UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

DOTTORATO DI RICERCA IN GENETICA CELLULARE E MOLECOLARE

TESI

"TACC3 mediates the association of MBD2 with histone acetyltransferases: a novel mechanism for reactivation of methylated promoters "

Coordinatore
Prof. C. B. Bruni

Candidato
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ANNO

2005

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1. INTRODUCTION

Symmetric methylation of cytosine in CpG dinucleotides is one of the widespread modifications in animal genomes. It is, generally, associated with “closed” (inactive) chromatin state and, therefore, negative regulation of transcription. To date, this modification has been found both in invertebrates (*Drosophila melanogaster)* (Hung et al., 1999) and in chordates (from *Ciona intestinalis* to mammals) (Simmen et al., 1999). It is known that DNA methylation plays an important role in so-called “epigenetic” regulation of gene expression-regulation that is not directly dependent on primary structure of DNA, but is maintained by many protein or non-protein factors (like histone modification, chromosome territory, etc., i.e., “epi”-genomic factors). DNA methylation affects gene expression directly or indirectly. Some transcriptional factors (i.e., Sp1) can interact only with non-methylated DNA sequences, whereas methylation of cytosine abolishes interaction (Clark et al., 1997). This in turn leads to less effective transcription of certain genes. On the other hand, there is a different mechanism of action of CpG methylation. So-called MBD (methyl-DNA-binding domain) proteins (Hendrich and Bird 1998) specifically recognize modified sequences and attract large multiprotein complexes that can change chromatin conformation

from “opened” to “closed”. It is generally accepted that DNA methylation is a unidirectional process. If any sequence acquires CpG methylation then this modification becomes stable and will be inherited after cell division. So, both daughter DNA molecules will have same pattern of methylation. However, CpG methylation is much more dynamic during early embryonic development. Epigenetic regulation of gene expression include embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability. Consistent with these important roles, a growing number of human diseases have been found to be associated with aberrant DNA methylation.

1.1 DNA methylation

In mammals, the considerable regulatory task of progressive gene repression has been aided by the covalent addition of a methyl group in position 5 of the cytosine base of CpG dinucleotides. Extensive investigation into the activities responsible for DNA methylation and their roles in a wide number of biological processes reinforces the importance of this extra layer of genomic complexity (Robertson and Wolffe, 2000; Li, 2002).

DNA methylation occupies up to 70% of the CpG dinucleotides in the genome, and represents one of the major epigenetic modifications in mammals (Robertson and Wolffe, 2000). The organization of the mammalian genome is such that there is a high density of CpG in the upstream promoter regions in most of the approximately 30,000 genes, as well as within gene introns and exons (Bird, 2002). This organizational feature and the capacity for DNA methylation in mammals lead to the early speculation that gene regulation might be highly sensitive to the methylation status of these so-called CpG islands (Holliday and Pugh, 1975). However, this proved not to be the case, as these potential targets for methylation were found to be unmethylated, at least for most genes under normal circumstances (Antequera and Bird, 1993; reviewed in Meehan and Stancheva, 2001). Notable exceptions to this general rule include imprinted genes and those of the inactive X-chromosome. Further genomic characterization identified other classes of sequence families with significant levels of CpG, leading to alternative proposals for the significance of this stable and heritable modification. The mammalian genome contains an extremely high burden of sequences that have arisen due to integration of retrotransposons. Uncontrolled expression of these sequences from their viral promoters would result in

transcriptional chaos, were it not for the susceptibility of these promoters to be repressed by DNA methylation (Yoder et al., 1997). This regulatory role for DNA methylation has been embodied in the genome defense hypothesis, and remains one of the significant functions of DNA methylation (Walsh et al., 1998).

One of the most comprehensively studied roles of DNA methylation is the marking of parental alleles by genomic imprinting. Imprinted genes are expressed in a non-Mendelian fashion, in which parent-of-origin specifies the active allele (Reik and Walter, 2001). These genes are essential for fetal growth and development, and have been shown to also influence postnatal growth trajectories and diverse biological processes, for example, affecting thermogenesis in offspring, maternal care and suckling behavior, and adult behavior and cognition (Li et al., 1999; Plagge et al., 2004). The marking of the active and inactive alleles is achieved though differential DNA methylation in critical regulatory regions. These differentially methylated regions (DMRs), are essential for expression or repression. It is interesting to note that a disproportionately high number of imprinted genes are found to be methylated on the maternal allele (Reik and Walter, 2001).

During development in mammals, there are at least two periods of genome-wide DNA methylation reprogramming (Reik

and Walter, 2001). The two periods best characterized include a time during primordial germ cell differentiation and one during preimplantation development. The extent of this reprogramming and whether, in fact, it is required for normal development in all mammalian species remain unknown (Bestor, 2000). In this regard, the details of DNA methylation reprogramming in humans are only just beginning to be appreciated. The degree to which this important regulatory mechanism operates in early development and germ cell differentiation in humans will influence our understanding of the potential impact of DNA methylation in human health and disease.

A growing body of evidence indicates that DNA methylation, together with chromatin modifications, are competent to specify transcriptional states and perhaps more importantly, mutually reinforce transcriptionally repressive states (Tamaru and Selker, 2001; Fuks et al., 2003; Tamaru et al., 2003).

1.2 Enzimatic activities and substrates

Introduction of the methyl group into the symmetrical dinucleotide 5-CpG-3 results in its positioning into the major groove of the DNA without interference with the base-pairing of nucleotides. Methylation of cytosine residues imposes a greater

level of risk to the stability of the genome as deamination of methyl-cytosine results in the transition of meC-T, a nucleotide base change less easily repaired and recognized than the deamination of cytosine to uracil (Hermann et al., 2004). The lack of high fidelity repair capacity for the meC-T transition explains the observation that CG sites are a major mutational hotspot, accounting for up to 30% of point mutations in the germline, and are decidedly underrepresented in mammalian genomes (Bird et al., 1985). This situation suggests that the maintenance of this modification and its functions have been the result of considerable evolutionary pressure, and that the continued maintenance of cytosine methylation must confer a significant advantage given the substantial costs. The complexity of DNA methylation in the genome suggests that there must be a number of activities responsible for its establishment and maintenance, able to operate in both specialized and generalized functions (Chen et al., 2003).

1.3 DNA Methyltransferases

DNA methyltransferases (MTases) represent a collection of three family groups numbered in order of their discovery (Bestor, 2000). These enzymes serve the two distinctive processes of

DNA methylation, the establishment of DNA methylation state by de novo methylation and, there after, the maintenance of those states by templating this information to daughter strands arising from replication (Lei et al., 1996; Okano et al., 1999). Despite some sequence similarities, the divisions of labor amongst this group can, in part, be inferred by their functional organization. Broadly, their organization can be resolved into two functional domains; the N-terminal domain comprising regulatory functions and the C-terminal catalytic domain (Fig.1) (Bestor, 2000; Robertson, 2002).

Dnmt1 was the first of the group of MTases identified, and is the largest of these activities with an extensive N-terminal regulatory domain and a smaller C-terminal domain (Bestor et al., 1988). This large regulatory domain contains a wide variety of functional motifs, including a nuclear localization signal, a proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997) interacting domain, and a replicating foci targeting region (Leonhardt et al., 1992). Dnmt1 does not function in the cell in an isolated manner, and is capable of interacting with many proteins via the N-terminus. This interaction is likely to be facilitated by the polybromo domain (PBHD), a hallmark of protein-protein interaction (Bestor and Verdine, 1994). Furthermore, this domain

