Regulation of MBD1-mediated transcriptional repression by SUMO and PIAS proteins

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In mammalian cells, DNA methylation is associated with heritable and stable gene repression, mediated in part by methyl-CpG-binding domain (MBD) proteins that recruit corepressors to modify chromatin. MBD1 protein, a member of the MBD family, forms a complex with SETDB1 histone methylase to silence transcription at target promoters by methylation of lysine 9 of histone H3. How MBD1-mediated transcriptional repression is regulated is currently unknown. Here we show that MBD1 is a target for sumoylation by PIAS1 (Protein Inhibitors of Activated STAT 1) and PIAS3 E3 SUMO (small ubiquitin-like modifier)-ligases, at two conserved lysine residues within the C-terminus of MBD1. Although sumoylated MBD1 binds to methylated DNA, it does not incorporate into a complex with SETDB1 and does not efficiently repress transcription of a target gene, p53BP2, in HeLa cells. Our data suggest that transcriptional silencing by MBD1 is regulated by a PIAS-mediated conjugation of SUMO1, which antagonizes the formation of a repressive complex with SETDB1.

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Introduction

Methylation of cytosine at CpG dinucleotides is the major epigenetic modification in vertebrate genomes and contributes to the maintenance of genome stability, regulation of gene expression and normal progression through development (Meehan and Stancheva, 2001; Bird, 2002). Approximately 70% of all CpG dinucleotides are methylated in mammalian somatic cells. Methylated CpGs are randomly distributed throughout the genome, but are normally excluded from promoter-associated CpG-rich sequences known as CpG islands (Bird, 2002). In human cancers, aberrant methylation of CpG islands results in stable and heritable gene silencing, mediated by a family of proteins that contain a methyl-CpG-binding domain (MBD) (Bird and Wolffe, 1999; Jones and Baylin, 2002). The MBD family comprises MBD1, MBD2, MBD3, MeCP2 and MBD4 (Hendrich and Bird, 1998). Transcriptional repression by MBD proteins operates through recruitment of corepressor complexes that modify chromatin into an inactive state (Bird and Wolffe, 1999; Prokhortchouk and Hendrich, 2002). Histone deacetylase and histone methylase (HMT) corepressor activities have been found stably or transiently associated with MBD proteins (Nan et al, 1998; Zhang et al, 1999; Fuks et al, 2003; Sarraf and Stancheva, 2004).

MBD1 is the largest protein in the MBD family. It contains a conserved N-terminal MBD domain, three centrally located CxxC motifs and a C-terminal transcriptional repression domain (TRD). The third CxxC motif has DNA-binding properties independent of the MBD domain, with preferential recognition of unmethylated CpG-rich DNA (Jorgensen et al, 2004). Several splice variants of MBD1 have been identified in human and mouse cells and shown to differ in the number of CxxC motifs and the length of the region between the last CxxC motif and the TRD (Fujita et al, 1999; Jorgensen et al, 2004). An isoform with two CxxC motifs (CxxC2 and CxxC3) is the most abundant MBD1 protein in HeLa cells and a variety of other human cell lines (Cross et al, 1997).

Transcriptional repression by MBD1 is largely histone deacetylation-independent and operates through the recruitment of histone H3 lysine 9 (H3K9) methylase SETDB1 and SETDB1 cofactor AM/MCAF1 to chromatin (Ng et al, 2000; Fujita et al, 2003a; Sarraf and Stancheva, 2004). An interaction between MBD1 and another H3K9 methylase, Suv39H1, has also been reported (Fujita et al, 2003b). H3K9 methylation was found at virtually all MBD1 binding sites, including silenced gene promoters, suggesting that the MBD1/SETDB1 complex silences transcription by introducing repressive methylation of H3K9 (Sarraf and Stancheva, 2004). It was further shown that migrating replication forks destabilize the interaction of MBD1 with methylated DNA to promote the assembly of a transient S-phase-specific complex between MBD1/SETDB1 and the major subunit p150 of chromatin assembly factor CAF-1. The MBD1-dependent recruitment of SETDB1 to the postreplicative chromatin assembly machinery is crucial for the maintenance of H3K9 methylation in synchrony with DNA methylation and for the stable epigenetic inheritance of silenced chromatin (Sarraf and Stancheva, 2004).

We wondered what mechanisms might permit regulation of MBD1-mediated maintenance of gene silencing. Such regulation may be particularly important during cell differentiation and development, when gene expression patterns and chromatin states undergo significant changes. Potentially, post-translational modifications of MBD proteins could either change the affinity of the MBD domain for methylated DNA or affect the assembly of MBDs into complexes with corepressor proteins. As an example of the first possibility, phosphorylation of MeCP2 has been observed as a result of calcium-dependent membrane depolarization in

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cortical neurons (Chen et al., 2003; Martinowich et al., 2003). Phosphorylated MeCP2 dissociates from methylated CpGs at the BDNF promoter, facilitating activation of BDNF transcription. However, there are no known examples of specific post-translational modifications that either promote or destabilize the interactions of MBDs with partner proteins.

