complex *in vivo* with the histone acetyltransferase pCAF. MBD2 could also associate with HDAC2, a component of MeCP1 repression complex. However, we found that the complexes formed by MBD2 with TACC3/pCAF and with HDAC2 were mutually exclusive. Moreover, HAT enzymatic assays demonstrated that HAT activity associates with MBD2 *in vivo* and that such association significantly increased when TACC3 was over-expressed. Overall our findings suggest that TACC3 can be recruited by MBD2 on methylated promoters and is able to reactivate transcription possibly by favoring the formation of an HAT-containing MBD2 complex and, thus, switching the repression potential of MBD2 in activation even prior to eventual demethylation.

## INTRODUCTION

Methylated DNA is generally associated with transcriptional silencing. Several recent studies have addressed the

mechanisms by which methyl-CpG signal is targeted, read and maintained by the different components of the DNA methylation system. A family of mammalian methyl-CpG-binding proteins, including MeCP2, MBD1, MBD2, MBD3, Kaiso and MBD4, has been found to be essential to interpret the methylation patterns and to mediate the biological consequences of DNA methylation (1–4). All methyl-binding proteins share a common structural stretch of 60–80 amino acids called mCpG-binding domain (MBD) and, with the exception of MBD4 (5), may mediate transcriptional repression by changing the local chromatin structure mainly through recruitment of histone deacetylases (HDACs) (1–3,6,7).

However, it seems likely that the activity of methyl-binding proteins depends on their association with different molecular partners. The best example of a multiple functions methyl-binding protein is MBD2. MBD2 exerts different activities depending on its association with different partners. MBD2 can be a component of a large protein complex, MeCP1, which represses transcription from densely methylated genes. MeCP1 includes HDAC1, HDAC2 and RbAp46/48 proteins (6,7). This way, MBD2 targets deacetylase activity at methylated sites. MBD2 can also recruit on methylated DNA a different corepressor complex, Mi-2/NuRD, through its heterodimerization with MBD3 (8,9). Other authors have identified a protein, MIZF, that associates with MBD2 and significantly enhances HDAC proteins recruitment and activity (10). In addition, MBD2 forms a complex with DNA methyltransferase 1 (DNMT1) on hemimethylated DNA at replication foci and may help to establish or maintain the repressed state of chromatin (11). While the above described functions result in transcriptional repression, recently it is emerging that when MBD2 associates with other partners, it may have opposite effects resulting in transcriptional activation of methylated genes. In a recent study, it was shown that the

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viral protein Tax can activate transcription from the methylated HTLV-1 long terminal repeat through the interaction with MBD2 (12). The transcription factor GATA-3 can displace MBD2 from a methylated promoter causing the transcriptional reactivation of GATA-3 responsive genes (13). We have recently described a novel MBD2 associating protein, *MBDin*, which shows the unique ability to reactivate MBD2-repressed genes still in methylated status (14). In the case of *MBDin*-mediated reactivation, as well as for GATA-3, transcriptional reactivation occurs prior to demethylation. However, *MBDin* is recruited by MBD2 on methylated DNA and acts by interfering with the ability of MBD2 to associate with the repressor complex rather than through displacement of MBD2 from methyl-CpG sites (14). Recently it has been shown that MBD2 interacts with RNA helicase A, a component of CREB transcriptional coactivator complex (15). All these mechanisms lead to activation of methylated genes not through a possible active demethylase function of MBD2, but, rather, through the association of MBD2 with a variety of factors that can be recruited on methylated DNA and determine different interpretations of DNA methylation signals.

In the present work we have identified TACC3 (transforming-acid-coiled-coil), as a novel MBD2 associating protein. We demonstrate that MBD2 and TACC3 may interact both at centrosomes in mitosis and in the interphase nucleus. We show that TACC3/MBD2 may form a complex with histone acetyltransferases and reactivate transcription from methylated genes. Our findings provide a possible general mechanism by which methylated genes can be reactivated through a switch from a closed to an open chromatin configuration.

## MATERIALS AND METHODS

### Construction of fusion genes and expression plasmids

Plasmids pBridge-MBD2, pGal4-MBD2, pBridge-ΔMBD2, pBridge-MUT1, pBridge-MUT3 and pBridge-MUT4, used for two-hybrid screening and specificity tests, and pMyc-MBD2 for immunoprecipitation experiments, have been described previously (14). The plasmid hTACC3-pCR2 Topo cloning (kindly provided by T. R. J. Lappin) containing the full length TACC3 coding sequence (16) was partially digested with EcoRI and the entire TACC3 cDNA was subcloned into the EcoRI sites of pCEFL-HA expression vector (17) to generate pHA-TACC3. pHA-ΔTACC3 expression vector was generated by cloning a 1290 bp TACC3 fragment (amino acids 409–837) into pCEFL-HA (EcoRI and XbaI sites). Primers used for amplification, containing restriction sites (underlined), were: TACC3Δ-EcoRI/F (5'-AGTC GAA TTC ATG GAT GAC CCA AAC TTC ATC-3') and TACC3-XbaI/R (5'-AGTC TCT AGA AGG TCA GAT CTT CTC CAT CTT G-3').