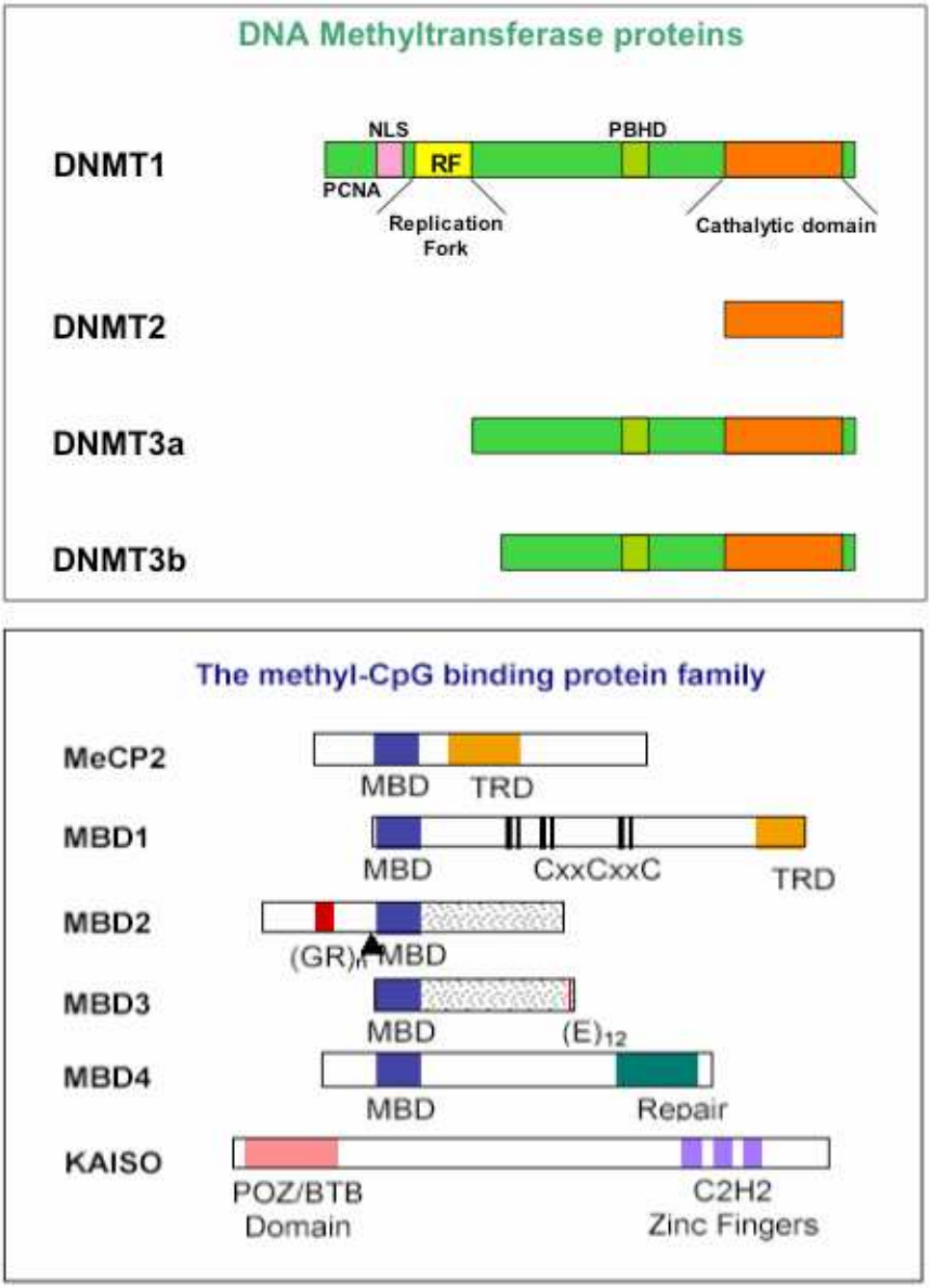

Fig.1 Schematic representation of proteins of the mammalian DNA methylation system.

has been implicated in a transport role for Dnmt1 to the replication foci (Liu et al., 1998).

Dnmt1 methylates DNA specifically at CG sites, with a strict preference for hemimethylated over unmethylated substrates in vitro (Pradhan et al., 1997, 1999). It is this preference for hemimethylated substrates that forms the basis for its function as a maintenance methylase. In this respect, it is not surprising that Dnmt1 expression is tightly coordinated with DNA replication. In addition to its function as a MTase, Dnmt1 has been shown to be associated with a wide variety of chromatin modifying activities, including histone methyltransferases, methyl CpG binding proteins, and heterochromatin binding protein HP1 (Hermann et al., 2004). Collectively, these associations share in common the properties of transcriptional repressors leading to the understanding that Dnmt1 and, hence, DNA methylation stably reinforces chromatin silencing (Bird, 2002).

The **Dnmt2** gene is the most highly conserved of the MTases in eukaryotes, found in both organisms that show methylation and those that have no detectable DNA methylation. Although it is ubiquitously expressed at low levels in most human and mouse tissues as well as mouse embryonic stem cells, mice homozygous for a Dnmt2 null mutation are viable, and display normal levels of

methylation at endogenous sequences (Okano et al., 1998b). Introduction of an inducible transgene containing Dnmt2 indicated that a genuine methylase activity could be demonstrated, albeit on CpT and CpA targets (Kunert et al., 2003). A weak but reproducible in vivo methyltransferase activity was recently demonstrated for the recombinant protein in mammalian cell lines (Liu et al., 2003).

Dnmt3 family. The highly related enzymes, Dnmt3a and Dnmt3b, are encoded by different genes but share the preference for methylation of unmethylated CG dinucleotides. This substrate preference identifies them as de novo DNA methylases (Okano et al., 1998a). Dnmt3a and Dnmt3b are thought to differ mechanistically due to inherent differences in the catalytic domains, suggesting that Dnmt3a is distributive while Dnmt3b is processive (Gowher and Jeltsch, 2002). These intrinsic differences allow for an effective division of labor between these related de novo methylases. The processive Dnmt3b is more suited to methylation of CG-rich regions of the genome, such as the CG-rich pericentromeric repeats (Hermann et al., 2004). The distributive nature of Dnmt3a requires that it adds back methylation to dinucleotides on a target by target basis, and is thus implicated in de novo methylation at single genetic loci (Hata

et al., 2002). Dnmt3a is the major form in adult tissues, where it colocalizes with heterochromatin. In contrast, the isozyme Dnmt3a2 is the major form during embryogenesis, and has been shown to localize with euchromatin (Chen et al., 2002).

The third significant member of the Dnmt3 family of enzymes is Dnmt3L. This highly degenerate protein shows clear homology to Dnmt3a and Dnmt3b, but despite conserved folding like MTases, Dnmt3L lacks any catalytic activity but is expressed together with Dnmt3a and 3b during gametogenesis and embryogenesis (Bourc'his et al., 2001; Hata et al., 2002). Dnmt3a and Dnmt3L are essential for establishment of imprinted regions in oocytes (Hata et al., 2002). The exact mechanism of this process is not fully worked out, but there is the suggestion that the sequence specific function of Dnmt3a may require an activator to enforce the accuracy of this targeting. In this regard, Dnmt3L may function as an activator protein in the methylation of single copy genes.

1.4 The methyl-binding proteins: linking DNA methylation and chromatine structure

A. Bird identified the first methyl-DNA-binding activity called MeCP1 (methyl-CpG-binding protein 1) (Meehan et al., 1989).

This activity included two complexes (400 and 800 kD) consisting of different components. It was shown that MeCP1 plays an important role in regulation of expression of reporter genes if these genes had methylated promotor regions. Then a protein called MeCP2 was identified (Lewis et al., 1992). It had a number of biochemical and functional characteristics different from MeCP1. For example, its molecular weight was about 70 kD and only one symmetrically methylated CpG was sufficient for specific interaction of MeCP2 with DNA sequence (MeCP1 needed 12 symmetrically methylated CpGs). Is was shown that for interaction with methylated DNA an 85-amino-acid domain in the N-terminal part of protein was responsible. This domain was called MBD (methyl-DNA-binding domain). An analogous domain was found for MBD1 protein (formerly PCM1).

In 1998, Hendrich and Bird published a paper about identification and characterization of a family of methyl-DNA-binding proteins (Hendrich and Bird 1998). Three new proteins MBD2, MBD3, and MBD4 were described in addition to previously characterized MBD1 and MeCP2. Expression of all MBD proteins was found in almost all somatic tissues. Moreover, methyl-specific interaction with DNA and co-localization with constitutive

heterochromatin were shown for MBD1, MBD2, and MBD4 (Fig.1).

MeCP2 is a protein with molecular weight ~70 kD. It has two functional domains-MBD and TRD (transcription repression domain). TRD is necessary for interaction of MeCP2 with the mSin3A/HDAC nucleosome remodeling complex and plays crucial role in repression of transcription of target genes. MeCP2 can also interact with other transcriptional factors and co-repressors (like SMRT) (Klose and Bird, 2004).