Modification of nuclear proteins by SUMO (Small Ubiquitin-like Modifier) has been shown to play a role in transcriptional regulation (Johnson, 2004; Hay, 2005). SUMO can be attached enzymatically to a variety of proteins, including transcription factors, histones and chromatin-related proteins (Girdwood et al., 2003; Shiio and Eisenman, 2003; Hay, 2005). Sumoylation of target proteins usually occurs at a lysine residue within the consensus sites KxExE, where X is a large hydrophobic amino acid (L, I or V) and x can be any amino acid. Conjugation of SUMO to target substrates is mechanistically similar to ubiquitination, but uses SUMO-specific enzymatic machinery, which includes E1 SUMO-activating enzyme formed by a heterodimer Aos1/Uba2, an E2 SUMO conjugation enzyme Ubc9 and E3 ligases such as RanBP2 and PIAS (Protein Inhibitors of Activated STAT) family proteins. E3 enzymes transfer SUMO from Ubc9 to specific protein substrates (Schwienhorst et al., 2000; Johnson, 2004; Hay, 2005). Unlike ubiquitination, sumoylation of target proteins does not promote protein degradation and in many cases stabilizes modified proteins (Desterro et al., 1998; Ulrich, 2005). However, the effects of SUMO conjugation are diverse and largely dependent on the function of the protein targeted for sumoylation. Like other post-translational protein modifications, sumoylation can either promote or inhibit the formation of specific protein complexes, affect subnuclear localization of proteins and regulate transcription both positively and/or negatively (Sachdev et al., 2001; Girdwood et al., 2003; Johnson, 2004; Long et al., 2004; Pastushok and Xiao, 2004; Gomez-del Arco et al., 2005; Hay, 2005).

In this report, we demonstrate that in human cells MBD1 protein is modified by SUMO1 at two conserved sites surrounding lysines K450 and K489 within the MBD1 C-terminus. We identify PIAS1 and PIAS3 as the E3 SUMO ligases, which directly interact with MBD1 and are required for sumoylation of MBD1 in vivo. Sumoylated MBD1 does not efficiently incorporate into a complex with SETDB1, resulting in derepression of the MBD1 target gene p53BP2. Our data suggest that assembly of the MBD1/SETDB1 repressive complex is controlled by a competition between SETDB1 and the SUMO conjugation machinery for binding to MBD1. The outcome of this competition is dependent on the protein levels of SETDB1 and PIAS1/3 SUMO ligases and provides a potential mechanism for flexible regulation of MBD1-mediated gene silencing.

**Results**

**MBD1 is modified by SUMO1**

We previously performed a yeast two-hybrid screen using human MBD1 as bait against a HeLa cDNA library to identify MBD1-interacting proteins (Sarraf and Stancheva, 2004). From this screen, we isolated and characterized two MBD1-binding partners—SETDB1 HMT and p150 subunit of chromatin assembly factor CAF-1. From the same screen, we also recovered nine plasmids that contained cDNAs encoding the E3 SUMO ligases PIAS1 and PIAS3. The putative interaction of MBD1 with PIAS proteins prompted us to investigate whether MBD1 protein is SUMO-modified in human cells. The conjugation of SUMO to target proteins creates branched protein molecules migrating with higher molecular weight than the unmodified protein in acrylamide gels. To investigate whether MBD1 is sumoylated in vivo, we prepared nuclear extracts, with or without the SUMO isopeptidase inhibitor N-ethyl maleimide (NEM), from HeLa, MRC5 primary human lung fibroblasts and NCI-H226 lung carcinoma cells. Western blots with anti-MBD1 antibodies detected a major 70 kDa MBD1 band in all nuclear extracts (Figure 1A). An additional ~130 kDa band could be seen only in extracts prepared with NEM, suggesting that it may represent a SUMO-modified form of MBD1. A third band, migrating slightly slower than the major 70 kDa MBD1 protein, could also be seen in all extracts, irrespectively whether prepared with or without NEM (Figure 1A, B, and G). This band is likely to represent a phosphorylated form of MBD1. Notably, the amounts of sumoylated MBD1 detectable by Western blots were low, estimated to be only about 10% of the total MBD1 protein, based on further experiments (not shown).

To investigate whether the 130 kDa protein recognized by anti-MBD1 antibodies represents sumoylated MBD1, we performed immunoprecipitations (IPs) with anti-MBD1, anti-SUMO1 and anti-SUMO2/3 antibodies from HeLa nuclear extracts. When anti-MBD1 antibodies were used to detect immunoprecipitated proteins on Western blots, both the 70 kDa and the 130 kDa proteins could be seen in anti-MBD1 IPs, while the anti-SUMO1 antibodies immunoprecipitated only the 130 kDa MBD1 (Figure 1B). In the reverse experiment, the anti-SUMO1 antibodies on Western blots also detected only the 130 kDa band of anti-MBD1 IPs, suggesting that this protein indeed represents SUMO1-modified MBD1 (Figure 1C). MBD1 was neither detectable among the proteins immunoprecipitated with anti-SUMO2/3 antibodies nor was the 130 kDa band in MBD1 IPs recognized by the anti-SUMO2/3 antibodies (Figure 1B and D). From these experiments, we conclude that MBD1 is SUMO1-modified in HeLa cells.

Additionally, we used small interfering (si)RNAs to deplete SUMO1, SUMO2 and SUMO3 or all SUMO proteins simultaneously in HeLa cells (Figure 1E and F). The 130 kDa MBD1 band was greatly reduced only when the cells received anti-SUMO1 siRNA, further reinforcing our conclusion that in HeLa MBD1 is modified by SUMO1 but not by SUMO2 or SUMO3 (Figure 1G).

**MBD1 has two functional sumoylation sites**

We next examined the amino-acid sequence of human MBD1 protein and found two potential sumoylation sites, both VKQE, surrounding lysines 450 (K450) and 489 (K489) within the C-terminus of MBD1 (Figure 2A). Alignment of MBD1 proteins from various mammalian species revealed that the two potential sumoylation sites are highly conserved (Figure 2B). Interestingly, although the C-terminus of Xenopus tropicalis MBD1 is not homologous to human MBD1 protein, it also contains two potential SUMO target sites (IKEE and VKTE) surrounding lysines 446 and 471 (Supplementary Figure S1). The conservation of SUMO conjugation sites in MBD1 proteins of all vertebrates is consistent...
with the idea that sumoylation of MBD1 may be functionally important.

