IN INVOLVEMENT OF SUMO MODIFICATION IN MBD1- AND MCAF1-MEDIATED HETEROCHROMATIN FORMATION

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Small ubiquitin-related modifiers, SUMOs, are involved in gene regulation and nuclear structures. However, little is known about the roles of SUMO-2/3 and SUMO-1 in heterochromatin formation of mammalian cells. Here, we demonstrate that SUMOs directly interact with human MCAF1, which forms complexes with either the methyl-CpG-binding protein MBD1 or SETDB1, which trimethylates histone H3 at lysine 9 (H3-K9) in the presence of MCAF1. Modification of MBD1 with either SUMO-2/3 or SUMO-1 facilitated the interaction between MBD1 and MCAF1, suggesting that SUMOylation links the methylation of DNA and histones. In a cultured human cell line, SUMOs were localized in MBD1- and MCAF1-containing heterochromatin regions that were enriched in trimethyl-H3-K9 and the heterochromatin proteins HP1β and HP1γ. Specific knockdown of either SUMO-2/3 or SUMO-1 induced dissociation of MCAF1, trimethyl-H3-K9 and the HP1 proteins from the MBD1-containing heterochromatin foci, suggesting a requirement for SUMOs for heterochromatin assembly. These findings provide insights into the roles of SUMOylation in the regulation of heterochromatin formation and gene silencing.

SUMOs are small ubiquitin-related modifiers that covalently attach to their target proteins, a process referred to as SUMOylation. This process involves an enzymatic pathway, which is similar to the pathway employed in the ubiquitin conjugation cascade (1-3). In contrast to ubiquitin, however, there are at least three SUMO paralogs in human cells, namely SUMO-2/SMT3A, SUMO-3/SMT3B and SUMO-1/SMT3C (4). SUMO-2 and -3 are more closely related to each other (95% amino acid identity) than to SUMO-1 (~50% identity). It has become increasingly clear that a wide variety of cellular proteins can be modified by either SUMO-2/3, SUMO-1 or both, leading to alterations in many signaling pathways associated with their target proteins. Most, but not all, of the SUMOylation target proteins appear to be involved in maintenance of nuclear integrity, regulation of nuclear transport or control of chromatin functions, such as transcription, replication, repair, recombination and chromatin modification (5,6).

Currently, there are numerous examples of co-repressor proteins that can be SUMOylated and transcription factors whose activities are down-regulated by SUMOylation, implying the involvement of a SUMO modification system in the regulation of transcriptional repression and maintenance of silenced heterochromatin (7-9). Although the exact mechanisms by which SUMO modification contributes to such anti-activation activities in chromatin remain poorly defined, it has been speculated that SUMOylation mediates changes in gene expression and chromatin assembly, at least, by serving as a binding platform for recruiting other chromatin proteins that may transmit or amplify the SUMO signal and maintain
the silenced state of chromatin (7-9). More recently, SUMO-mediated recruitment of histone deacetylases has been reported to play an important role (10-14), implying the possibility that histone-modifying enzymes may act as effectors for SUMO modification signals.

Covalent addition of a methyl group to the 5 position of cytosine (5mC) is the major modification of DNA in vertebrate genomes. This modification predominantly occurs within CpG dinucleotides, and is involved in a wide range of biological phenomena, including genomic imprinting, X-chromosome inactivation and tissue-specific gene expressions (15,16). Furthermore, it is well established that sites of DNA methylation are recognized by a family of proteins designated methyl-CpG-binding proteins (16,17). These proteins utilize transcriptional co-repressors or mediators to silence transcription and also modify the surrounding chromatin, thereby providing a link between DNA methylation and chromatin remodeling and modifications. Among these proteins, MBD1 is relatively well characterized, and has been implicated in regulating chromatin structure and gene silencing through a currently unknown mechanism that probably involves histone modifications, such as deacetylation and H3-K9 methylation (16-21).

Considering the biological consequences of the effect of MBD1 on DNA methylation, the roles of an MBD1-interacting protein, designated MBD1-containing chromatin-associated factor 1 (MCAF1; also known as ATFa-associated modulator (AM)), are intriguing. Specifically, this protein has been reported to interact with MBD1 via the transcriptional repression domain (TRD) at the carboxyl-terminal region of MBD1 (21), and it also forms a tight complex with a histone H3-K9 methyltransferase, SETDB1/ESET, that appears to facilitate SETDB1-dependent conversion of dimethyl-H3-K9 to the trimethyl state (22). In addition to MBD1 and SETDB1, MCAF1 also interacts with several transcriptional factors, including ATFa (23), Sp1 (24) and the homeobox-containing zinc finger protein ZHX1 (25), suggesting that it acts as an recruiter for a wide range of proteins that can modulate gene regulation and chromatin formation. However, the mechanisms involved in regulating the assembly of macromolecular complexes containing MCAF1 and how such chromatin complexes contribute to the regulation of gene silencing and heterochromatin formation remain largely uncharacterized.