MeCP2 is the most intensively studied MBD protein because conditional brain-specific deletion of this factor leads to generation of symptoms analogous to symptoms of Rett syndrome (Johnston et al., 2003). Some of these symptoms are mental retardation, heavy breathing, stereotyping, memory dysfunctions, etc. Patients suffering from Rett syndrome in most cases have point mutations in the MeCP2 gene. It is now known that MeCP2 regulates expression of *BDNF* (brain-developed neurotrophic factors) that is important for proper development of the nervous system as well as memory formation, etc.

MBD1 is the largest member of the MBD family. It consists of ~640 amino acid residues and has molecular weight ~75 kD. MBD1 interacts with DNA in a methylation-dependent manner in

EMSA experiments. But it can repress either methylated or non-methylated transitory transfected constructs. Moreover, MBD1, unlike MBD2 and MBD4, can co-localize with regions of constitutive heterochromatin even in cell with aberrant DNA methylation maintenance system (i.e., without DNMT1) (Hendrich and Bird 1998).

In early works, only two functional domains of MBD1 were described-MBD and a domain consisting of three CxxC-motifs (homologous to motifs found in DNA-methyltransferase DNMT1), but another domain was described quite soon. This domain (TRD, by analogy to transcriptional repression domain of MeCP2) is involved in transcriptional repression of reporter constructs as well as one of the CxxC-motifs (Ng et al., 2000).

It was shown at the same time that MBD1 can utilize different functional domains for interaction with different proteins. For example, TRD is important for interaction with MCAF (MBD1-containing chromatin-associated factor) (Fujita et al., 2003a). Complexes Suv39h1-HP1*alpha* (Fujita et al., 2003b) and p150-CAF-HP1 (Reese et al., 2003) interact with the MBD domain. MBD1 can also interact with SETDB1 histone-methyltransferase.

Protein p150-CAF was characterized as a partner of MBD1 in a yeast two-hybrid screen. Complex CAF (chromatin associated

factor) takes part in nucleosome assembly after DNA replication and maintenance of active/inactive chromatin state. One of three subunits of this complex, p150, interacts with HP1 protein and is involved in maintenance of inactive heterochromatin. Moreover, p150-CAF can interact with PCNA (proliferating cell nuclear antigen) during DNA replication. PCNA, in turn, interacts with DNMT1. p150-CAF attracts MBD1-SETDB1 complex that leads to methylation of lysine 9 of histone H3 and to formation of inactive chromatin.

MBD2 and MBD3 are related proteins. There is a hypothesis that genes coding these factors diverged from a common precursor. The genes have common exon-intron structure and amino acid sequences of MBD2 and MBD3 are identical by 70% (Hendrich et al., 1999a).

But despite these similarities, MBD2 and MBD3 have different functions. MBD3 is a structure subunit of one of the major chromatin-remodeling complexes-NuRD (nucleosome remodeling and histone deacetylase complex) (Zhang et al., 1999), whereas MBD2 is only one of the DNA-binding subunits of MeCP1 (Ng et al., 1999). Knockout of MBD3 leads to embryonic lethality in mice immediately after implantation of the embryo (Hendrich et al., 2001). Deletion of MBD2 does not have such serious

consequences. Moreover, there are no abnormalities either in imprinting/X-inactivation or repression of mobile elements in MBD2-/- mice. There is only one phenotypic effect of knockout of MBD2-abnormal maternal behavior (Hendrich et al., 2001).

MBD4 is a protein with molecular weight ~60 kD having two functional domains-MBD and a glycosylase domain. MBD4 co-localizes with regions of constitutive heterochromatin and can bind methylated DNA *in vitro*, but despite other MBD-proteins does not participate in regulation of gene expression.

This protein belongs to the mismatch repair system. Methylated cytosines are so-called “hot spot” of mutagenesis they can be converted to thymines after spontaneous deamination. MBD4 is involved in processes of recognition and correction of such mutations (Hendrich et al., 1999b). Deletion of MBD4 leads to accumulation of mutations and higher frequency of carcinogenesis (Miller et al., 2002).

Kaiso is a unique methyl-DNA-binding protein. It does not have classical methyl-DNA-binding domain and interacts with DNA via a zinc finger domain consisting of three zinc fingers of C2H2 type. Another functional domain of Kaiso is the N-terminal 120 amino acid BTB/POZ-domain (Prokhortchouk et al., 2001). This domain is usually located in the N-terminal part of BTB-

proteins and serves for homo- or heterodimerization during protein-protein interactions. Most BTB-proteins are transcriptional repressors. Kaiso was initially identified by a yeast two-hybrid screen as a partner of p120-catenin. p120-catenin is an important predominantly cytoplasmic protein interacting with and stabilizing E-cadherin (Davis et al., 2003).

It was also shown that Kaiso is a component of double MeCP1 complex. It is a part of a rapidly migrating band called Kaiso-generated band (KGB) (Prokhortchouk et al., 2001). The molecular weight of KGB is ~700 kD. There is a possibility that Kaiso-containing complex serves as an effector in signal-transduction pathway from cell membrane to nucleus and is responsible for repression of target genes in response to extracellular signals.

1.5 Different functions of MBD2 protein

The activity of methyl-binding proteins likely 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 (Ng and Bird 1999; Ng et al., 1999). By 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 hetrodimerization with MBD3 (Hendrich et al., 2001; Bowen et al., 2004). Other authors have identified a protein, MIZF, that associates with MBD2 and significantly enhances HDAC proteins recruitment and activity (Sekimata et al., 2001)**.** In addition, MBD2 form 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 (Tatemat et al., 2000). 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 viral protein Tax can activate transcription from the methylated HTLV-1 long terminal repeat (LTR) through the interaction with MBD2 (Ego et al., 2005).

The transcription factor GATA-3 can displace MBD2 from a methylated promoter causing the transcriptional reactivation of GATA-3 responsive genes (Hutchins et al., 2002). We have recently described a novel MBD2 associating protein, MBD*in*,

showing the unique ability to reactivate MBD2-repressed genes still in methylated status (Lembo et al., 2003). In the case of MBD*in*-mediated reactivation, as well as for GATA-3, transcriptional reactivation occurs prior to demethylation. However, MBD*in* 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 (Lembo et al., 2003)**.** Recently it has been shown that MBD2 interacts with RNA helicase A, a component of CREB transcriptional co-activator complex (Fujita et al., 2003). All these mechanims 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 factors that can be recruited on methylated DNA and determine different interpretations of DNA methylation signals.

2 THE AIM OF THIS STUDY

At current stage, it seems likely that the activity of methyl-CpG-binding proteins can depend on their association with different molecular partners.

Despite recent studies mainly propose methyl-CpG-binding proteins in the hystone deacethylases-mediated transcriptional repression it is also possible their role in alternative mechanisms and their involvement in other biological functions. This could be particulary true for the methyl-CpG-binding protein MBD2 for which both repression ability and demethylation activity have been described.

The aim of this study was to identify, using yeast two-hybrid screening, molecular partners of MBD2. Here we describe a novel MBD2 interacting protein, TACC3, which is able to modulate the MBD2-mediated transcriptional functions. We demonstrate that MBD2 and TACC3 may interact both at centrosomes in mitosis and in the interphase at 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.