### Yeast two-hybrid assay

We performed yeast two-hybrid screens by yeast mating according to the manufacturer's instructions (Clontech). We used a human bone marrow and a brain cDNA libraries

pretransformed in the Y187 *Saccharomyces cerevisiae* strain cloned downstream of the Gal4AD in the pGADGH plasmid (Clontech). pBridge-MBD2 (14) was used as a bait and transformed in the PJ692A *S.cerevisiae* strain using the lithium acetate method and then treated according to the manufacturer's instructions. The diploid strains were cultured on high stringency plates lacking histidine, adenine, tryptophan and leucine. The resultant clones were tested for β-galactosidase activity using a colony-lift filter assay (18). Purification and sequencing of positive clones, tests for interaction specificity and measurement of protein interaction were performed as described (14).

### Cell culture, transient transfections and CAT assays

293T, MCF7 and Cos-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (Life Technologies). Cells were plated at a density of  $\sim 5 \times 10^5$  per 100 mm Petri dish 24 h before transfections. DNA transfections were carried out by calcium phosphate precipitation using Calphos (Clontech) or lipofectAMINE™ plus reagents (Invitrogen) according to the manufacturer's instructions. For transcription assays, cultures were cotransfected with test plasmids (5 μg) and different amounts of effector plasmids as indicated in the text. The β-galactosidase expression plasmid, pSVβ-gal (Promega) (2 μg), was used as an internal control for transfection efficiency. Chloramphenicol acetyltransferase (CAT) assays were performed with different amounts of extracts to ensure linear conversion of the chloramphenicol with each extract and the results are presented as the means of at least three independent transfection experiments. The CAT activity was quantified using the Molecular Dynamics PhosphorImager System TM. Total protein extracts (5 μg) were assayed for β-galactosidase activity as previously described (19).

Rat thyroid PC Cl3 cell lines were maintained in Coon's modified F12 medium (EuroClone, Milano, Italy) supplemented with 5% newborn calf serum (HyClone, Logan, UT) and six growth factors (6H), including TSH (1 mU/ml), and insulin (10 μg/ml) as previously described (20). DNA transfections were carried out by lipofectAMINE™ plus reagents (Invitrogen) according to the manufacturer's instructions.

### Drug treatments and quantitative RT-PCR analysis

PC Cl3 cells were treated with different amounts of trichostatin A (TSA, Invitrogen) or of 5-aza-2'-deoxycytidine (ICN Biomedical Inc.) for 36 h. Total RNA was isolated with TRIzol Reagent (Invitrogen). QantiTect Reverse Transcription kit (QUIAGEN) was used to generate cDNA. The following galectin-1-specific primers were used: Gal-1/Fw (5'-CTCG-GGTGGAGTCTTCTGAC-3') and Gal-1/Rv (5'-TCCCCAG-GTTTGAGATTGAG-3'). Amplification of rat G6PD cDNA was used as the endogenous control using the following primers: G6/fw (5'-TCCTCTATGTGGAGAATGAACG-3') and G6/rev (5'-TCATTCAGAGCTTTGCCACA-3'). Quantitative PCR amplifications were then performed using sybr green (Applied Biosystems) in a Chromo4 Real Time thermocycler (BIORAD). Calculations of relative expression levels were performed using the  $2^{-\Delta\Delta Ct}$  method (21).

### Immunoprecipitation and immunoblotting

293T and MCF7 cell extracts were prepared in Nonidet P-40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl) with proteinase inhibitors (Roche Molecular Biochemicals). Immunoprecipitations were performed as described previously (17). The samples were immunoprecipitated with mouse monoclonal anti-HA (Roche Molecular Biochemicals), mouse anti-myc, rabbit anti-HDAC2 and mouse anti-pCAF antibodies (Santa Cruz), and resolved on 12 and 10% SDS-PAGE. Immunoprecipitated (IP) proteins were analysed by immunoblotting performed by using goat polyclonal anti-HA, mouse anti-myc, goat anti-TACC3, rabbit anti-HDAC2, mouse anti-pCAF (Santa Cruz) and rabbit anti-MBD2a (Abcam) antibodies. Immunoblots were stained with correspondent secondary antibodies (Amersham Biosciences) and revealed with the enhanced chemiluminescence detection system (Amersham Biosciences).

### *In vitro* DNA methylation

pGAT1700 plasmid (50 µg) (22) were treated with 50 U of SssI methylase (New England Biolabs) at 37°C in the presence of 5 mM adenosylmethionine for 8 h. Complete methylation of treated plasmids was confirmed by HpaII restriction enzyme digestion (data not shown). After transfections, cells were harvested and used for transcription assays. Methylation status of pGAT1700 plasmid was determined by bisulfite analysis at different times after transfections, as previously described (14).

### Immunocytofluorescence

Cos-7 and NIH3T3 cells were incubated in 8% BSA in phosphate-buffered saline (PBS) for 1 h at room temperature (RT). Cells were then incubated with primary antibodies: goat polyclonal anti-MBD2 antibodies (1:200), mouse monoclonal anti-γ-tubulin (Sigma) (1:2000), rabbit polyclonal anti-MBD2a (Abcam) antibodies and goat polyclonal anti-TACC3 and were diluted in 2% BSA-PBS incubated 2 h at RT. For double immunofluorescent staining, the primary antibodies were incubated in unison. After extensive washing with PBS, these antibodies were detected by appropriate secondary antibodies as follows: Alexa Fluor 488-conjugated goat anti-mouse Ig antibodies (Molecular probes), Alexa Fluor 568-conjugated rabbit anti-goat Ig antibodies (Molecular probes), Alexa Fluor 568-conjugated goat anti-rabbit Ig antibodies and Alexa (Molecular probes) and Fluor 488-conjugated rabbit anti-goat Ig antibodies (Molecular probes). Secondary antibodies were diluted 1:100 in 2% BSA-PBS and incubated for 45 min at RT. Cells were then incubated in PBS containing 5 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 10 min at RT. After incubation, the cells were washed in PBS and coverslips were mounted with 4.8% Mowiol in 50 mM Tris pH 8.5. Images were obtained using a Zeiss Axioskop MC 100 fitted with a camera and all the images were acquired with a 60× Plan-Neofluar objective. Images were subjected to scale adjustment using image software (Photoshop, Adobe Systems, Inc., San Diego, CA).