To investigate whether the VKQE sequences of human MBD1 are essential for conjugation of SUMO1, we mutated K450 and K489 or E452 and E491 to alanine and coexpressed monomeric RFP-tagged wild-type and mutant proteins with Flag-SUMO1 in HeLa cells. Ectopically expressed MBD1 proteins were analysed on Western blots with anti-RFP antibodies (Figure 2C). Subsequently, we immunoprecipitated the wild-type and the mutant RFP-MBD1 proteins and confirmed the identity of sumoylated forms on Western blots with anti-SUMO1 antibodies (Figure 2D). As predicted, some of the 130 kDa RFP-MBD1 was sumoylated, producing two slow migrating bands of ~190 and ~140 kDa, while the single mutations (K450A, K489A, E452A and K491A) produced only single additional band of ~140 kDa (Figure 2C and D). Therefore, we conclude that the 190 kDa protein is MBD1 sumoylated at both lysine residues, while the 140 kDa proteins are the monosumoylated forms of MBD1. We were unable to detect SUMO1 conjugated to MBD1 carrying double K to A or E to A point mutations (RFP-MBD12(K–A) and RFP-MBD12(E–A); Figure 2C and D). Similar sumoylation patterns could be observed with GST-MBD1 (wild type and mutants) coexpressed in Escherichia coli (Supplementary Figure S2). Collectively, these experiments demonstrate that the VKQE sequences are the only functional sumoylation sites within MBD1.

Figure 1 Sumoylation of MBD1 protein in vivo. (A) The anti-MBD1 antibodies detect an additional 130 kDa band in nuclear extracts prepared with SUMO isopeptidase inhibitor NEM. (B) The anti-MBD1 antibodies recognize a 130 kDa band in SUMO1 but not in SUMO2/3 IPs. (C, D) The 130 kDa band in MBD1 IPs is recognized by anti-SUMO1 but not by anti-SUMO2/3 antibodies. (E) SUMO1 siRNA efficiently reduces the ladder of SUMO1-modified proteins in HeLa nuclear extracts. An arrowhead indicates the major SUMO1-modified protein RanGAP1. (F) siRNA against SUMO2/3 leads to substantial loss of SUMO2/3-modified proteins in HeLa nuclear extracts. (G) SUMO1 but not SUMO2/3 siRNA reduces the sumoylation MBD1.
GST-MBD1 in *E. coli* is comparable to the ∼60 kDa shift of sumoylated MBD1 observed in human cells. This suggests that the endogenous MBD1 is normally modified by SUMO1 at both lysine residues.

**SUMO conjugation sites are not essential for the localization of MBD1 to nuclear foci**

It has been reported that SUMO-modified transcription factors are recruited to promyeloid leukaemia protein bodies.
and nuclear matrix attachment regions (Zhong et al., 2000; Sachdev et al., 2001). Therefore, we asked whether sumoylation of MBD1, although seemingly low on Western blots, might be important for the recruitment of MBD1 to some specific nuclear compartments. In human cells, the endogenous MBD1, as well as GFP-tagged MBD1 expressed from a transfected plasmid, accumulate in 6–7 nuclear foci, against a background of diffuse nuclear staining (Figure 2E and H). When we coexpressed GFP-tagged MBD1 and RFP-tagged single (K450A, K489A) or double (2(K–A)) lysine mutants of MBD1 in HeLa cells, the wild-type and mutant MBD1 proteins showed identical nuclear localization (Figure 2E–G and H–J). Therefore, it seems unlikely that sumoylation facilitates the recruitment of MBD1 protein to specific nuclear domains.

**PIAS1 and PIAS3 interact with the central portion of MBD1**

PIAS1 and PIAS3 proteins share ~65% homology and contain a SAR/Acinus/PIAS (SAP) putative DNA-binding domain, a nuclear localization signal, a catalytic domain known as SP-RING and a SUMO interaction motif (SIM) (Figure 3A). To characterize in more detail the interactions between MBD1 and PIAS proteins, we generated series of PIAS1 and PIAS3 deletion constructs and tested them in yeast two-hybrid assays for binding to full-length MBD1 (Supplementary Figures S3 and S4). These interactions were independently confirmed by GST-MBD1 pull-down assays with in vitro translated HA-tagged full-length and truncated PIAS1 (Supplementary Figure S3). From these experiments, we found that the C-terminus of PIAS1 (amino acids 510–651) and the C-terminus of PIAS3 (amino acids 500–619) are required for binding to MBD1 in vitro and in vivo.

To investigate further whether PIAS1 and PIAS3 bind to a specific region of MBD1, we tested various MBD1 deletion constructs in yeast two-hybrid assays with full-length PIAS1 and PIAS3 proteins (Figure 3B). As above, these interactions were independently confirmed by pull-down assays with GST-tagged truncated MBD1 proteins and in vitro translated HA-tagged PIAS1 and PIAS3 (Figure 3C and D). From all MBD1 deletion constructs, a protein containing the second CxxC motif and the adjacent region of MBD1 (amino acids 221–480) was sufficient for binding of PIAS1 and PIAS3 in vitro and in vivo (Figure 3B–D). Notably, PIAS1 and

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**Figure 3** PIAS1 and PIAS3 bind to the second CxxC motif and adjacent region of MBD1. (A) Schematic representation of human PIAS1 protein and its known domains. The regions conserved between PIAS1 and PIAS3 (~65% homology) are indicated. (B) MBD1 deletion constructs were used to identify regions of MBD1 that bind PIAS1 and PIAS3 in yeast two-hybrid assays. The interactions were scored by growth on -Leu/-Trp/-His/-Ade (QDO) medium. (C–D) GST-MBD1 deletion proteins were used to pull down in vitro translated HA-tagged PIAS1 and PIAS3 proteins.
PIAS3 binding overlaps with the first sumoylation site of MBD1 at K450 and is in a close proximity to the second sumoylation site at K489. This suggested to us that MBD1 may be a direct target for conjugation of SUMO1 by PIAS1 and PIAS3.