3. MATERIALS AND METHODS

3.1 Construction of fusion genes and expression plasmids

For construction of Gal4Binding-Domain (Gal4BD) and Gal4Activation-Domain (Gal4AD) fusion genes, different fragments were amplified by PCR with pairs of primers linked to restriction sites and cloned in pBridge, pSG424 (Sadovsky et al., 1995) and pGADGH (Clontech) plasmids: pBridge-MBD2 and pGal4-MBD2 were obtained by cloning the entire MBD2b coding sequence (786 bp) in the pBridge and pSG424 plasmids respectively; pBridge-ΔMBD2, pBridge-MUT1, pBridge-MUT3 and pBridge-MUT4 are deletion mutants of pBridge-MBD2 and were obtained by cloning respectively a 471 bp (amino acids positions 103 to 260), 177 bp (amino acids 103 to 162), 153 bp (amino acids 209 to 260), 180 bp (amino acids 156 to 216) fragment of the human MBD2 coding sequence in the pBridge plasmids. Used primers containing restriction sites (underlined) were: for pBridge-MBD2, MBD2-1 (5'-AGTCGAATTCATGGATTGCCCGGCCCTCC CC-3') and MBD2-2 (5'-AGTCGGATCCTTAGGCTTCATCTCCAC TGTC-3'); for Gal4-MBD2, MBD2-1 and MBD2-3 (5'-AGTCGAATTCTTAGGCTTCATCTCCACTGTC-3'); for pBridge-ΔMBD2, MBD2-7 (5'-AGTCGAATTCAAACAACCGGTAACCAAG-3') and MBD2-2; for pBridge-MUT1, MBD2-7 and MBD2-9 (5'-

AGTCGGATCCTTATCCTTGAAGACCTTTGGGTAG-3'); for pBridge-MUT3, MBD2-11 (5'-AGTCGAATTCCTCTGCAAAGCTTTTATTGTC-3') and MBD2-2; for pBridge-MUT4, MBD2-12 (5'-AGTCGAATTCC TACCCAAAGGTCTTCAAGGA-3') and MBD2-10 (5'-AGTCGGATCCTTAGAC AATAAAAGCTTTGCAGAG-3') (Lembo et al., 2003). For the expression of red fluorescent protein (RFP) fused to, pRFPMBD2: was obtained by cloning the entire MBD2b coding sequence (786 bp) in the pDsRedN1 plasmid (Clontech). Used primers containing restriction sites (underlined) were: for RFPMBD2, MBD2-4 (5'-AGTCGAATTCAGCACAATGG ATTGCCCGGCCCTCCCC-3') and MBD2-14 (5'-AGTCGGATCC AAGGCTTCATCTCCACTGTCCAT-3'). To construct pMyc-MBD2 expression vectors, PCR-generated full-length MBD2 was cloned into pcDNA3.1A (Invitrogen) plasmid. Used primers containing restriction sites (underlined) were: for pMyc-MBD2, MBD2-4 and MBD2-5 (5'-AGTCGGATCCGGCTTCATCTCCACTGTCCAT-3'). The plasmid hTACC3-pCR2 Topo cloning (kindly provided by T.R.J. Lappin) containing the full length TACC3 coding sequence (McKeveney et al., 2001), was partially digested with *Eco*RI and the entire TACC3 cDNA was subcloned into the *Eco*RI sites of pCEFL-HA expression vector (Fedele et al., 2000) to generate pHA-TACC3. pHA-ΔTACC3 expression vector was generated by

cloning a 1290 bp TACC3 fragment (amino acids 409 to 837), into pCEFL-HA (*Eco*RI and *Xba*I sites). Primers used for amplification, containing restriction sites (underlined), were: TACC3-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'). All PCR-derived products as well as subcloning junctions were verified by sequence analysis.

3.2 Yeast two-hybrid assay

We performed yeast two-hybrid screens by yeast mating according to the manufacturer's instructions (Clontech). In the screens we used a human HeLa S3 cell line cDNA library pretransformed in the Y187 *S. cerevisiae* strain cloned downstream of the Gal4AD in the pGADGH plasmid (Clontech). We cloned 'bait' sequences downstream of the Gal4BD in the pBridge vector (Clontech) and transformed the PJ692A *S. cerevisiae* strain using the lithium acetate method and then treated according to the manufacturer's instructions. The diploid strains were plated on high stringency synthetic medium lacking histidine, adenine, tryptophan and leucine. His(+), Ade(+) diploid colonies were patched on selective plates and assayed for β-galactosidase activity using a colony-lift filter assay (Breeden and

Nasmyth, 1985). Total DNA was prepared from colonies displaying a His(+)/Ade(+)/LacZ(+) phenotype and pGADGH library plasmids were then rescued after transformation of a HB101 –Leu *E. coli* strain. The isolated pGADGH library plasmids were tested for specificity by cotransformation into PJ692A either alone or in combination with the pBridgeMBD2 construct or in combination with plasmids containing different unrelated baits (syntenin, HMGA1 and galectin-1 cDNAs) fused to sequences encoding the Gal4BD. The cDNA inserts from specific clones were sequenced using the dideoxy termination method (Sanger et al., 1977). For measurement of protein interaction, plasmids were cotransformed into a yeast strain Y187 and plated on synthetic medium lacking tryptophan and leucine. After four days of growth colonies were inoculated in liquid SD –Leu/-Trp for 24 hours and a liquid assay for β-galactosidase activity was performed according to the manufacturer's instructions (Clontech).

3.3 Isolation of a human shortest TACC3 cDNA clone

A bone marrow and a brain cDNA libraries in λgt11 were purchased from Clontech. A total of about $3x10^6$ clones were screened by an *in situ* plaque hybridization technique (Sambrook et al., 1989). The fragment of pGADGH-TACC3 was labeled by

random priming procedure (Feinberg and Vogelstein, 1983) and used as a probe. Hybridizations were carried out at 60℃, and filters were washed under low-stringency conditions (0.6x SSC, 0.4%SDS at 40℃). Phage DNA was isolated by small-scale purification procedure (Sambrook et al., 1989). Inserts from positive clones were sequenced using the dideoxy termination method (Sanger et al., 1977).

3.4 Cell culture and transient transfections

293T, MCF7 and Cos-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (Life Technologies).

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 mg/ml) as previously described (Ambesi-Impiombato et al., 1980).

Cells were plated at a density of about 5 x 10^5 per 100 mm Petri dish 24 hours before transfections. DNA transfections were carried out by calcium phosphate precipitation using Calphos (Clontech) or lipofectAMINE^{TM} 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 (β-Gal) expression plasmid, pSVβ-Gal (Promega) (2μg), was used as an internal control for transfection efficiency.

3.5 Methylation analysis

50 μg of pGAT1700 plasmid (Salvatore et al., 1995) were treated with 50 U of *Sss*I methylase (New England Biolabs) at 37℃ in the presence of 5 mM adenosylmethionine for 8 h. Complete methylation of treated plasmids was confirmed by *Hpa*II 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 (Lembo et al., 2003).

3.6 CAT assays

Chloramphenicol acetyltransferase (CAT) assays were performed with different amounts of extracts to ensure linear conversion of the chloramphenicol with each extract and results are presented as the means of at least three independent transfection experiments. For normalization of transfection efficiencies we used a β-Gal expression plasmid was included in

the co-transfection. Total protein extracts (5 µg) were assayed for β-galactosidase activity as previously described (Majello et al., 1994). The CAT activity was quantified by counting the amount of [^{14}C]chloramphenicol converted to the mono-acetylated form using the Molecular Dynamics Phoshorimager^{TM} system.

3.7 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 (Fedele et al., 2000). The samples were immunoprecipitated with mouse monoclonal anti-HA (Roche Molecular Biochemicals), mouse anti-myc, rabbit anti-HDAC2, goat anti-TACC3 and mouse anti-pCAF antibodies (Santa Cruz), anti-MBD2a antibodies (Abcam), and resolved on 12% and 10% SDS-PAGE. Immunoprecipitated (IP) proteins were analyzed 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 (ECL) (Amersham Biosciences).

3.8 Drug Treatments and Quantitative RT-PCR analysis

PC Cl3 cells were treated with different amounts of trichostatin A (50-100 μM) (TSA, Invitrogen) or of 5-aza-2'-deoxycytidine (10-100 μM) (ICN Biomedical Inc.) for 36 hours. Total RNA was isolated with TRIzol Reagent (Invitrogen), as described in the RNA isolation protocol. QantiTect Reverse Transcription kit (QUIAGEN) was used to generate cDNA. The following galectin-1-specific primers were used: Gal-1/Fw (5'-CTCGGGTGGAGTCTTCTGAC-3') and Gal-1/Rv (5'-TCCCCAGGTTTGAGATTCAG-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). Equal amounts of total RNA samples (1 μg) were used for cDNA conversion. After a 15 min incubation at 50℃, RT was inactivated by he ating at 95℃ for 3 min. The PCR amplifications were then performed using sybr green (Applied Biosystems), in a Chromo4 Real Time thermocycler (BIORAD) under the following conditions: 1 cycle, 1,5 min denaturation at 95℃ and then 40 cycle of 1 5 s at 95℃

and 1 min at 60℃. Each sample was tested in duplic ate, and mean values were calculated. Copy number quantification was depending on the number of PCR cycle required for threshold detection of the fluorescence signal cycle threshold (C_t values) (Aerts et al. 2004). The fold induction Galectin-1 mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The average C_t was calculated for G6PD and Galectin-1 and ΔC_t ($C_{t\ G6PD}$ - $C_{t\ Gal\text{-}1}$) was determined. The $\Delta\Delta C_t$ was used for relative quantification using the expression in PC Cl3 cells as a reference control.