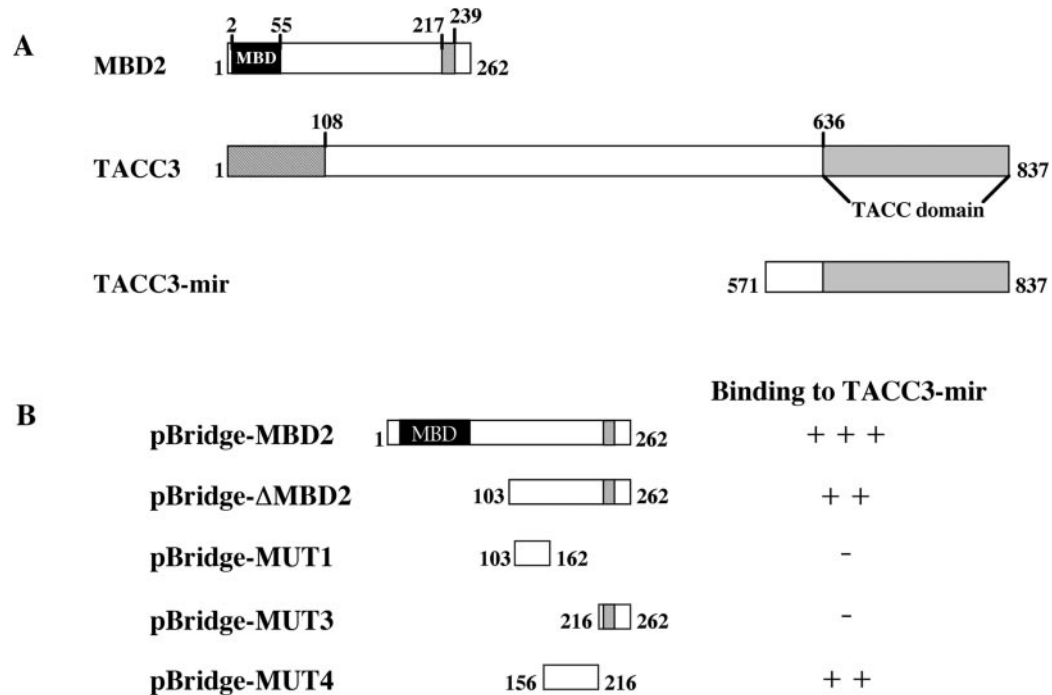
### Non-radioactive Histone Acetyltransferase Activity Assay

293T cells were transfected and nuclear extracts immunoprecipitated with mouse monoclonal anti-Myc, and subjected to the assay. Nuclear proteins were differentially extracted by lysing cells in ice-cold hypotonic buffer (0.2% NP-40, 10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl). Nuclei were separated through a 60% sucrose cushion and lysed in HB buffer (250 mM Tris-HCl, pH 7.8, 60 mM KCl) by freeze-and-thaw treatment. Non-radioactive HAT assay was done using a HAT assay kit (Upstate Biotechnology, Inc.), according to the manufacturer's protocol. Briefly, each immunoprecipitated fraction of nuclear extract, mixed with 100 mM acetyl-CoA and 1×HAT assay buffer, was incubated on an enzyme-linked immunosorbent assay plate precoated with histones H3 and H4 for 5 or 10 min. After several washes with Tris-buffered saline, acetylated histones were detected using an anti-acetyl-lysine rabbit polyclonal antibodies followed by the horseradish peroxidase-based colorimetric assay. A recombinant pCAF (25 ng) (Upstate Biotechnology, Inc.) was used as a positive control. Data were expressed as optical density (OD) at 450 nm.

## RESULTS

### Interaction screening

To identify new partners of the MBD2 protein, we performed interaction screening using the two-hybrid system in yeast (Clontech). We have simultaneously analysed a bone marrow and a brain cDNA libraries. A total of  $\sim 3 \times 10^6$  clones were tested for each library using the short form of MBD2 cDNA, MBD2b, as bait (14). After the specificity of interaction was assessed (see Materials and Methods), we have identified a total of 92 positive clones (41 and 51 from bone marrow and brain, respectively). These clones contained cDNA inserts between 512 and 2779 bp, and, after sequencing, we could divide them in 11 different groups each containing 3–12 clones with different insert length but containing the same cDNAs. Most groups contained clones derived from the same library, possibly reflecting the tissue-specific nature of the cDNAs, while some others contained identical cDNAs derived from both libraries. Interestingly, most groups contained cDNAs encoding for transcriptional repressors or activators. We chose to analyse in detail Group F, which included eight clones, all derived from bone marrow library and each containing part of the coding sequence of transforming acid coiled-coil protein 3, TACC3 (DDBJ/EMBL/GenBank accession number AJ243997). TACC3 was a very interesting candidate MBD2 interactor because of its capacity to associate with chromatin remodeling factors (23). TACC3 is a 100 kDa protein belonging to the family of transforming-acidic coiled-coil containing (TACC) proteins including three human members (24–26). TACC3 contains three domains (Figure 1A): a conserved N-terminal region of 108 amino acids, encoded by exons 2 and 3 in each vertebrate TACC3 gene, an highly variable central region and the conserved TACC domain (C-terminal region from amino acids 636 to 837) that share  $\sim 60\%$  identity with the C-terminal region of TACC1 and