**PIAS1 and PIAS3 are required for sumoylation of MBD1 in vivo**

Because PIAS proteins interact with MBD1, we asked whether PIAS1 and PIAS3 are responsible for sumoylation of MBD1 in vivo. We transfected HeLa cells with pools of siRNA against PIAS1, PIAS3 or both PIAS1 and PIAS3 and analysed the levels of PIAS proteins and sumoylation of MBD1 on Western blots (Figure 4A–C). GL2 siRNA against firefly luciferase was used as a control for nonspecific effects. PIAS1 and PIAS3 siRNAs efficiently reduced the protein levels of the respective PIAS protein compared to the controls (Figure 4A). In both cases, the loss of a PIAS protein was accompanied by a decrease in SUMO1-modified MBD1 (Figure 4B). However, MBD1-SUMO1 was no longer detectable on Western blots only when we depleted PIAS1 and PIAS3 simultaneously (Figure 4B). This implies that both PIAS proteins contribute to conjugation of SUMO1 to MBD1.

To investigate further whether an increase in the levels of PIAS1 and PIAS3 would stimulate sumoylation of MBD1, we transfected HeLa with plasmids expressing Flag-tagged SUMO1 or co-transfected them with Flag-SUMO1 and GFP-tagged either wild-type PIAS1 and PIAS3 or catalytically inactive PIAS1<sup>C350S</sup> and PIAS3<sup>C5/H4A</sup> (Long et al., 2004; Munarriz et al., 2004) (Figure 4D). The cells were collected 2 days after the transfection and the levels of PIAS1, PIAS3 proteins and SUMO-modified MBD1 in the nuclear extracts were examined. Western blots with anti-PIAS1 antibodies detected both endogenous and GFP-tagged PIAS1, indicating that the overall PIAS1 levels in transiently transfected cells were approximately two-fold higher than in the controls (Figure 4D, top panel). Both GFP-PIAS1 and GFP-PIAS3 were detectable with anti-GFP antibodies (Figure 4D, bottom panel). Consistent with our binding assays and siRNA experiments, we observed a significant increase in SUMO-modified MBD1 in nuclear extracts of cells co-transfected either with GFP-PIAS1 and Flag-SUMO1 or with GFP-PIAS3 and Flag-SUMO1 (Figure 4E and F). We did not detect accumulation of MBD1-SUMO1 in cells transfected with catalytically inactive PIAS proteins (Figure 4E and F). The sumoylation of MBD1 by PIAS1 and PIAS3 seems to be specific, since transfections with GFP-PIASy, another PIAS family protein, or Flag-SUMO1 alone did not induce accumulation of MBD1-SUMO1 (Supplementary Figure S5). We also noticed that transfection of GFP-PIAS1 or GFP-PIAS3 without Flag-SUMO1 was sufficient to stimulate the sumoylation of MBD1 (Figure 5A and B and Supplementary Figure S5). This indicates that the endogenous concentration of PIAS proteins rather than SUMO1 itself is limiting the sumoylation of endogenous MBD1. It also implies that both PIAS1 and PIAS3 SUMO ligases are functionally equivalent and can target MBD1 independently. In principle, PIAS1 and PIAS3 could either directly sumoylate MBD1 or stimulate sumoylation of MBD1 by some indirect mechanism. Since PIAS proteins interact with MBD1 in vitro and in vivo (see below), and their catalytic activities are required for sumoylation of MBD1, these data collectively suggest that MBD1 is a direct target for SUMO conjugation by PIAS1 and PIAS3.

**Sumoylation inhibits the formation of a complex between MBD1 and SETDB1**

Sumoylation of target proteins can affect the assembly of specific protein complexes by either promoting or disrupting

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**Figure 4** PIAS1 and PIAS3 are required for sumoylation of MBD1 in vivo. (A) siRNA depletion of PIAS1, PIAS3 and both PIAS proteins in HeLa. GL2 is a control siRNA against firefly luciferase. (B) MBD1 and MBD1-SUMO1 detected in the extracts of PIAS-depleted cells (as in A). MBD1-SUMO1 is lost completely only when both PIAS1 and PIAS3 were depleted. (C) PCNA was used as a loading control for the experiments shown in (A) and (B). (D) HeLa cells were transfected with plasmids expressing the indicated proteins. PIAS1, GFP-PIAS1 and GFP-PIAS3 (both wild-type and catalytic mutants PIAS1<sup>C350S</sup> and PIAS3<sup>C5/H4A</sup>) were detected with anti-PIAS1 and anti-GFP antibodies. (E) A Western blot of the same extracts as in (D) probed with anti-MBD1 antibodies demonstrates that MBD1-SUMO1 accumulates in cells transfected with GFP-PIAS1 and GFP-PIAS3 but not in cells transfected with catalytically inactive PIAS proteins. (F) MBD1 was immunoprecipitated from the same extracts as in (D) and (E), and MBD1-SUMO1 was detected with anti-Flag antibody.
protein–protein interactions (Johnson, 2004). Our evidence that SUMO1 is usually conjugated to both K450 and K489 of MBD1 in human cells led us to investigate whether sumoylated MBD1 is able to interact with its partner proteins, SETDB1 and CAF-1. We have previously shown that in HeLa cells MBD1 forms a stable complex with SETDB1 throughout the cell cycle, and with chromatin assembly factor CAF-1 specifically in S-phase (Sarraf and Stancheva, 2004). The region of MBD1 interacting with SETDB1 (amino acids 221–480) contains the first sumoylation site of MBD1 (Figures 2A and 3B). The TRD of MBD1 (amino acids 221–480) contains the first sumoylation site of MBD1 at K450. Notably, the SETDB1 binding region of MBD1 also overlaps with the amino acids required for PIAS1/PIAS3 binding (Figures 2A and 3B). The TRD of MBD1 (amino acids 480–546) includes the second sumoylation site at K489