3.9 Nonradioactive 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. Nonradioactive 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 100mM acetyl-CoA and 1X 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 TBS, acetylated histones were detected using an antiacetyl-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 OD at 450 nm.

3.10 Immunocytofluorescence

Cos-7 and NIH3T3 cells were incubated in 8% Bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour 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 and 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 phosphate buffered saline 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 50mM 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 60x Plan-Neofluar objective. Images were subjected to scale adjustment using image software (Photoshop, Adobe Systems, Inc., San Diego, CA).

4. RESULTS

4.1 Interaction screening

To identify news partners of the MBD2 protein, we performed interaction screening using the two-hybrid system in yeast (Clontech) (Fig.2). We have simultaneously analyzed a bone marrow and a brain cDNA libraries. A total of about $3x10^6$ clones was tested for each library using the short form of MBD2 cDNA, MBD2b, as bait (Lembo et al., 2003). 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 from 3 to 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 analyze in detail Group F, which included 8 clones, all derived from bone marrow library and each containing part of the coding sequence of transforming acid coiled-coil protein 3, TACC3

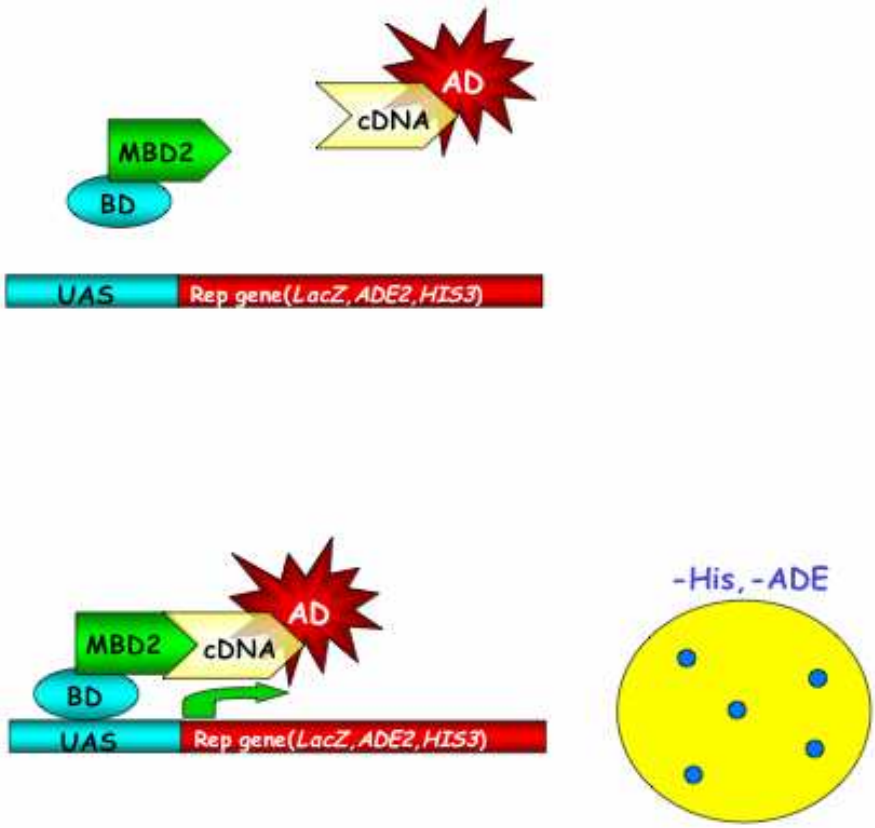

Fig.2 Schematic representation of the yeast two -hybrid system.

(DDBJ/EMBL/GenBank accession number AJ243997). TACC3 was a very interesting candidate MBD2 interactor because of its capacity to associate with chromatin remodeling factors (Gangisetty et al., 2004). TACC3 is a 100 kDa protein belonging to the family of TACC proteins including three human members (Still et al 2004; Lappin et al., 2002). TACC3 contains three domains (Fig. 3A): 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 about 60% identity with the C-terminal region of TACC1 and TACC2 (Still et al., 2004). All the 8 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 (Fig. 3A).

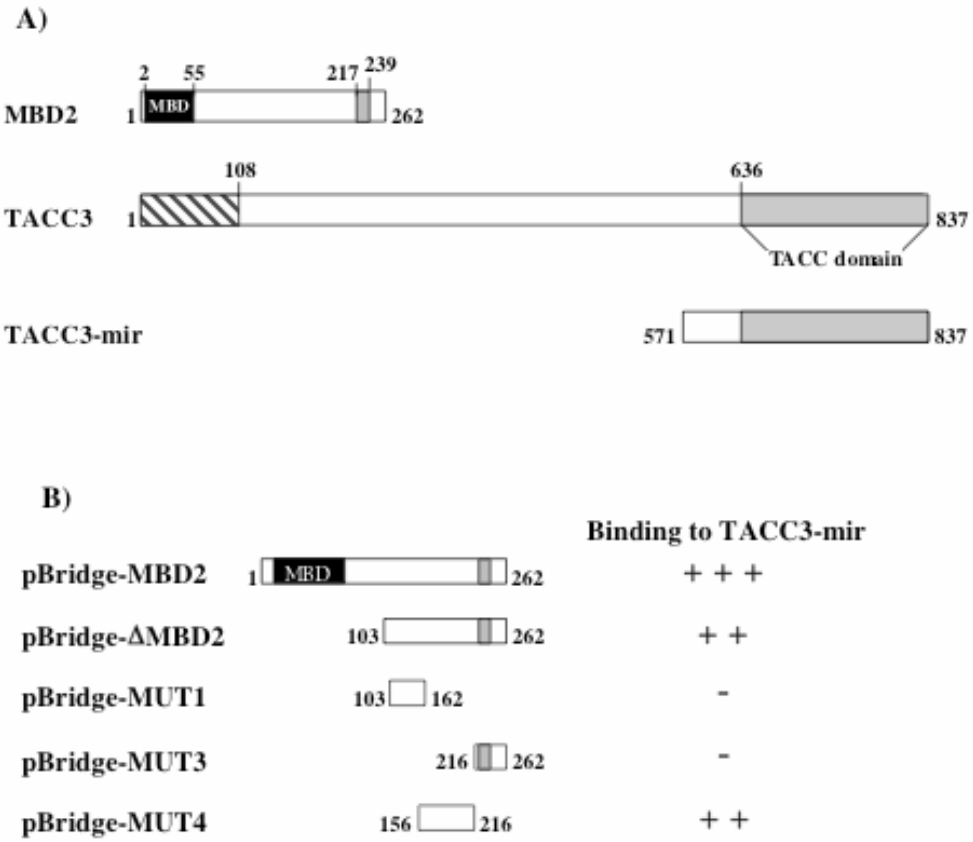

Fig. 3 Definition of MBD2 and TACC3 binding region in yeast. (**A**) Schematic representation of structural and functional domains of MBD2 and TACC3 proteins. For MBD2, the methyl-binding-domain (MBD) is indicated as a black box; the putative coiled-coil domain is indicated as a gray box. For TACC3, 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 b-galactosidase assay.

4.2 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 Fig. 3B, deletion of MBD2 N-terminal 103 amino acids (pBridge-ΔMBD2) 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.

4.3 Immunoprecipitation experiments

Coimmunoprecipitation experiments were performed to investigate whether MBD2 and TACC3 form a complex in mammalian cells (Fig. 4). 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-ΔTACC3). 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 Fig. 4, both TACC3 and ΔTACC3 coimmunoprecipitated with MBD2 (Fig. 4A and 4B 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 to 837), containing the TACC domain, is sufficient for the interaction.