**Figure 1.** Definition of MBD2 and TACC3 binding region in yeast. (A) Schematic representation of structural and functional domains of MBD2 and TACC3 proteins. For MBD2, the methyl-binding-domain (MBD) is indicated as a black box; the putative coiled-coil domain is indicated as a gray box. For TACC3, the striped box contains a conserved N-terminal region and the TACC domain is indicated as a gray box. TACC3-mir (minimal interacting region) represents the minimal TACC3 region interacting with MBD2 in yeast. (B) Mapping of the TACC3-binding region of MBD2 by yeast two-hybrid experiments. Full length and deletion mutants of MBD2 were fused to GAL4 DNA binding domain (bait) and the TACC3-mir (amino acids from 571 to 837) was fused to the GAL4 activation domain (pGAD-F3 plasmid clone). The interaction between the bait proteins and prey was tested on the basis of the His and Ade reporter gene activation and by quantitative liquid  $\beta$ -galactosidase assay.

TACC2 (24). All the eight positive clones of group F contained the entire TACC domain. The shortest one (pGAD-F3) contained TACC3 sequences encoding a 267 amino acids polypeptide (from amino acids 571 to 837) indicating that this region of TACC3 is sufficient for the interaction with MBD2 (Figure 1A).

#### Interaction of MBD2 deletion mutants with TACC3 in yeast

To narrow the regions of MBD2 able to interact with TACC3, deletion experiments were performed in yeast. As shown in Figure 1B, deletion of MBD2 N-terminal 103 amino acids (pBridge- $\Delta$ MBD2) did not affect the ability of MBD2 to associate with TACC3, demonstrating that the methyl-binding domain (MBD) is dispensable for the interaction. The further removal of 100 amino acids at the C-terminus (pBridge-MUT1) abolished the capacity of MBD2 to bind TACC3 showing that the C-terminal region is necessary for the interaction. The analysis of pBridge-MUT3 and pBridge-MUT4 demonstrated that the MBD2 region from amino acids 156 to 216 is sufficient for the interaction with TACC3, while the extreme C-terminus (amino acids 216–262) is dispensable.

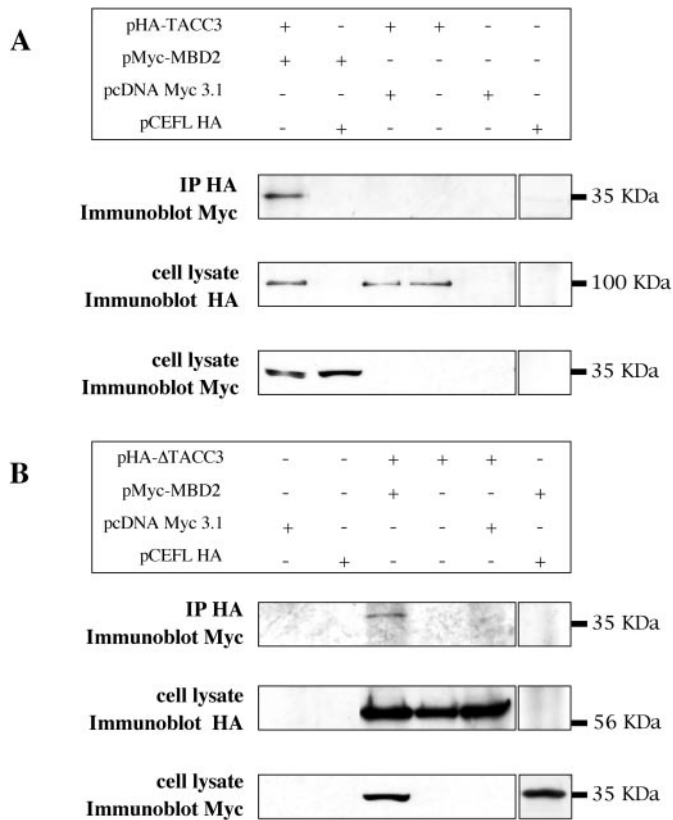
#### Immunoprecipitation experiments

Coimmunoprecipitation experiments were performed to investigate whether MBD2 and TACC3 form a complex in mammalian cells (Figure 2). 293T cells were transfected with expression vectors containing the myc-tagged full length

MBD2 cDNA (pMyc-MBD2), together with HA-tagged full length TACC3 cDNA (pHA-TACC3) or a TACC3 mutant form containing the C-terminal 429 amino acids (pHA- $\Delta$ TACC3). Subsequently, cell lysates were immunoprecipitated with anti-HA antibodies. The immunoprecipitated fractions and whole cell extracts were then immunoblotted and hybridized with anti-myc or anti-HA antibodies. As shown in Figure 2, both TACC3 and  $\Delta$ TACC3 coimmunoprecipitated with MBD2 (Figure 2A and B, respectively). Empty vectors were also cotransfected as negative controls. These results confirm that in mammalian cells MBD2 and TACC3 form a complex and that the TACC3 C-terminal region (amino acids 409–837), containing the TACC domain, is sufficient for the interaction.