To establish whether sumoylated MBD1 interacts with SETDB1 and CAF-1, we transfected HeLa with either GFP-PIAS1, GFP-PIAS3 or co-transfected them with GFP-PIAS1 and Flag-SUMO1 to achieve accumulation of MBD1-SUMO1. Both unmodified MBD1 and sumoylated MBD1 were easily detectable in the transfected cells with anti-MBD1 antibodies (Figure 5A and B, IP supernatants). We synchronized the cells in the S-phase, prepared nuclear extracts and immunoprecipitated SETDB1 and CAF-1 (Figure 5A and B, IP pellet). In SETDB1 IPs, the nonsumoylated MBD1 readily co-immunoprecipitated with SETDB1, as detected by anti-MBD1 antibodies (Figure 5A, IP pellet). By contrast, the MBD1-SUMO1 that SUMO1 is usually conjugated to both K450 and K489 of MBD1 in human cells led us to investigate whether sumoylated MBD1 is able to interact with its partner proteins, SETDB1 and CAF-1. We have previously shown that in HeLa cells MBD1 forms a stable complex with SETDB1 throughout the cell cycle, and with chromatin assembly factor CAF-1 specifically in S-phase (Sarraf and Stancheva, 2004). The region of MBD1 interacting with SETDB1 (amino acids 221–480) contains the first sumoylation site of MBD1 (Figures 2A and 3B). The TRD of MBD1 (amino acids 221–480) contains the first sumoylation site of MBD1 at K450. Notably, the SETDB1 binding region of MBD1 also overlaps with the amino acids required for PIAS1/PIAS3 binding (Figures 2A and 3B). The TRD of MBD1 (amino acids 480–546) includes the second sumoylation site at K489 and is known to recruit the SETDB1 cofactor AM/MCAF1 (Fujita et al., 2003a). Thus, it is reasonable to speculate that sumoylation of MBD1 may affect SETDB1 and/or AM binding. On the other hand, CAF-1 binds to the N-terminus of MBD1, which is far from the sumoylatable lysines and may not be affected by conjugation of SUMO to MBD1 (Figure 2A).
IP with anti-CAF-1 antibodies (Figure 5B, IP supernatant). From these experiments, it is clear that MBD1-SUMO1 does not interact efficiently with SETDB1 but still can bind to CAF-1 in the S-phase.

**Downregulation of SETDB1 leads to accumulation of MBD1-SUMO**

In earlier studies, we found that most of the MBD1 co-immunoprecipitates with SETDB1 from HeLa nuclear extracts (Sarraf and Stancheva, 2004). Since PIAS proteins and SETDB1 share the same region of MBD1 for binding, we hypothesized that the formation of a complex between MBD1 and SETDB1 might protect MBD1 against conjugation of SUMO1 by the endogenous PIAS1 or PIAS3. To test this, we used siRNA to reduce SETDB1 protein levels in HeLa and asked whether MBD1-SUMO1 accumulates in these cells. GL2 siRNA served as a control. Western blots with anti-SETDB1 antibodies showed that 2 and 4 μg of SETDB1 siRNA, but not the equivalent amounts of GL2 siRNA, reduced SETDB1 protein to about 40 and 10%, respectively, compared to the controls (Figure 5C). In the same extracts, Western blots with anti-MBD1 antibodies detected a dose-dependent accumulation of MBD1-SUMO1 in cells treated with SETDB1 siRNA but not with GL2 siRNA (Figure 5C). Neither SETDB1 nor GL2 siRNA led to the upregulation of PIAS1 or PIAS3 protein levels, or had a general effect on SUMO1-modified proteins as the sumoylation of RanGAP1 remained unchanged (Figure 5C). Consistent with accumulation of MBD1-SUMO1 in cells treated with SETDB1 siRNA, MBD1 co-immunoprecipitated more efficiently with PIAS proteins when SETDB1 levels were reduced (Supplementary Figure S6).

In summary, these experiments demonstrate that SETDB1 protein, when present in HeLa cell, competes against PIAS1 and PIAS3 for binding to MBD1 and thus protects MBD1 from targeting by the SUMO modification machinery. Given that most of the MBD1 in HeLa is in complex with SETDB1, this may explain why only a small proportion of MBD1 is sumoylated in these cells (Figure 1A).

The inhibition of SETDB1 binding to MBD1 by SUMO1 should result in a detectable decrease of MBD1-associated HMT activity comparable to that observed in SETDB1-depleted cells (Sarraf and Stancheva, 2004). To test this, we immunoprecipitated MBD1 from nuclear extracts prepared from cells transfected either with GFP-PIAS1, GFP-PIAS3, GFP-PIAS3CS/HA or from cells treated with SETDB1 and GL2 siRNAs. These IPs were further used for *in vitro* histone methylation assays using recombinant histone H3 N-terminal tails as a substrate for incorporation of 3H-labelled methyl groups (Figure 5D). In agreement with our previous results, MBD1 IPs from cells transfected with GFP-PIAS1 or GFP-PIAS3 or from cells treated with SETDB1 siRNA showed significantly reduced histone methylation activity compared to the controls (Figure 5D). This in turn led us to investigate whether transcriptional repression by MBD1 is compromised in cells with elevated levels of MBD1-SUMO1.