Here, we report that MCAF1 interacts with SUMO-2/3 and SUMO-1, with a preference for SUMO-2/3, via short peptide sequences similar to the SUMO-binding motif. Using a human cultured cell line, we demonstrate SUMOylation of MBD1 both in vitro and in vivo, as well as facilitated anchorage of MCAF1 to SUMOylated MBD1. Furthermore, RNA interference (RNAi) experiments directed against SUMO-2/3 or SUMO-1 reveal that depletion of the SUMO pathways perturbs the assembly of MCAF1, trimethyl-H3-K9, HP1β and HP1γ at MBD1-containing heterochromatin. Taken together, our results indicate that SUMOs function as epigenetic modulators for heterochromatin formation, at least in part, by regulating the MCAF1-MBD1 interaction.

**EXPERIMENTAL PROCEDURES**

**Plasmids** - To generate pAS2-1 SUMO-3G-SUMO-3G, human SUMO-3 was amplified by polymerase chain reaction (PCR) using the following oligonucleotides: forward primer: 5’-CATGCCATGGCCGACGAAAGCCAAGGAGCAGCAGAC-3’ (an NcoI site is underlined); reverse primer: 5’-CTGCAGAACCAGCACAATGGTCCCGTGCTGTCGTGGAAAC-3’ (a BstXI site is underlined); forward primer: 5’-CTGCAGAACCATTGTGCTGGCCGACGAAGCCCCAGAAC-3’ (a BstXI site is underlined); and reverse primer: 5’-CGCAGATCCATCCATCCGTCCTGGGCGCAAGAAC-3’ (a BamHI site is underlined). The generated PCR fragments were digested with NcoI, BstXI and BamHI, and cloned into pAS2-1 predigested with NcoI and BamHI. To generate pGEX4T-1 SUMO-1/2/3, SUMO sequences were PCR-amplified, and subcloned into pGEX4T-1 (Amersham Biosciences). To generate pET30 SUMO-1/2/3, SUMO sequences were PCR-amplified, and subcloned into pGEX4T-1 (Amersham Biosciences). To generate pET30 SUMO-1/2/3, SUMO sequences were PCR-amplified, and subcloned into pGEX4T-1 (Novagen). To generate pcDNA3 DsRed MCAF1965-975, MCAF1965-975 was annealed using...
the following oligonucleotides: 5′-AATTCGGTGTCAATCTCACATGGA TGATGAGAGTGC-3′ and 5′-TCGAGTCACTCTTCATCATCCATTGTA ATCAATGACACC -3′, and subcloned into pcDNA3 DsRed monomer (Aoto and Nakao, manuscript in preparation). To construct plasmids expressing MCAF1-D968A and -L969A, site-directed mutagenesis was performed. MBD1373-605 was PCR-amplified, and subcloned into pMAL-c2X (New England BioLabs) using the following oligonucleotides: forward primer: 5′-CGGAATTC
CGTTGGCGCCAATGCCTGCA
GTTT-3′ (an EcoRI site is underlined); and reverse primer: 5′-CCGTCTAGA
CTACTGCTTTCTAGCTCCAG
GTTT-3′ (an XbaI site is underlined). MCAF1 and MBD1 expression plasmids were used as previously described (20,21,24,26).

Yeast two-hybrid screening - The yeast strain AH109 carrying pAS2-1 SUMO-3G-SUMO-3G was transformed with a mouse 11-day embryo cDNA library constructed in pACT2 (Clontech). Plasmids harboring cDNAs were recovered from histidine-positive colonies.

Cell culture, small interfering RNAs (siRNAs) and transient transfection - HeLa and C-33A cells were cultured in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 Ham (Sigma) supplemented with 5% fetal calf serum (FCS) and antibiotics under 5% CO2. HeLa cells were transfected with plasmid DNAs using FuGENE 6 (Roche). For siRNA experiments, C-33A cells were transfected with 20 nM siRNA duplex oligoribonucleotides using Oligofectamine (Invitrogen), and harvested at 60 h post-transfection. The siRNA duplexes used were designed to target the mRNAs encoding human SUMOs. The sequences were as follows: SUMO-1#1: 5′-GAAUCAUACUGUAAAGACAGGGUG-3′ (Stealth™ siRNA; Invitrogen); SUMO-1#2: 5′-CACAUCUCAAGAAACUCAA-3′ (21-mer siRNA; Japan Bioservice); SUMO-2#1: 5′-GGCCUAUCUGCGAGGCAGGGCUUGC-3′ (Stealth™ siRNA; Invitrogen); SUMO-2#2: 5′-UGAGGCAGAUCAGAUUCAG (21-mer siRNA; Japan Bioservice); SUMO-3#1: 5′-AGCCUAUUGUGAACGACAGGGAUUG-3′ (Stealth™ siRNA; Invitrogen); SUMO-3#2: 5′-GAUUAAGAGCGCAUACACCA (21-mer siRNA; Japan Bioservice). The siRNAs for GL3 were described previously (24).