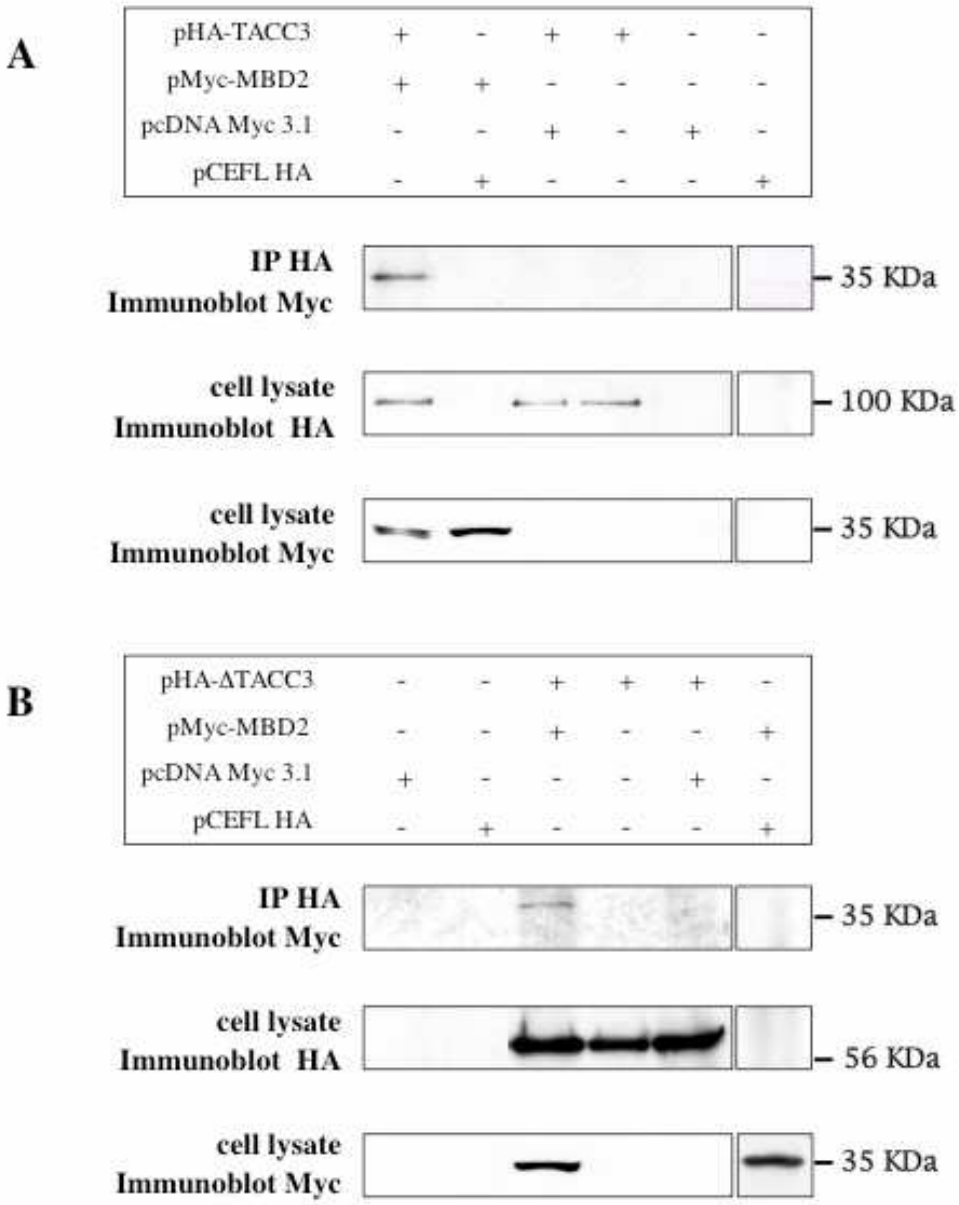

Fig. 4 In vivo association of MBD2 with TACC3 and DTACC3. 293T cells were transfected in the indicated combinations with the following expression plasmids: (A) pHA-TACC3, pMyc-MBD2 and control empty vectors; (B) pHA-DTACC3, pMyc-MBD2 and control empty vectors. The whole-cell extracts were prepared and subjected to immunoprecipitation with goat polyclonal anti-HA antibodies. Immunoprecipitated (IP) proteins were analyzed by immunoblotting with mouse monoclonal anti-Myc and anti-HA antibodies.

4.4 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) (Salvatore et al., 1995) containing 1700 bp of the mouse galectin-1 promoter that can be repressed by in vitro DNA methylation (Benvenuto et al., 1996) and, when methylated, is bound by MBD2 (Lembo et al., 2003). 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 (Fig. 5) clearly showed that TACC3 was able to reactivate, in a dose dependent manner, the methylated promoter (Fig. 5). Interestingly, the reactivation was much stronger in the presence of overexpressed 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.

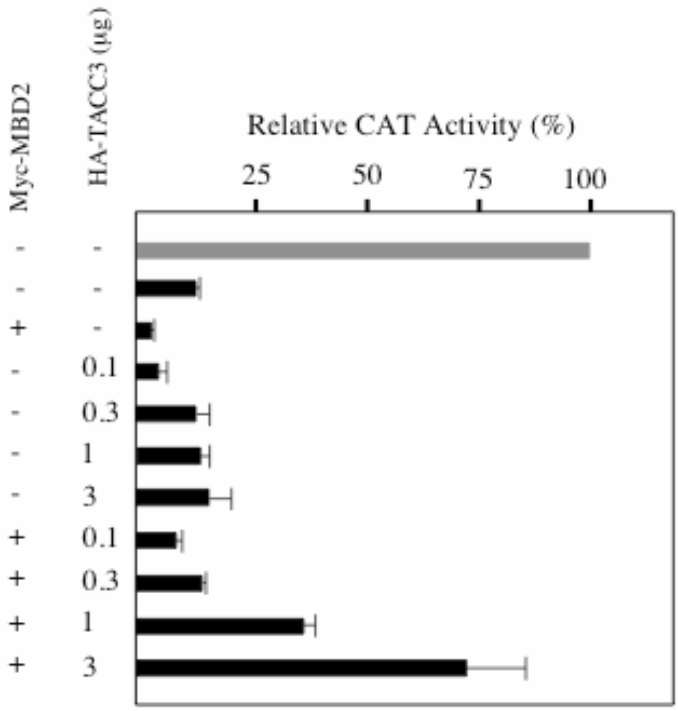

Fig. 5 Influence of MBD2 and TACC3 overexpression on activity of methylated transfected. 293T cells were transfected with 3 μg of unmethylated (gray bar) or in vitro methylated (black bars) pGAT1700 reporter plasmid. Cotransfected expression plasmids were: pSVβ (2 μg) and, where indicated, pMyc-MBD2 (2 μg) 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 β-galactosidase activity. The results are presented as an average of multiple experiments +/- SD. Each histogram bar represents the mean of at least four independent transfections.

Seven CpG sites contained in the -177/+11 region of the transfected mouse galectin-1 promoter, whose methylation state is critical for transcriptional activity (Lembo et al., 2003; Salvatore et al., 1998; Chiariotti et al., 1999), were analyzed using mouse-specific bisulfite-modified primers. No significant change of methylation pattern was observed up to 60h 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 (Benvenuto et al., 1996). Our results showed that exogenous expression of TACC3, as well as treatment of cells with the deacethylase inhibitor TSA or the demethylating drug azacytidine, induced a significant increase of endogenous galectin-1 gene expression within 36 hours in a dose dependent manner (Fig. 6). Moreover our results showed that TACC3 displays a synergistic effect with TSA treatment (Fig. 6).

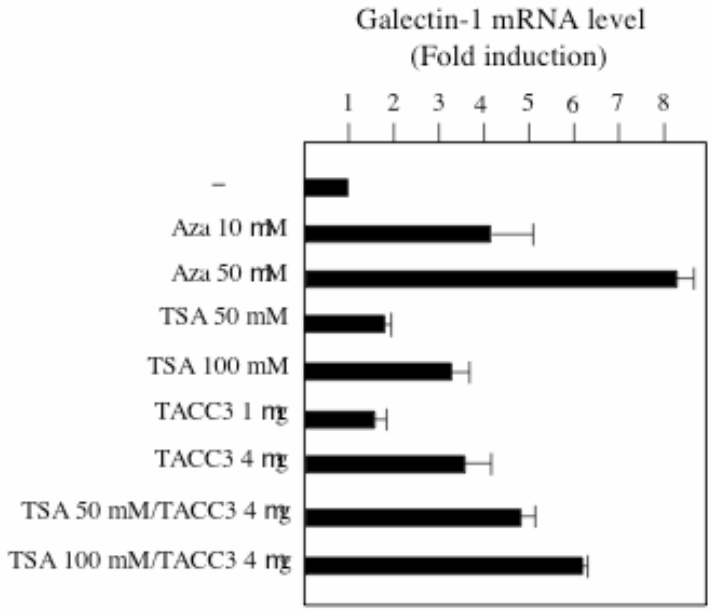

Fig. 6 Quantitative RT-PCR assays of endogenous galectin-1. mRNAs were performed on PC Cl3 cells untreated or treated with the indicated concentrations of 5-aza-2'-deoxycytidine (Aza) or Trycostatin A (TSA) for 36 hours. Where indicated (TACC3), cells were transfected with pHA-TACC3 expression vector. mRNA expression levels are shown as fold-induction compared to the PC Cl3 value, arbitrarily set as 1. The results are presented as an average of at least three independent experiments +/- SD.