#### TACC3 affects the activity of methylated promoters

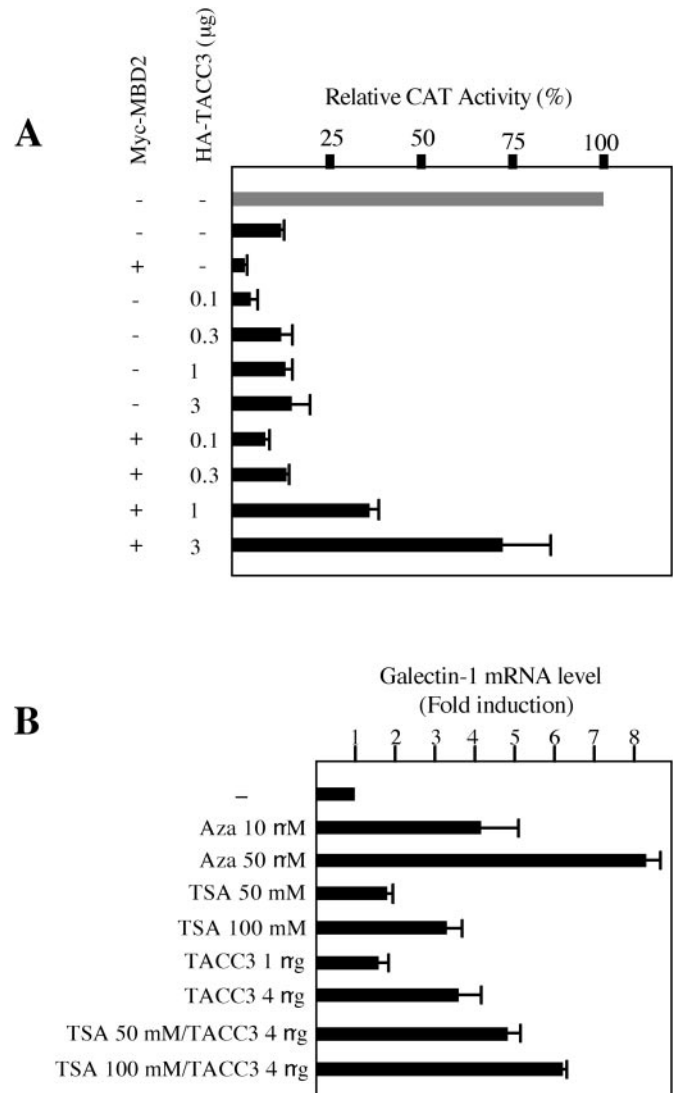
To explore whether TACC3 could affect the transcriptional repression potential of MBD2, we performed transient transfection assays into human 293T cells using a methylated promoter. To this aim we have used a CAT reporter plasmid (pGAT1700) (22) containing 1700 bp of the mouse galectin-1 promoter that can be repressed by *in vitro* DNA methylation (27) and, when methylated, is bound by MBD2 (14). Transcription assays were performed after transfection of MBD2 and TACC3 expression vectors, along with either unmethylated or *in vitro* methylated pGAT1700 construct in 293T cells. The results (Figure 3A) clearly showed that TACC3 was able to reactivate, in a dose dependent manner,



**Figure 2.** *In vivo* association of MBD2 with TACC3 and  $\Delta$ TACC3. 293T cells were transfected in the indicated combinations with the following expression plasmids: (A) pHA-TACC3, pMyc-MBD2 and control empty vectors; (B) pHA- $\Delta$ TACC3, pMyc-MBD2 and control empty vectors. The whole cell extracts were prepared and subjected to immunoprecipitation with goat polyclonal anti-HA antibodies. Immunoprecipitated (IP) proteins were analysed by immunoblotting with mouse monoclonal anti-Myc and anti-HA antibodies.

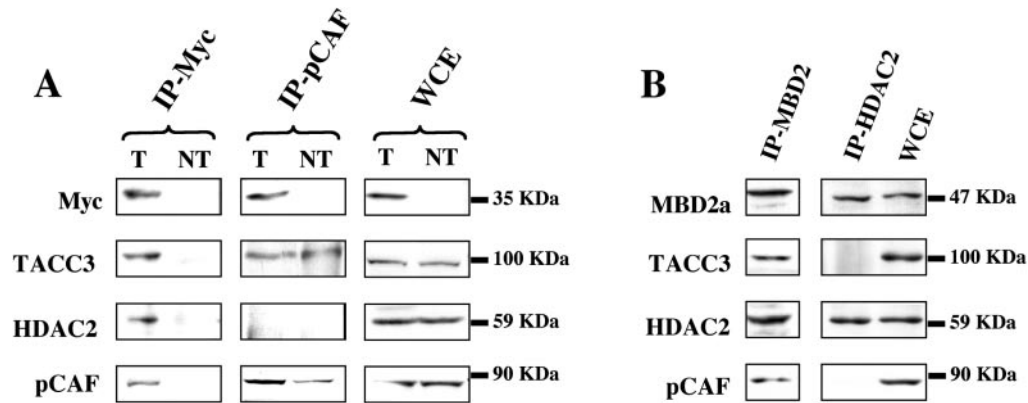
the methylated promoter (Figure 3A). Interestingly, the reactivation was much stronger in the presence of over-expressed MBD2, indicating that MBD2 is essential for the recruitment of TACC3 on the methylated promoter. In contrast, the activity of unmethylated pGAT1700 was not significantly affected by either TACC3 or MBD2 exogenous expression (data not shown). To exclude that reactivation of the methylated promoter was a consequence of a demethylation event, we performed, in parallel with transcriptional assays, a sodium bisulfite methylation analysis. Seven CpG sites contained in the -177/+11 region of the transfected mouse galectin-1 promoter, whose methylation state is critical for transcriptional activity (14,28,29), were analysed using mouse-specific bisulfite-modified primers. No significant change of methylation pattern was observed up to 60 h after transfection of the reporter gene alone or in combination with TACC3 and/or MBD2 (data not shown) indicating that the reactivation observed in the transcriptional assays occurred when the promoter was still in a fully methylated status.