Bacterial SUMOylation, protein expression and purification - SUMO-modified MBP-MBD1373-605 was obtained using an E. coli SUMOylation system. E. coli strain BL21(DE3) co-transformed with pMAL-MBD1373-605 and pT-E1E2S3 (or pT-E1E2S1) protein expression vectors (27) was used to express MBP-tagged and SUMO-modified MBD1373-605. Expression of recombinant proteins was induced with 0.2 mM isopropyl-β-D-galactopyranoside (IPTG) at 25°C for 18 h. Purification of GST fusion proteins, MBP fusion proteins and (His)6 fusion proteins was carried out as previously described (27).

GST pull-down assay - The standard conditions used were as follows: bacterially expressed GST and GST fusion proteins (3 μg) were immobilized on glutathione-Sepharose beads (Amersham Biosciences) and incubated with (His)6-tagged proteins (3 μg) or E. coli lysates in a buffer (500 μl) consisting of 20 mM HEPES pH 7.9, 20 mM KCl, 1.5 mM MgCl2, 0.01 mM ZnCl2, 10% glycerol, 0.1% Triton X-100 and 0.1 mM dithiothreitol (DTT) for 1 h at 4°C. For the binding assay described in Fig. 5A, the pull-down assay was performed in a buffer (500 μl) consisting of 25 mM HEPES pH 7.5, 150 mM NaCl, 0.05% NP-40 and 1 mM DTT (28).

Antibodies - Two rat monoclonal antibodies, 4D12 against SUMO-1 and 3H12 against SUMO-2/3, were produced using a rat medial iliac lymph node method (29). The other antibodies used in this study were: anti-SUMO-1 (Alexis Co.), anti-SUMO-2/3 (4), anti-MCAF1 (21), anti-HP1α (24), anti-HP1β (24), anti-HP1γ (24), anti-MBD1 (Santa Cruz Biotechnology), anti-trimethyl-H3-K9 (Upstate), anti-trimethyl-H3-K27 (Upstate), anti-trimethyl-H3-K4 (Abcom), anti-SC35 (BD Biosciences), anti-hnRNP-U (ImmuQuest), anti-PML (Santa Cruz Biotechnology), anti-GST (Santa Cruz Biotechnology), anti-T7 (Novagen), anti-MBP (New England BioLabs), anti-Myc (Roche) and anti-FLAG M5 (Sigma).

Immunoprecipitation - C-33A and HeLa cells were treated with phosphate-buffered saline (PBS) containing 0.1% NP-40 and 20 mM N-ethylmaleimide (NEM) on ice for 10 min, rinsed with ice-cold PBS and lysed on ice with RIPA buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 1%...
NP-40, 0.1% sodium deoxycholate, 20 mM NEM, 0.1 mM DTT and protease inhibitors). After sonication and centrifugation for 15 min, nuclear cell lysates were incubated with specific antibodies or control immunoglobulin G (IgG) for 30 min at 4°C. Next, 30 μl of protein G-agarose beads (Amersham Biosciences) was added and the samples were incubated for a further 1 h. The beads were extensively washed with RIPA buffer as the binding buffer. The proteins associated with the beads were separated by SDS-PAGE, and subjected to immunoblot analysis.

Indirect immunofluorescence assay - For immunofluorescence labeling, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.2% Triton X-100 in PBS for 5 min. Following 3 rinses with PBS, the cells were sequentially incubated with a specific primary antibody, followed by an appropriate secondary antibody. The secondary antibodies used were: Alexa-488-conjugated donkey anti-rat IgG (Molecular Probes), Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch), Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) and Cy5-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) antibodies. After 3 further washes with PBS, the cells were mounted under coverslips and analyzed using an Olympus IX71 microscope and the Lumina Vision software (Mitani Co.).

RESULTS

MCAF1 binds to SUMO-1 and SUMO-2/3 with a preference for SUMO-2/3. To identify possible chromatin proteins that may specifically recognize the SUMO moiety and be recruited to sites of SUMOylation, we performed a yeast two-hybrid screening using the Gal4-SUMO-3G-SUMO-3G fragment, which was incapable of conjugation, as bait. After multiple rounds of screening of a mouse 11-day embryo cDNA library, we isolated a clone encoding amino acids 787-1306 of the ATFa-associated factor AM, a mouse homolog of human MCAF1 (Fig. 1A).