4.5 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 (Gangisetty et al., 2004), 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 (Gangisetty et al., 2004). In this cell line, MBD2a and HDAC2 endogenous proteins were expressed at detectable levels, as evidenced by western blot analysis (Fig. 7). 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 (Fig. 76). Results showed that both exogenous Myc-tagged MBD2b (Fig. 7A) and endogenous MBD2a (Fig. 7B) proteins form a complex with pCAF and TACC3. MBD2 could also

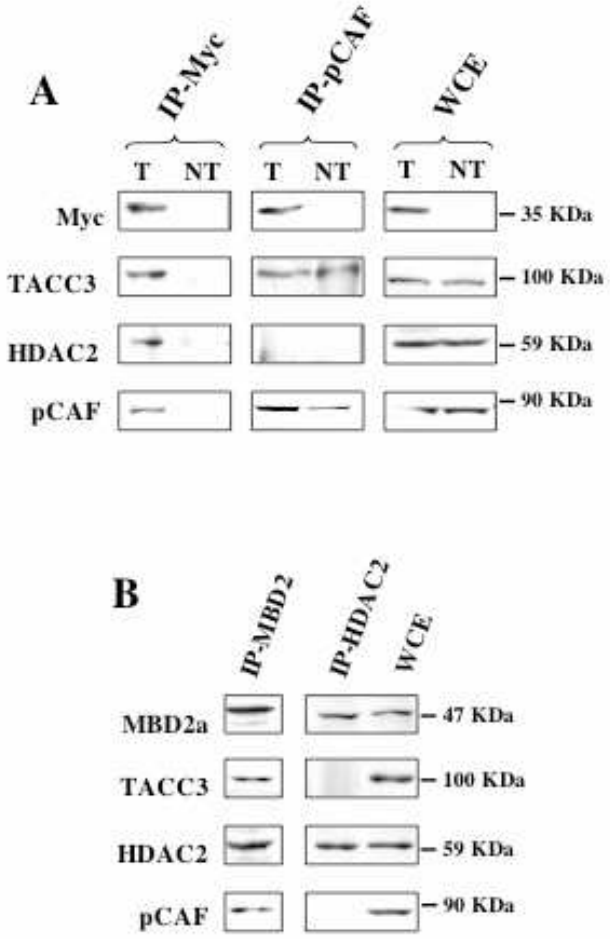

Fig. 7 In vivo interactions of MBD2, TACC3, pCAF and HDAC2. (**A**) Cell lysates from MCF7 untransfected cells (NT) or transfected with pMyc-MBD2 plasmid (T) were immunoprecipitated (IP) and immunoblotted with the indicated antibodies. Anti-myc antibodies were used to detect Myc-MBD2 fusion protein. 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.

bind HDAC2, as extensively reported (Ng and Bird, 1999; Ng et al., 1999). However, our experiments show that the complexes formed with MBD2 by HDAC2 and by TACC3/pCAF were mutually exclusive (Fig. 7B).

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 12 hours in the presence of 0.5% serum. Immunoprecipitation experiments were performed at time points 0, 1, 3, 6, 12, 18 and 24 hours after 10% serum was restored. Results showed 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.

4.6 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 analyzed whether HAT enzymatic activity could be 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 8, showed that HAT activity consistently associated with MBD2 protein even in the absence of exogenous TACC3 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.

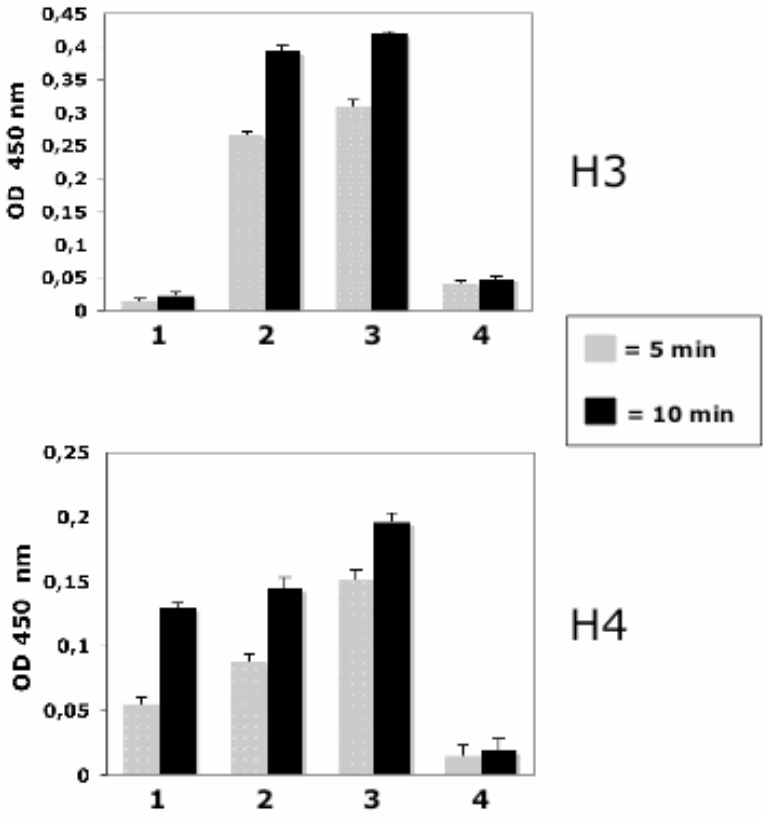

Fig. 8 Analysis of MBD2-associated acetyltransferase activity. 293T cells were transfected with pMyc-MBD2 (4 μg) (lane 2) alone or in combination with pHA-TACC3 (2 μg) (lane 3). 48 hours after transfection nuclear extracts were immunoprecipitated with anti-Myc antibodies. Recombinant pCAF (25 ng) (lane 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 optical density (OD) values at 450 nm.

4.7 Centrosomal localization of MBD2

Finally, because TACC3 localizes at centrosomes in mitosis (Gergely et al., 2000), we tested the possibility that MBD2 protein is recruited at centrosomes as well. Asyncronous Cos-7 cells were subjected to immunostaining study using goat anti-MBD2 antibodies (red) and mouse anti-γ-tubulin antibodies (green) as centrosomes indicator. In interphase, MBD2 localizes in the nucleus (Fig. 9A-a), as exstensively reported (Hendrich et al., 1998; Hutchins et al., 2002; Lembo et al., 2003). By contrast, during different phases of mitosis, MBD2 mainly localizes at centrosomes (Fig. 9A-b and 9A-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 (Fig. 9B). 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 (Sakai et al., 2002). Our findings further support the hypotesis 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.