Finally, we investigated whether TACC3 was able to reactivate endogenous methylated genes. We used a rat thyroid cell line, PC Cl3, in which galectin-1 endogenous gene is heavily methylated and barely expressed (27). Our results showed that exogenous expression of TACC3, as well as treatment



**Figure 3.** Influence of MBD2 and TACC3 over-expression on activity of methylated transfected or endogenous galectin-1 promoter activity. (A) 293T cells were transfected with 3  $\mu$ g of unmethylated (gray bar) or *in vitro* methylated (black bars) pGAT1700 reporter plasmid. Cotransfected expression plasmids were pSV $\beta$  (2  $\mu$ g) and, where indicated, pMyc-MBD2 (2  $\mu$ g) and pHA-TACC3 at different amounts. The level of transcription is given as a percentage of CAT activity seen in the presence of unmethylated pGAT1700 reporter plasmid alone (set as 100%). Percent relative activity of the promoters is expressed as the ratio of CAT to  $\beta$ -galactosidase activity. The results are presented as an average of multiple experiments  $\pm$  SD. Each histogram bar represents the mean of at least four independent transfections. (B). Quantitative RT-PCR assays of endogenous galectin-1 mRNAs were performed on PC Cl3 cells untreated or treated with the indicated concentrations of 5-aza-2'-deoxycytidine (Aza) or Trycostatin A (TSA) for 36 h. Where indicated (TACC3), cells were transfected with pHA-TACC3 expression vector. mRNA expression levels are shown as fold-induction compared with the PC Cl3 value, arbitrarily set as 1. The results are presented as an average of at least three independent experiments  $\pm$  SD.

of cells with the deacetylase inhibitor TSA or the demethylating drug azacytidine, induced a significant increase of endogenous galectin-1 gene expression within 36 h in a dose dependent manner (Figure 3B). Moreover our results showed that TACC3 displays a synergistic effect with TSA treatment (Figure 3B).



**Figure 4.** *In vivo* interactions of MBD2, TACC3, pCAF and HDAC2. (A) Cell lysates from MCF7 untransfected cells (NT) or transfected with pMyc-MBD2 plasmid (T) were immunoprecipitated (IP) and immunoblotted with the indicated antibodies. Anti-myc antibodies were used to detect Myc-MBD2 fusion protein. WCE: whole cell extract. (B) whole cell lysates from MCF7 cells were subjected to immunoprecipitation with anti-HDAC2 antibodies, as indicated. Western blots were performed with the indicated antibodies. Anti-MBD2a antibodies were used to detect endogenous MBD2a protein.

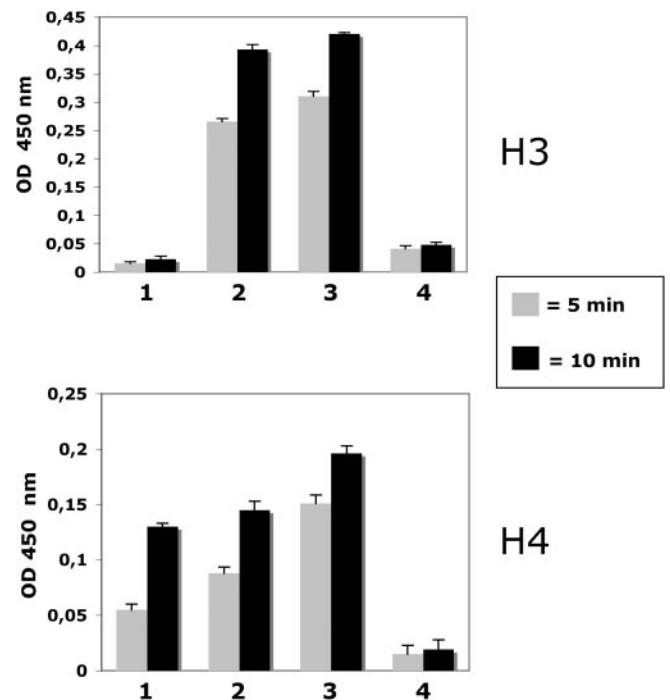
### MBD2/TACC3 complex interact with a nuclear histone acetyltransferase (pCAF)

We next investigated potential mechanisms that could account for the observed effects of MBD2/TACC3 complex on the activity of methylated promoters. Because TACC3 was recently found in a protein complex containing endogenous histone acetyltransferases (23), we hypothesized that TACC3 might favor a switch to an open chromatin configuration on MBD2-bound promoters. We therefore investigated the possibility that MBD2, through the interaction with TACC3, may recruit HATs on methylated promoter. To test this hypothesis, we used a breast cancer cell line (MCF7) in which endogenous TACC3 forms a complex with pCAF (23). In this cell line, MBD2a and HDAC2 endogenous proteins were expressed at detectable levels, as evidenced by western blot analysis (Figure 4). Immunoprecipitation experiments were performed using anti-Myc, anti-MBD2a, anti-pCAF and anti-HDAC2 antibodies, respectively, and western blots were performed using the same antibodies and, in addition, the anti-TACC3 antibodies (Figure 4). Results showed that both exogenous Myc-tagged MBD2b (Figure 4A) and endogenous MBD2a (Figure 4B) proteins form a complex with pCAF and TACC3. MBD2 could also bind HDAC2, as extensively reported (6,7). However, our experiments show that the complexes formed with MBD2 by HDAC2 and by TACC3/pCAF were mutually exclusive (Figure 4B).