**Sumoylation antagonizes MBD1-mediated transcriptional repression**

We have previously shown that MBD1/SETDB1 complex methylates chromatin at K9 of histone H3 to silence transcription of the MBD1 target gene p53BP2 in HeLa (Sarraf and Stancheva, 2004). Efficient transcriptional repression by MBD1 requires interaction with SETDB1 protein, since p53BP2 is aberrantly expressed in cells treated with SETDB1 siRNA (Sarraf and Stancheva, 2004). Given that MBD1-SUMO1 does not interact with SETDB1, we asked whether accumulation of MBD1-SUMO1 in cells overexpressing PIAS1 might lead to the loss of p53BP2 silencing and thus mirror the effect of SETDB1 siRNA. Therefore, we compared the expression of p53BP2 in cells co-transfected with SETDB1 siRNA to that in cells co-transfected with GFP-PIAS1 and Flag-SUMO1 (Figure 6B, p53BP2). As a control, we co-transfected cells with catalytically inactive GFP-PIAS1CS/350S and Flag-SUMO1. In all experiments, we used the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a control for nonspecific effects (Figure 6B, GAPDH). Consistent with our previous observations, p53BP2 expression was detectable in cells treated with SETDB1 siRNA. Neither PIAS1/3 siRNA nor SUMO1/2/3 siRNA led to aberrant expression of p53BP2. In contrast, transfections with PIAS1 and Flag-SUMO1 but not with PIAS1CS/350S and Flag-SUMO1 upregulated p53BP2 transcription to levels comparable to those seen in SETDB1 siRNA-treated cells (Figure 6B). Thus, sumoylation of MBD1 by PIAS1 not only inhibits SETDB1 binding but also disrupts transcriptional repression of p53BP2 gene normally mediated by the MBD1/SETDB1 complex.

We further hypothesized that a form of MBD1 with both sumoylatable lysines mutated to alanine (MBD12K(A)) should not be a target of the SUMO conjugation machinery and might rescue the repression of p53BP2 in PIAS1-overexpressing cells. We co-transfected cells with GFP-PIAS1, Flag-SUMO1 and either RFP-MBD12K(A) or RFP-MBD1 and analysed them for p53BP2 expression. In parallel, we investigated the single lysine mutants of MBD1 (MBD1K450A and MBD1K489A) to determine the contribution of individual sumoylation sites for the regulation of MBD1-mediated repression by SUMO. All GFP-MBD1 proteins showed comparable expression in the transfected cells (Supplementary Figure S7). However, only the nonsumoylatable MBD12K(A) but not RFP-MBD1 could successfully rescued p53BP2 repression in PIAS1-overexpressing cells (Figure 6B). MBD1K450A and MBD1K489A were not as efficient as MBD12K(A) in silencing p53BP2 (Figure 6B). Collectively, these experiments demonstrate that conjugation of SUMO to either one of the two sumoylatable lysines of MBD1 results in disruption of MBD1-mediated repression of p53BP2.

**SETDB1-mediated H3K9 methylation of p53BP2 promoter is lost in PIAS1-overexpressing cells**

Recruitment of the MBD1/SETDB1 complex to the p53BP2 promoter in HeLa cells establishes transcriptionally repressive chromatin trimethylated at K9 of histone H3 (Sarraf and Stancheva, 2004). Given that sumoylation of MBD1 by PIAS1 inhibited binding of SETDB1 to MBD1 and induced p53BP2 transcription, we examined whether this is accompanied by loss of MBD1, SETDB1 and H3K9 methylation from the promoter of the p53BP2. Chromatin immunoprecipitations (ChIP) with anti-MBD1 antibodies detected approximately equal amounts of MBD1 bound to the p53BP2 promoter in nontransfected cells, cells transfected with GFP-PIAS1, GFP-PIAS1 and Flag-SUMO1, and in control cells transfected with PIAS1CS/350S and Flag-SUMO1 (Figure 6C, α-MBD1). Thus, accumulation of MBD1-SUMO1 in cells overexpressing...
PIAS1 does not affect MBD1 binding to the p53BP2 promoter. A sequential pull down of MBD1-enriched chromatin (immunoprecipitated with antibodies against MBD1) with anti-SUMO1 and anti-Flag antibodies detected SUMO1-modified MBD1 bound to p53BP2 sequences in cells transfected with PIAS1 and PIAS1/SUMO1 but not in the control cells (Figure 6C, α-SUMO1, α-Flag). This indicates that MBD1-SUMO is not impaired in binding to methylated DNA and localizes to the endogenous MBD1 binding sites. However, ChIP with anti-SETDB1 and anti-trimethyl H3K9 antibodies detected reduced amounts of SETDB1 at the p53BP2 promoter, as well as reduced H3K9 methylation of p53BP2 promoter chromatin, in cells transfected with PIAS1 and PIAS1/SUMO1 as compared to the controls (Figure 6C, α-SETDB1, α-H3K9me3). In summary, these experiments demonstrate that sumoylated MBD1, although capable of binding to methylated DNA, is unable to recruit SETDB1 HMT to chromatin. This results in the loss of the repressive histone modification and aberrant expression of p53BP2.

Discussion

**MBD1 is targeted for sumoylation by PIAS1 and PIAS3 proteins**

Post-translational modifications such as phosphorylation, ubiquitination, acetylation and sumoylation often contribute to the regulation of protein function and stability. Here we demonstrate that the methyl-CpG-binding protein MBD1 is SUMO1-modified in human cells at lysines K450 and K489 within two conserved SUMO conjugation sites (VKQE). These two sequences seem to be the only functional sumoylation sites within MBD1, since mutant MBD1 proteins with either lysines 450 and 489 or glutamates 452 and 491 mutated to alanine cannot be sumoylated in HeLa or in E. coli expressing mammalian SUMO conjugation enzymes. The conservation of sumoylation sites between amphibian and human MBD1, within a region that shows overall poor amino-acid similarity, suggests that SUMO conjugation to MBD1 might be functionally important in all vertebrate species.