As a step toward understanding the role of MCAF1 binding to SUMO, we first performed in vitro GST pull-down assays to investigate whether the binding was direct and whether the binding affinities varied among the SUMO paralogs. When beads bound to GST-SUMO-1, GST-SUMO-2, GST-SUMO-3 or GST alone were incubated with a lysate from C-33A human cervical cancer cells, MCAF1 associated more efficiently with GST-SUMO-2/3-beads than with GST-SUMO-1-beads (Fig. 1B). MCAF1 binding to SUMO-1 and SUMO-2/3 was direct, since bacterially expressed recombinant full-length (His)₆-MCAF1 protein was retained on GST-SUMO-conjugated beads (Fig. 1C).

MCAF1 contains a conserved amino acid sequence similar to the SUMO-binding motif. Mouse and human MCAF1 proteins have been demonstrated to belong to an evolutionarily conserved family of MCAF/AM proteins (24). A comparison of MCAF family members revealed two highly conserved domains in both mouse and human MCAF1 proteins, referred to as Domain 1 and Domain 2. To determine the region responsible for binding to SUMOs, we generated a series of deletion mutants of human MCAF1, and performed GST pull-down assays. The results revealed that 11 amino acid residues (amino acids 965-975) of MCAF1 (MCAF1 965-975) located between Domain 1 and Domain 2 were sufficient for a specific interaction with (His)₆-SUMO-3 (Fig. 1A). A semi-quantitative binding analysis revealed that the affinity of MCAF1 965-975 for (His)₆-SUMO-2/3 was approximately 5-fold higher than that for (His)₆-SUMO-1 (Fig. 1D).

A database search for MCAF1 965-975 using the basic local alignment search tool algorithm BLAST detected several peptide sequences encoded in mouse, rat, dog and chicken MCAF1 (Supplemental Fig. 1A), suggesting evolutionary conservation of this amino acid sequence. We also found that the N-terminal half of this sequence partially overlapped with a recently described hydrophobic amino acid cluster in the SUMO-binding motif present in Pias1, Piasx, Sae2, Pml1, Ranbp2/Nup358 and thymine DNA glycosylase (Fig. 1E) (6,28,30-32). It should be mentioned that our alanine scanning analysis revealed that valine (V) at residue 966, isoleucine (I) at 967, aspartic acid (D) at 968, leucine (L) at 969 and threonine (T) at 970 in MCAF1 965-975 were essential for the binding (Supplemental Fig. 1B). In addition, we found that peptides in which D at 973
or glutamic acid (E) at 975 was substituted for asparagine (N) or alanine (A), respectively, showed dramatically reduced affinities for SUMO-2/3 (Supplemental Fig. 1B) as well as SUMO-1 (data not shown), suggesting an important role of this acidic amino acid cluster for the binding. Although several previously described SUMO-interacting peptides, including PIAS1, PIASx, SAE2 and PML IV, also contain an acidic cluster at their C-terminus (Fig. 1E) (6,30), the roles of the negatively-charged residues in the SUMO binding have not been investigated in detail.

The SUMO-MCAF1 interaction occurs in vivo. To examine the importance of the amino acid residues in MCAF1\textsubscript{965-975} for the binding to SUMO-2/3, two MCAF1 point-mutants in which D968 and L969 were substituted for alanine (A) were generated and designated (His\textsubscript{6})\textsubscript{2}-MCAF1-D968A and (His\textsubscript{6})\textsubscript{2}-MCAF1-L969A, respectively. GST pull-down assays revealed that neither of the mutants showed stable binding to GST-SUMO-2/3 (Fig. 2A), despite the observed association between wild-type (His\textsubscript{6})\textsubscript{2}-MCAF1 and GST-SUMO-2/3, indicating that D968 and L969 are critical for the interaction with SUMO-2/3 in vitro. Importantly, when the MCAF1 mutants were fused to EGFP (EGFP-MCAF1-D968A and EGFP-MCAF1-L969A) and co-expressed with Myc-SUMO-3G in HeLa cells, Myc-SUMO-3G was barely enriched in EGFP foci in cells expressing either the EGFP-MCAF1-D968A mutant (Fig. 2B, lower panel) or EGFP-MCAF1-L969A mutant (data not shown), whereas a substantial amount of Myc-SUMO-3G accumulated in EGFP foci in cells expressing wild-type EGFP-MCAF1 (Fig. 2B, upper panel). Given that Myc-SUMO-3G is a SUMO-3 mutant incapable of conjugation, these data provide evidence that MCAF1 interacts non-covalently with SUMO-2/3 in vivo, and further confirm that D968 and L969 are necessary components for the binding to SUMO-2/3. Similar results were obtained when Myc-SUMO-1G was used (data not shown).