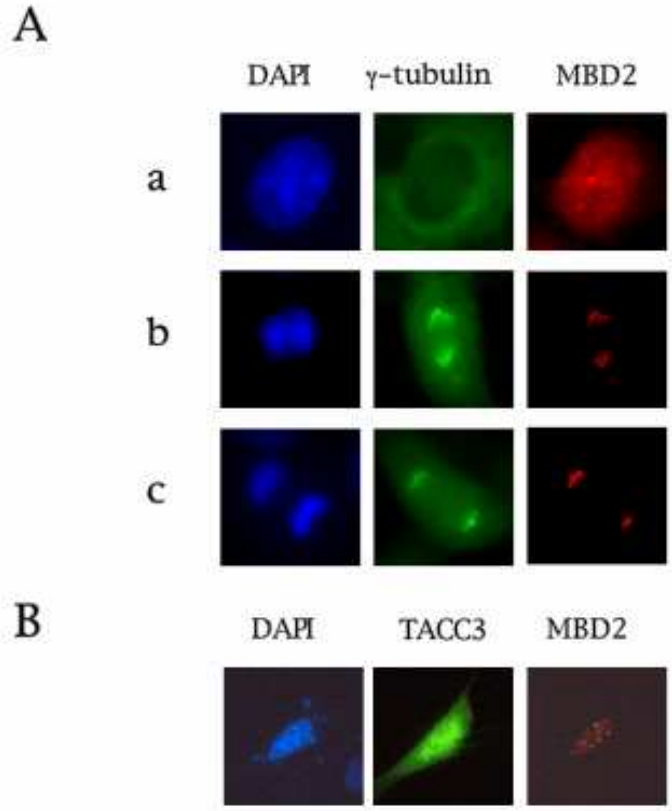

Figure 6. (**A**) MBD2 localizes at centrosomes in M phase. Asyncronous Cos-7 cells were subjected to immunofluorescence study using goat anti-MBD2 antibodies (red) and mouse anti-γ-tubulin antibodies (green). DNA was stained with DAPI (blue). (a) interphase; (**b**) metaphase and (**c**) anaphase. (**B**)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

5. 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 MBD*in*, a novel GTPase protein, is able to associate with MBD2 and to reactivate methylated genes without altering the methylation pattern of the target genes (Lembo et al., 2003). However, the mechanisms underlying this phenomenon remain poorly understood. In the present study we have specifically selected other MBD2 partners that might share with MBD*in* 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 (Gangisetty et al., 2004). Compatibly with a physilogical relevance of their interaction, TACC3 and MBD2 are ubiquitously expressed in mammalian tissues, even if the relative proportion may vary among different tissues. (Hendrich and Bird, 1998; Still et al., 2004).

TACC3 belongs to a recently identified and evolutionary conserved protein family referred as TACC (transforming-acidic

coiled-coil containing) family (Still et al., 2004; Lappin et al., 2002). 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 of this domain. All the TACC proteins are associated with the mitotic centrosome/spindle apparatus (Gergely et al., 2000), 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 coactivator of different nuclear factors (Gangisetty et al., 2004; Sadek et al., 2000).

We show that TACC3, as well as MBD*in*, has the capacity to relief 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 MBD*in* and the interaction with MBD2 occurs through different regions of this methyl-binding protein. While MBD*in* interacts with the extreme C-terminal of MBD2 (amino

acids 216 to 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 to 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 to 837) is sufficient for the interaction with MBD2 both in yeast and in mammalian cells. This region include 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 (Gangisetty et al., 2004), 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 (Fig. 4 and Fig. 7). 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 (Cress and Seto, 2000). Recently, it has been also demonstrated that HAT and HDACs directly interact and immunoprecipitate in the same fractions (Yamagoe et al., 2003). Our data indicate that, although both TACC3/pCAF and HDAC2 may associate with MBD2 in MCF7 cells, they are part of distinct complexes (Fig. 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 (Yamagoe et al., 2003) 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.

According to the current models, two possible mechanisms account for the reactivation of previously silent methylated genes: i) the promoter can be demethylated after interaction of MBD2 with MBD*in* and a co-activator protein complex (Lembo et al., 2003); ii) the TACC3/pCAF complex, can be to compete with repressor complex for the association with MBD2 on methylated promoter, it is possible that the acetylation might trigger promoter demethylation in a longer period. In Figure 10 we have described

a possible model of how TACC3 mediates reactivation of methylated promoters.

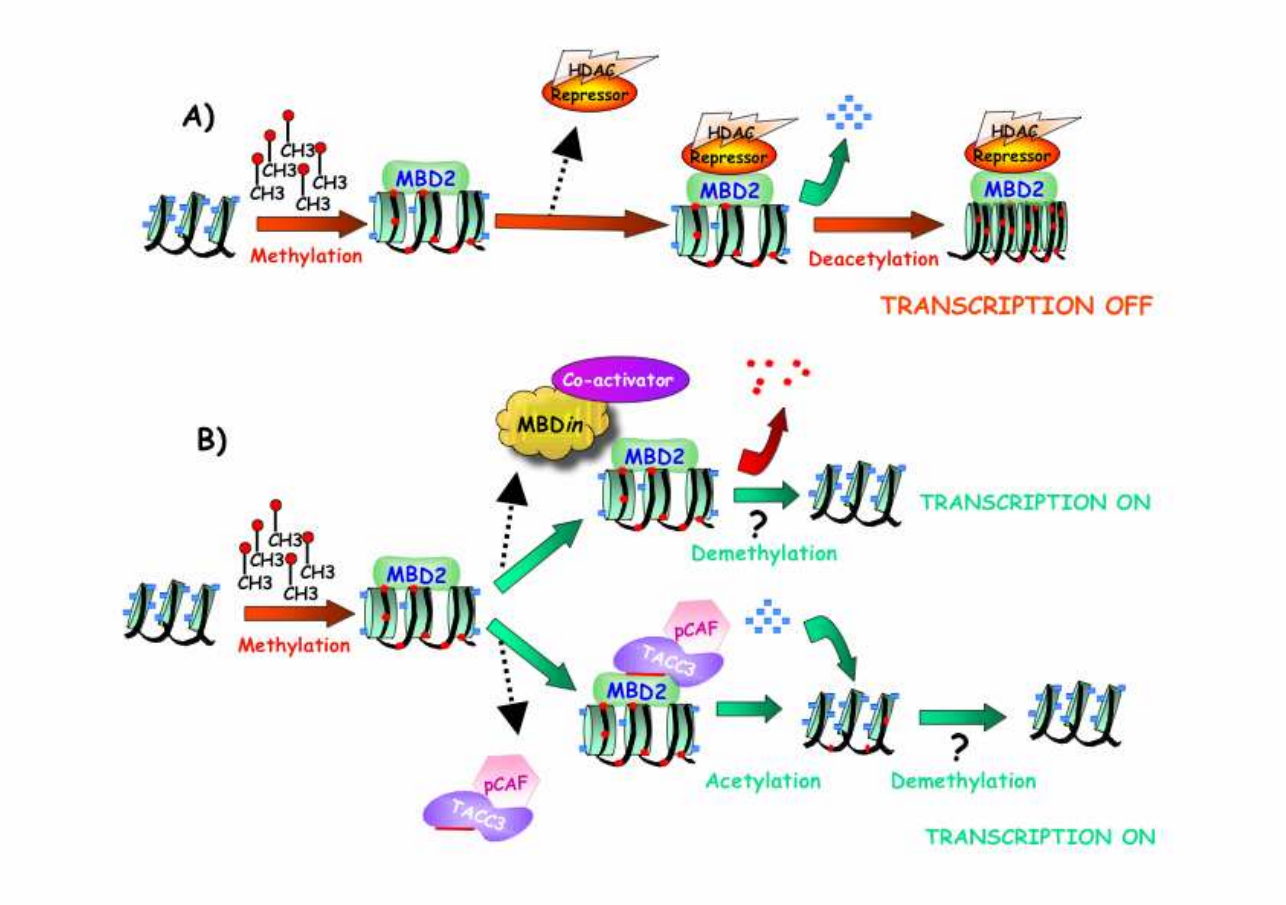

Fig. 10 Possible models for reactivation of silent methylated genes. Nucleosomes are shown as green cylinders representing the core histone complexes with DNA indicated by the black-line wrapped around the cylinders. Blue ovals denote acetylation of histones. DNA methylation is indicated by red circles. A) A transcriptionally inactive promoter adopt a tightly packed chromatin structure by the recruitment of methyl-CpG-binding proteins and their associated co-repressors and histone deacetylases. B) TACC3/pCAF, activator complex, compete with repressor complex for the association with MBD2. Consequently the chromatin is acetylated and acquires an open conformation and the gene is transcribed although still methylated. As a second hypothesis MBD2 can bind a demethylating protein and the promoter can be reactivated by a demethylation process mediated by MBD2.

6. ACKNOWLEDGEMENTS

I am especially grateful to Prof. Carmelo Bruno Bruni in whose laboratory this experimental work was performed.

A very special thank goes to Prof. Lorenzo Chiariotti, who always encouraged me handing on me his enthusiasm for scientific research and providing me his support and critical advices.

I would like also to thank all my colleagues, Dr. Raffaela Pero, Dr. Francesca Lembo and Dr. Francesco Natale, who supported me on my research projects.

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