In search of enzymatic activities that target MBD1 for sumoylation, we found that two E3 SUMO ligases, PIAS1 and PIAS3, which we initially identified in a yeast two-hybrid screen, are required for the conjugation of SUMO1 to MBD1 as revealed by RNAi experiments. Both PIAS proteins, when expressed above their endogenous levels in HeLa, efficiently target MBD1 for SUMO conjugation. It is likely that SUMO is transferred directly from PIAS1 or PIAS3 to MBD1 since catalytically inactive PIAS1C350S and PIAS3CS/HA did not lead to accumulation of MBD1-SUMO in vitro. PIAS1 and PIAS3 share significant homology within their functional SAP and SP-RING domains (Figure 3A). However, the least conserved C-terminal regions of both PIAS proteins bind to MBD1 in vitro and in yeast two-hybrid assays. On the other hand, another PIAS family protein, PIASY, neither interacts with MBD1 nor stimulates sumoylation of MBD1 in HeLa cells. Characteristically for transient enzyme–substrate interactions, the endogenous MBD1 and PIAS1 or PIAS3 do not immunoprecipitate efficiently from HeLa nuclear extracts. We could detect an interaction between the endogenous MBD1 and PIAS proteins only when the levels of SETDB1 protein...
were reduced by siRNA (Supplementary Figure S6). Therefore, it is unlikely that PIAS1 and PIAS3 form a stable complex with MBD1.

**Properties of sumoylated MBD1 protein**

A number of studies demonstrate that sumylation of nuclear proteins can affect their localization and function (Johnson, 2004; Hay, 2005). The presence of sumoylated MBD1 in all human cell lines examined, and accumulation of MBD1-SUMO in cells overexpressing PIAS1 or PIAS3, prompted us to investigate the properties of SUMO1-modified MBD1. As a starting point, we established that the sumoylation sites of MBD1 are not essential for the localization of MBD1 to nuclear foci. A mutant MBD1 protein MBD1\(^{2(K-A)}\), with both sumoylatable lysines replaced by alanine, displays localization indistinguishable from that of the wild-type MBD1 including in cells that overexpress PIAS proteins (not shown). Our subsequent analyses by ChIP demonstrate that MBD1-SUMO binds as well as unmodified MBD1 to known endogenous MBD1 binding sites such as the p53BP2 promoter. Given that the sumoylatable lysines K450 and K489 are not located within the two DNA-binding domains of MBD1, it is unlikely that MBD1-SUMO1 has DNA-binding properties unique to the modified protein. However, it is still possible that conjugation of SUMO1 induces subtle conformational changes that affect the affinity of MBD1 for some specific genomic sequences. In the future, it might be of interest to investigate in more detail the binding preferences of MBD1-SUMO1.

As post-translational protein modifications, including sumoylation, can either promote or inhibit the assembly of specific protein complexes, we investigated whether MBD1-SUMO1 binds its partner proteins. Interestingly, we found that MBD1-SUMO1 readily co-immunoprecipitates from HeLa nuclear extracts with chromatin assembly factorCAF-1 p150, but not with SETDB1 HMT. Consistently, MBD1 immunoprecipitates from PIAS1- or PIAS3-overexpressing cells have reduced HMT activity. Given that the sumoylation sites of MBD1 overlap with or are in close proximity to the region of SETDB1 binding (Figure 2A), one could hypothesize that SUMO1 conjugated to MBD1 interferes sterically with SETDB1 binding. However, preliminary in vitro binding experiments indicate that bacterially expressed GST-MBD1 and GST-MBD1-SUMO1 interact with SETDB1 equally well (Supplementary Figure S8). Therefore, the inhibition of SETDB1 binding to MBD1-SUMO in vivo may either involve additional proteins that preferentially recognize MBD1-SUMO1 but do not bind to MBD1 or, alternatively, sumoylation may promote subsequent post-translational modifications of MBD1 that are inhibitory to SETDB1 binding.

While this manuscript was under review, it was reported that AM/MCAF1, a protein that binds to and regulates SETDB1, preferentially interacts with SUMO2/3-modified MBD1 in vitro (Uchimura et al., 2006). These findings have led to the suggestion that conjugation of SUMO2/3 to MBD1 could promote the recruitment of SETDB1 to MBD1 via MCAF1 and thus result in the stabilization of heterochromatic loci, in contrast to our observations that conjugation of SUMO1 to MBD1 disrupts the interaction with SETDB1. Whether SUMO2/3 modification of MBD1 is required for MCAF1 binding in vivo clearly requires further investigation, because of the interesting possibility that conjugation of SUMO1 and SUMO2/3 may have opposing effects on MBD1-associated corepressor complexes. However, we note that, unlike Uchimura et al. (2006), we failed to detect any SUMO2/3-modified MBD1 under our experimental conditions. The reasons for these differing results may be due to the specific cell types and/or growth conditions used.