Endogenous SUMO-2/3, SUMO-1 and MCAF1 are colocalized. Thus far, our results had implied that MCAF1 acts as a SUMO-binding protein. Therefore, we next attempted to assess the physiological relevance of the endogenous SUMO-MCAF1 interaction using mammalian cultured cells. As shown in Fig. 3A, indirect immunofluorescence experiments revealed a large number of C-33A cells containing discrete nuclear foci in which SUMO-2/3 and MCAF1 were colocalized, although some SUMO-2/3 and MCAF1 may also be distributed throughout the nucleoplasm, except for the nucleolus. Both the sizes and numbers of the SUMO-2/3-MCAF1 foci varied among individual cells with an average of 2-6 clear and large foci per cell. Double-staining with anti-SUMO-2/3 and anti-SUMO-1 antibodies (Fig. 3B, upper panel) or anti-MCAF1 and anti-SUMO-1 antibodies (data not shown) revealed that the merged signals of the nuclear foci were almost indistinguishable, suggesting enrichment of SUMO-1 in SUMO-2/3-MCAF1 foci as well. Therefore, these data indicate the relevance of not only SUMO-2/3-MCAF1, but also SUMO-1-MCAF1, interactions in vivo. Although it is possible that a larger amount of SUMO-2/3 may be enriched with MCAF1 via the preferential association of SUMO-2/3 with MCAF1, our present indirect immunofluorescence technique could not quantitatively compare the levels of SUMO-2/3 versus SUMO-1 accumulation in MCAF1 foci.

SUMOs and MCAF1 are enriched in heterochromatin proteins. Since MCAF1 was enriched in heterochromatin foci that usually contain HP1\(\beta\), HP1\(\gamma\), trimethyl-H3-K9 and MBD1 (24), we tested whether SUMO foci overlapped with these heterochromatin proteins in C-33A cells. Staining with anti-SUMO-2/3 (Fig. 3B) or anti-SUMO-1 (data not shown) antibodies demonstrated that the SUMOs were colocalized with heterochromatin marker proteins. In contrast, other non-heterochromatic nuclear proteins, including the RNA splicing factor SC35 (Fig. 3B), nuclear scaffold protein hnRNP-U/SAF-A (data not shown) and promyelocytic leukemia (PML)-bodies (data not shown) did not show significantly merged signals with SUMO-MCAF1 foci. In particular, the transcriptionally active chromatin marker trimethyl-H3-K4, facultative heterochromatin marker trimethyl-H3-K27 and HP1 family protein HP1\(\alpha\) did not shown any
significantly merged signals with SUMO-MCAF1 foci (Fig. 3B). Taken together, these observations suggest that SUMO-MCAF1 foci represent MBD1-containing constitutive heterochromatic regions in C-33A cells.

**SUMOylated forms of MCAF1 are barely detectable.** Since MCAF1 and SUMOs interacted *in vitro* and colocalized *in vivo*, one could argue that MCAF1 itself could be an efficient substrate for SUMOylation. To elucidate whether MCAF1 was SUMOylated in C-33A cells, we performed an immunoprecipitation analysis of endogenous MCAF1 (Supplemental Fig. 2). When the proteins immunoprecipitated with the anti-MCAF1 antibody were probed with either anti-MCAF1, anti-SUMO-2/3 or anti-SUMO-1 antibodies, none of the antibodies detected any SUMOylated bands, which were expected to migrate more slowly than the 240-kDa band of non-modified full-length MCAF1 (Supplemental Fig. 2, *arrowhead*). Since more than 95% of the total cellular pool of MCAF1 could be extracted under our experimental conditions (data not shown), these results indicate that either most of the endogenous MCAF1 is modified very poorly, if at all, by SUMOs or SUMOylated MCAF1 is unstable in C-33A cells.

**MBD1 is an efficient substrate for SUMOylation.** We therefore hypothesized that there may be SUMOylated proteins in MBD1-containing heterochromatin, and that SUMOylation of such proteins may provide sufficient anchoring of MCAF1 to heterochromatin regions. A straightforward test of this idea was to determine the SUMOylated proteins residing in heterochromatin, and to investigate whether SUMOylation of such proteins provided a sufficient binding platform for MCAF1. For these purposes, we undertook a “candidate screening” approach to identify proteins that could be SUMOylated and should be present in heterochromatin.