In order to investigate whether the formation of MBD2/TACC3/pCAF complex is cell cycle specific, similar immunoprecipitation experiments were performed in synchronized MCF7 cells. Cells were grown for 12 h in the presence of 0.5% serum. Immunoprecipitation experiments were performed at time points 0, 1, 3, 6, 12, 18 and 24 h after 10% serum was restored. Results show that the MBD2/TACC3/pCAF complex was present at all time points (data not shown) indicating that the formation of the complex is not cell cycle specific.

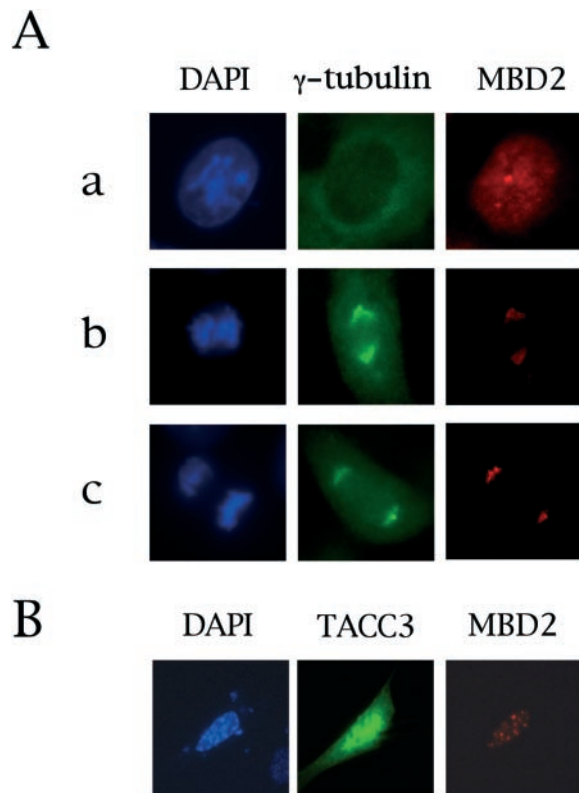
### HAT activity associates with MBD2

The above experiments indicate that MBD2 and the acetyltransferase pCAF may be part of a protein complex. Thus, we next analysed whether HAT enzymatic activity could be



**Figure 5.** Analysis of MBD2-associated acetyltransferase activity. 293T cells were transfected with pMyc-MBD2 (4  $\mu$ g) (lane 2) alone or in combination with pHA-TACC3 (2  $\mu$ g) (lane 3). After 48 h transfection nuclear extracts were immunoprecipitated with anti-Myc antibodies. Recombinant pCAF (25 ng) (lane 1) and immunoprecipitated fractions from untransfected cells (lane 4) were used as controls. The HAT activity of each immunoprecipitated fractions was determined by *in vitro* colorimetric HAT assay. Incubation times of the assays were 5 min (gray columns) and 10 min (black columns). The acetylation status of histones H3 (top panel) and H4 (bottom panel) is represented as OD values at 450 nm.

associated with MBD2 and whether TACC3 may favor such association. For this purpose, 293T cells were transfected with pMyc-MBD2 alone or in combination with pHA-TACC3 expression vector. Then, the HAT enzymatic activity, coprecipitating with MBD2, was assayed. The assays were performed both on histones H3 and H4. The results, presented in Figure 5, show that HAT activity consistently associated with MBD2 protein even in the absence of exogenous TACC3



**Figure 6.** (A) MBD2 localizes at centrosomes in M phase. Asynchronous Cos-7 cells were subjected to immunofluorescence study using goat anti-MBD2 antibodies (red) and mouse anti- $\gamma$ -tubulin antibodies (green). DNA was stained with DAPI (blue). (a) interphase; (b) metaphase and (c) anaphase. (B) MBD2/TACC3 colocalization in NIH3T3 cells in interphase nucleus.

expression. However, TACC3 over-expression significantly enhanced the MBD2-associated HAT activity, in particular when H4 was used as a substrate.

These data suggest that MBD2 associates with HATs in a direct manner or, more likely, through different bridge proteins and that TACC3 may actively contribute in providing the MBD2 complex with HAT activity.

#### Centrosomal localization of MBD2

Finally, because TACC3 localizes at centrosomes in mitosis (25), we tested the possibility that MBD2 protein is recruited at centrosomes as well. Asynchronous Cos-7 cells were subjected to immunostaining study using goat anti-MBD2 antibodies (red) and mouse anti- $\gamma$ -tubulin antibodies (green) as centrosomes indicator. In interphase, MBD2 localizes in the nucleus (Figure 6A, a), as extensively reported (1,13,14). In contrast, during different phases of mitosis, MBD2 mainly localizes at centrosomes (Figure 6A, b and c).

In order to establish whether MBD2 and TACC3 co-localize in interphase nuclei, we performed an immunofluorescence analysis in NIH3T3 mouse cells. Results showed that TACC3 partially colocalizes with MBD2 at nucleus (Figure 6B). However, localization of TACC3 was diffuse and not limited to the spots of hypermethylated DNA which was seen for MBD2 and in concordance with possible multiple functions of TACC3 in interphase nuclei.