**Regulation of MBD1-mediated repression by a competitive binding of PIAS and SETDB1 proteins to MBD1**

The functional significance of SUMO1 conjugation to MBD1 became apparent when we investigated the expression of an MBD1 target gene, p53BP2, in cells overexpressing PIAS1. Normally, MBD1 binds to methylated DNA at the promoter of p53BP2 and represses transcription via recruitment of SETDB1, which methylates chromatin at K9 of histone H3. In cells overexpressing PIAS1, MBD1-SUMO1 is still bound to p53BP2 promoter but no longer recruits SETDB1. This results in a decrease of H3K9 methylation and aberrant transcription of p53BP2 gene. Interestingly, only the MBD1\(^{2(K-A)}\) protein with both sumoylatable lysines replaced by alanine, but not the MBD1 proteins carrying single lysine mutations, could rescue the repression of p53BP2 in PIAS1-overexpressing cells. This implies that sumoylation of either lysine, K450 or K489 is sufficient to abrogate transcriptional silencing mediated by MBD1. On the other hand, MBD1 seems to be protected from conjugation of SUMO when it is in complex with SETDB1. Thus in cells treated with SETDB1 siRNA, MBD1 is more readily available to the sumoylation machinery. Notably, the upregulation of p53BP2 in PIAS1-overexpressing cells is comparable to the level of p53BP2 expression in cells treated with SETDB1 siRNA. Taken together, these observations suggest the existence of a finely balanced mechanism to regulate transcriptional repression by MBD1, which operates through SETDB1 and PIAS proteins competing for binding to MBD1 (Figure 7). Assuming that MBD1 levels are constant, as they were throughout our experiments, the outcome of this competition, and therefore the ability of MBD1 to repress transcription, would be dependent on the concentration of SETDB1 and PIAS proteins. Thus, the formation of a repressive MBD1/SETDB1 complex should be favored in cells where either SETDB1 protein levels are high or PIAS1 and PIAS3 levels are low (Figure 7A). Conversely, high levels of PIAS1 and/or PIAS3 or low levels of SETDB1 should result in SUMO ligases competing more efficiently against SETDB1 for binding to MBD1 (Figure 7B). Ultimately, the interaction of MBD1 with PIAS proteins should lead to sumoylation of MBD1, inhibition of complex formation between MBD1 and SETDB1, and inefficient repression of MBD1 target genes. It is to be expected that the activation of transcription following sumoylation of MBD1 would require the repressive H3K9 methylation to be removed from chromatin. Given that sumoylated MBD1 binds to CAF-1 but no longer recruits SETDB1 to methylate newly assembled nucleosomes, it is likely that the efficient expression of MBD1 target genes may require the passage of the cells through the S phase. Alternatively, H3K9 methylation may be removed by either histone H3 exchange concomitant with transcriptional activation or by an enzymatic H3K9 demethylation activity that is recruited to MBD1-SUMO1. Further experiments will address these interesting possibilities.
In conclusion, we have shown that conjugation of SUMO1 to MBD1 by PIAS family proteins inhibits the formation of a complex between MBD1 and SETDB1 HMT and antagonizes MBD1/SETDB1-mediated repression. Our data are consistent with a model where the function of MBD1 in transcriptional repression and maintenance of silenced chromatin is regulated by SETDB1 and PIAS proteins competing for binding to MBD1. Such a flexible mechanism to regulate MBD1-mediated repression may be particularly important during embryonic development, when gene expression in various lineages of differentiating cells is switched on or off. Additionally, aberrant methylation of CpG islands in cells undergoing tumorigenic transformation may require a reinforced potential of MBD1 to silence transcription, which could be achieved by downregulation of PIAS proteins. Notably, aberrantly high or aberrantly low expression of PIAS1 and PIAS3 has been detected in human tumours and may contribute to the progression of the disease (Beer et al., 2002; Lapointe et al., 2004; Wang and Banerjee, 2004).

Materials and methods

Plasmids and transient transfections
Point mutations in MBD1, PIAS1 and PIAS3 were generated by mutagenic PCR. To generate catalytically inactive PIAS proteins, we mutated cysteine 350 in the SP-RING domain of PIAS1 to serine (PIAS1C350S) and cysteine 334 and histidine 336 in the catalytic domain of PIAS3 to serine and alanine respectively (PIAS3 CS/HA).

Antibodies and co-IP assays
The preparation of nuclear extracts and co-IPs were performed according to standard protocols. The antibodies used for IPs and Western blots were anti-MBD1 IMG-306 (TCS Cell Works), anti-Flag M2 (Sigma), anti-CAF-1, anti-GFP, anti-HA (provided by CRUK), anti-SETDB1 (Upstate), anti-RFP (Clontech), anti-PIAS1 sc8152, anti-SUMO1 sc5308, anti-SUMO2/3 sc52873 (Santa Cruz Biotechnology) and anti-PIAS3 (Abcam).

In vitro pull downs
GST-tagged MBD1 deletion constructs and pull-down procedure were described previously (Sarraf and Stancheva, 2004). pGADT7-PIAS1 and pGADT7-PIAS3 were translated in rabbit reticulocyte lysates (TnT T7 kit, Promega). Hundred nanograms of each GST-MBD1 protein and 1 μl of translated PIAS1 or PIAS3 were used in the pull-down experiments.

Histone methylation assays
Histone methylation assays with MBD1 immunoprecipitates were performed as described previously (Sarraf and Stancheva, 2004). Recombinant GST-tagged histone H3 N-terminal peptide containing amino acids 1–54 was used as a substrate (Tachibana et al., 2001). The incorporation of [3H]-methyl groups by the immunoprecipitated MBD1 complexes was measured by scintillation counting (PerkinElmer) and analysed on 12% SDS gels by autoradiography.

RT-PCR
RNA purification, reverse transcription and PCRs were performed as described elsewhere (Sarraf and Stancheva, 2004).

ChIP
Chromatin IPs were performed as described in Sarraf and Stancheva (2004). For re-ChIP immunoprecipitated chromatin from the first IP (scaled 3 × 1) was removed from protein G sepharose by adding SDS to 1% and incubation for 15 min at room temperature. The samples were diluted to 0.1% SDS and subjected to a second round of IP and washes. Additional antibodies (to those above) used for ChIP assays were anti-H3K9me3 (provided by P Singh) and anti-H3 (Upstate).

Microscopy
Transfected HeLa cells were grown on 15 mm cover slips in DMEM, fixed with 4% paraformaldehyde in PBS, mounted in vectashield and observed by Olympus BX61 microscope equipped with ColorView CCD camera and AnalySIS software for image capture.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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