Among the proteins tested, the methyl-CpG-binding protein MBD1 was identified as a good candidate. As shown in Fig. 4A and B, the bacterial SUMOylation system (27) revealed that a GST protein fused to the full-length form of MBD1 (GST-MBD1) was efficiently SUMOylated, further demonstrating that the C-terminal region of MBD1 (MBD1$_{373-605}$), which contained the TRD, was responsible for SUMOylation. Moreover, we revealed SUMOylation of MBD1 using an ectopic expression system in mammalian cultured cells (Fig. 4C). When we expressed FLAG-tagged MBD1 in HeLa cells and performed an immunoprecipitation analysis with an anti-FLAG antibody, multiple high-molecular mass bands were detected by the anti-SUMO-2/3 antibodies, suggesting that FLAG-MBD1 was efficiently modified by endogenous SUMO-2/3 (lanes 2 and 5). Multiple SUMOylated bands were also detected when FLAG-MBD1 was transfected along with Myc-SUMO-3 (lanes 3 and 6). Of note, FLAG-MBD1 proteins modified by Myc-SUMO-3 migrated slightly more slowly than FLAG-MBD1 proteins modified by endogenous SUMO-2/3, due to the addition of Myc-tag moieties. We also found that ectopically expressed FLAG-MBD1 was efficiently modified by either endogenous SUMO-1 or ectopically expressed Myc-SUMO-1 (data not shown). Taken together, these results demonstrate that MBD1 is efficiently modified by either SUMO-2/3, SUMO-1 or both *in vitro* as well as *in vivo*.

**Endogenous MBD1 is SUMOylated.** To formally confirm that MBD1 is indeed a physiological SUMOylation substrate, we immunoprecipitated endogenous MBD1, and investigated the existence of the SUMOylated form of MBD1. As shown in Fig. 4D, in addition to the 85- and 90-kDa bands corresponding to non-modified forms of the endogenous spliced variants of MBD1 (26,33), an anti-MBD1 antibody precipitated multiple high-molecular mass bands, particularly around 140- and 180-kDa (*white circles*). Furthermore, these bands could be superimposed with bands detected by the anti-SUMO-2/3 and anti-SUMO-1 antibodies, demonstrating SUMOylation of endogenous MBD1. It should be noted that a single molecule of SUMO is expected to migrate at around approximately ~15-kDa. Therefore, the band shifts from 85–90-kDa (non-modified forms) to 140–180-kDa (modified forms) suggested that 4-6 molecules of either SUMO-2/3, SUMO-1 or both were appended to a single molecule of MBD1. Taken together, these results indicate that a significant proportion of endogenous MBD1 is modified by either SUMO-2/3, SUMO-1 or both *in
SUMO modification of MBD1 enhances the association between MBD1 and MCAF1. Next, we performed GST pull-down assays to test whether SUMOylation of MBD1 increased its affinity for MCAF1 compared with the affinity of the non-modified form of MBD1 for MCAF1. When recombinant maltose-binding protein (MBP) fused to MBD1373-605 (MBP-MBD1373-605) was modified with SUMO-3 in the bacterial SUMOylation system (27), the total bacterial lysate contained a mixture of SUMO-3-modified MBP-MBD1373-605, in which different numbers of SUMO-3 were appended to the MBD1 373-605 moiety of the MBP fusion protein (Fig. 5A, lane 7). Upon incubation of GST-MCAF1 or GST-MCAF1965-975 with a total lysate containing the mixture of SUMOylated MBD1373-605, followed by a GST pull-down assay, we found that SUMO-3-modified MBP-MBD1373-605 proteins, rather than the non-modified form of MBP-MBD1373-605, were efficiently enriched in the fraction precipitated with GST-MCAF1 beads (lane 2) or GST-MCAF1965-975 beads (lane 4). Comparisons between the 80-kDa band of the non-modified form of MBD1373-605 (Fig. 5A, white arrows) and the 120-kDa (Fig. 5A, black arrowheads) and 160-kDa (Fig. 5A, white arrowheads) bands of SUMO-3-modified MBD1373-605 revealed enrichment of p120 and p160, respectively. It should be noted that, given that the free form of SUMO-3 was expected to be ~15-kDa, p120 and p160 may represent MBD1373-605 containing 2 and 5 molecules of SUMO-3, respectively.

Using GST pull-down assays, we also tested the binding of GST-MCAF1 to SUMO-1-conjugated forms of MBP-MBD1373-605. As shown in Fig. 5A (lanes 8–14), both GST-MCAF1 and GST-MCAF1965-975 were able to bind to SUMO-1-modified MBP-MBD1373-605. However, their interactions appeared somewhat weaker than the interactions of GST-MCAF1/MCAF1965-975 with SUMO-3 modified MBP-MBD1373-605 (Fig. 5A and B). Collectively, these results suggest that the SUMOylated form of MBD1 provides a more stable association with MCAF1 than the non-modified form of MBD1.