These observations indicate that TACC3 and MBD2 associate both in interphase nucleus and at centrosomes during cell division.

Interestingly, other components of the NuRD complex, such as MBD3 and HDAC1, have been recently localized at mitotic centrosomes (30). Our findings further support the hypothesis that the NuRD complex play a yet uncharacterized, cell cycle controlled, role at the centrosomes and suggest that TACC proteins may be involved in this subcellular localization during mitosis.

#### DISCUSSION

In the present work we show that TACC3 associates with MBD2 and may form a complex with histone acetyltransferases switching the MBD2-mediated transcriptional repression to activation without involving promoter demethylation.

Searching for MBD2 partners, we have recently demonstrated that *MBDin*, a novel GTPase protein, is able to associate with MBD2 and to reactivate methylated genes without altering the methylation pattern of the target genes (14). However, the mechanisms underlying this phenomenon remain poorly understood. In the present study we have specifically selected other MBD2 partners that might share with *MBDin* a similar activity. Among several clones positive for MBD2 interaction in yeast, TACC3 was chosen because of its potential ability to associate with histone acetyltransferases, as recently reported (23). Compatible with a physiological relevance of their interaction, TACC3 and MBD2 are ubiquitously expressed in mammalian tissues, even if the relative proportion may vary among different tissues. (1,24).

TACC3 belongs to a recently identified and evolutionary conserved protein family referred as TACC family (24,26). Mammalian TACC genes encode highly acidic proteins characterized by a conserved and unique 200 amino acids coiled-coil domain termed the 'TACC domain', but lacking significant homology outside this domain. All the TACC proteins are associated with the mitotic centrosome/spindle apparatus (25) and localize in the nucleus during interphase. In particular, for TACC3, the nucleus represents the major interphase subcellular localization. Noticeably, as mentioned above, it has been recently demonstrated that TACC2 and TACC3 proteins may form a complex with different histone acetyltransferases, including pCAF. Thus, it has been proposed that such a mechanism could account for the observed function of TACC3 as a transcriptional co-activator of different nuclear factors (23,31).

In the present paper we show that TACC3, as well as *MBDin*, has the capacity to relieve the MBD2-mediated transcriptional repression of methylated genes even in the absence of demethylation events. The protein structure of TACC3 does not display any evident feature in common with *MBDin* and the interaction with MBD2 occurs through different regions of this methyl-binding protein. While *MBDin* interacts with the extreme C-terminal of MBD2 (amino acids 216–262) here we found that the same region is not essential for the association with TACC3 which, instead, occurs through a more proximal portion of MBD2 (amino acids 156–216). This is compatible with a simultaneous binding of the two partners to MBD2 and with eventual synergistic activities. Moreover, we show that

the C-terminal region of TACC3 (amino acids 571–837) is sufficient for the interaction with MBD2 both in yeast and in mammalian cells. This region includes the TACC domain, raising the possibility that other members of the TACC family might associate with MBD2. Further investigation will be necessary to verify the occurrence and the functional role of such potential multiple interactions.

While it is well known that MBD2 is an essential component of the chromatin repressor complex such as the MeCP1 complex, which includes different histone deacetylases (HDAC1 and HDAC2), the possibility that MBD2 could form alternative complexes with HATs, natural antagonist of deacetylases, has been to date poorly investigated. Because TACC3 associates with the histone acetyltransferase pCAF (23), we have addressed the intriguing possibility that MBD2, as a partner of TACC3, could also be part of the same complex. Coimmunoprecipitation experiments of both exogenous and endogenous proteins showed that this was the case (Figures 2 and 4). It has been reported that some transcription factors (YY1 and Sp1) may interact with both HATs and HDACs, thereby acquiring an activator or repressor function depending on the promoter context or on the presence of other factors (32). Recently, it has also been demonstrated that HAT and HDACs directly interact and immunoprecipitate in the same fractions (33). Our data indicate that, although both TACC3/pCAF and HDAC2 may associate with MBD2 in MCF7 cells, they are part of distinct complexes (Figure 4). In any case our findings reinforce the general concept that the actual activity of a promoter depends on a dynamic equilibrium of HATs and HDACs recruitment (33) and extend this view to the mechanisms regulating the activity of methylated promoter. It is likely that methyl-binding proteins play a central role in the establishment of such equilibrium on methylated genes. In fact, our data show that, in addition to HDAC, also HAT enzymatic activity associates with MBD2 *in vivo*. Interestingly, we observed that a large amount of HAT activity associates with MBD2 even in the absence of TACC3 indicating that TACC3 may favor such association but it is not essential. Although our data show that the reactivation of methylated galectin-1 gene occurs in a short time in the absence of demethylation events, we cannot exclude the intriguing possibility that the acetylation might trigger promoter demethylation in a longer period. In conclusion, our data suggest that MBD2 is not just a simple mediator of transcriptional repression functioning by recruitment of chromatin repressor complexes on densely methylated promoters. We wish to propose that methyl-binding proteins may mediate both transcriptional repression and reactivation of methylated genes depending on the dynamic equilibrium of enzymatic activities recruited on methylated promoters. Different proteins, including TACC3, might favor a prevailing recruitment of HAT activity, therefore leading to changes of chromatin configuration sufficient to reactivate methylated genes even in the absence of demethylation events.

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