Depletion of either the SUMO-2/3 or SUMO-1 pathway perturbs the assembly of MCAF1, trimethy-H3-K9 and HP1 foci. Our finding that SUMOylation of MBD1 facilitates its association with MCAF1 supports the idea that MCAF1 is enriched in heterochromatin regions, at least in part, via anchoring to SUMO-modified MBD1. Therefore, we used RNAi directed against SUMO-2/3 to investigate whether SUMO-2/3 is critical for the assembly of MCAF1 at MBD1-containing heterochromatin regions in vivo. To demonstrate the specificity of the RNAi technique, we used two different siRNA duplexes for SUMO-1 (SUMO-1#1 and #2 siRNAs) and 2 independent combinations of SUMO-2 and -3 siRNA duplexes (SUMO-2/3 #1 and #2 siRNAs). For control experiments, a siRNA against firefly luciferase GL3 was used. In each siRNA experiment, judging from immunofluorescence analysis using anti-SUMO-2/3 or anti-SUMO-1 antibodies, ~60% of the cells showed no detectable levels of either SUMO-1 or SUMO-2/3 at 60 h post-transfection with either SUMO-2/3 or SUMO-1 siRNAs, respectively, whereas no reduction in the signals was observed in the control experiments (Fig. 6A–E, and data not shown).

A comparison of the SUMO knockdown cells and control cells revealed no significant differences in terms of the localization of MBD1 or the number of MBD1 foci (data not shown), implying that depletion of either the SUMO-2/3 or SUMO-1 pathway may not completely impair the ability of MBD1 to localize at specific DNA regions, possibly via interactions between the methyl-CpG-binding domain of MBD1 and methylated DNA. In contrast, 52% and 61% of SUMO-2/3 and SUMO-1 knockdown cells, respectively, showed no significant accumulation of MCAF1 at MBD1-containing foci (Fig. 6A and F). Moreover, 75% and 67% of SUMO-2/3 and SUMO-1 knockdown cells, respectively, showed decreased signals for trimethyl-H3-K9 around the MBD1-containing regions (Fig. 6B and F). In agreement with these observations, 57% and 43% of SUMO-2/3 and SUMO-1 knockdown cells, respectively, showed HP1β delocalization from the MBD1 foci (Fig. 6C and F), implying that the formation of MBD1-containing heterochromatin was perturbed. We further tested the subcellular
localizations of other HP1 proteins, HP1γ and HP1α, and found delocalization of HP1γ, but not HP1α, in SUMO-2/3 and SUMO-1 siRNA-treated cells (Fig. 6D and E, and data not shown). Taken together, we conclude that the SUMO-2/3 and SUMO-1 pathways are involved in the anchorage of MCAF1, and are also critical for methylation of histone H3-K9 and targeting of HP1β and HP1γ to MBD1-containing heterochromatin.

Perturbation of the SUMO pathway by ectopic expression of MCAF1965-975 induces delocalization of MCAF1 and HP1β. The idea of facilitated anchorage of MCAF1 to MBD1-containing heterochromatin by SUMOylation was further tested using cells that ectopically overexpressed MCAF1965-975. When a monomeric red fluorescent protein fused to MCAF1965-975 (DsRed-MCAF1965-975) was transiently overexpressed in C-33A cells, the signals for both endogenous SUMO-2/3 (Fig. 7A) and SUMO-1 (data not shown) were remarkably reduced, suggesting that overproduction of monomeric DsRed-MCAF1965-975 effectively perturbed both SUMO pathways, possibly via sequestration of the SUMOs and/or competition with pre-existing SUMO-binding proteins. Significantly, in these DsRed-MCAF1965-975-expressing cells, the numbers of cells in which both MCAF1 and HP1β were delocalized from MBD1-containing foci increased (Fig. 7B and C), reminiscent of the depletion of the SUMO pathway by siRNAs described above. Since overexpression of monomeric DsRed alone had no apparent effect on the localizations of MCAF1 and HP1β at MBD1 foci (Fig. 7A-C, upper panels), these results indicate the importance of the SUMO-binding region, MCAF1965-975, for regulating the anchorage of MCAF1 to MBD1-containing heterochromatin and further support the idea that SUMO-MCAF1 interactions are required for the proper assembly of MBD1-containing heterochromatin.

**DISCUSSION**

**MCAF1 is a SUMO-binding protein.** In the present paper, we have identified a previously unknown SUMO-binding region in MCAF1 (MCAF1965-975) that facilitates its interaction with SUMOs. Significantly, we have revealed that MCAF1965-975 shows similarity to a previously characterized SUMO-binding motif (6,28,30-32). Moreover, we found that MCAF1 preferentially interacts with SUMO-2/3 rather than SUMO-1 in vitro. These results imply a previously unappreciated function of MCAF1 as a modulator for SUMO modification signals with the potential to preferentially transmit signals derived from SUMO-2/3-modified proteins. In addition, we found that following multisite-modification and/or poly-chain formation of SUMOs on MBD1373-605, MCAF1 appeared to further increase its affinity for MBD1. This finding further supports the idea that MCAF1 functions as a modulator of SUMO signaling and suggests that MCAF1 may be able to amplify SUMO signals to downstream events. Thus, our results define MCAF1 as a novel class of SUMO regulator and predict the existence of a previously undescribed SUMO-MCAF1-based regulatory network.

**MBD1 is a SUMOylation substrate.** In the present study, we have shown for the first time that the methyl-CpG-binding protein MBD1 is SUMOylated in mammalian cells, suggesting direct linkage of the SUMO modification pathway with a wide variety of important epigenetic cellular phenomena regulated by DNA methylation, including gene silencing and heterochromatin formation. It is feasible that other methyl DNA-binding proteins, besides MBD1, that reside in heterochromatin may also be SUMOylated. We are currently investigating whether other methyl DNA-binding proteins, including other MBD family proteins (16), MeCP2(34) and kaiso (35), are SUMOylated using the bacterial SUMOylation system.

It is currently unclear how SUMOylation of MBD1 is regulated and whether a SUMO E3 ligase that upregulates SUMOylation of MBD1 is present. Since previous studies have shown direct interactions of MBD1 with MCAF1 (21,24), we first suspected that MCAF1 may act as a SUMO E3 ligase toward MBD1. However, neither binding of a SUMO E2 enzyme (Ubc9) to MCAF1 nor the ability of MCAF1 to enhance SUMOylation of MBD1 in vitro was observed (Supplemental Fig. 3A and B). In addition, while many of the SUMO E3 ligases reported to date have the ability to
become auto-SUMOylated (36-39), MCAF1 appears to be inefficiently auto-SUMOylated. Thus, there is still poor evidence to support a role for MCAF1 as a SUMO E3 ligase. It will be interesting in the near future to identify the SUMO E3 ligases for MBD1 and to elucidate how such E3 ligases regulate the SUMOylation of MBD1 during cell cycle progression and cellular differentiation.

The SUMO modification pathway cross-talks with the DNA and histone methylation pathways. We have shown that SUMOylation of MBD1 provides sufficient anchoring of MCAF1 at heterochromatin regions in C-33A cells and demonstrated that depletion of either the SUMO-2/3 or SUMO-1 pathway results in delocalization of trimethyl-H3-K9, HP1β and HP1γ from MBD1 foci, suggesting the possibility that destabilization of the interaction between MCAF1 and MBD1 perturbs the histone methylation pathway and the proper assembly of heterochromatin proteins at MBD1-containing DNA regions.

The molecular mechanism for how the SUMO pathway is linked to histone methylation and heterochromatin formation in mammalian cells currently remains to be fully elucidated, although two possibilities appear feasible. First, augmented recruitment of MCAF1 via an interaction with SUMOylated MBD1 may contribute to the maintenance of a stable assembly of H3-K9 methyltransferases at MBD1-containing heterochromatin regions, thereby stabilizing the association of HP1 with MBD1-containing heterochromatin. In the present study, we have not identified any such putative H3-K9 methyltransferases that can be recruited by MCAF1 complexed with SUMOylated MBD1. However, we suggest that SETDB1 is a likely candidate for this scenario, since previous reports have described that MCAF1 is a regulatory subunit of the SETDB1 histone H3-K9 methyltransferase complex (22,24) and that MCAF1 complexed with SETDB1 modulates the histone methylase activity of SETDB1, converting it from an H3-K9 dimethylase to a trimethylase (22,24). Second, anchorage of MCAF1 to SUMOylated MBD1 may enhance the recruitment of chromatin remodeling activity and/or histone chaperone activity that somehow preferentially incorporate trimethyl-H3-K9. The chromatin assembly factor CAF1, which interacts with MBD1, represents a likely candidate for this scenario (18,40), although neither facilitated association of CAF1 with MCAF1-MBD1 complexes nor accumulation of CAF1 at SUMO-MCAF1 foci in C-33A cells have yet been demonstrated.

Regardless of the mechanism, our results implies a direct link between the SUMO pathway and the methylation of DNA and histone methylation. In addition, the dramatic effects on the nuclear localizations of several heterochromatin proteins, including trimethyl-H3-K9 and HP1 proteins, in either SUMO-2/3- or SUMO-1-depleted cells reveal highly dynamic features of the SUMO modification pathway in the context of heterochromatin formation. It is intriguing that HP1α, a well-described constitutive heterochromatin protein in mammalian cells (41), seems to exhibit a different role in terms of the regulation of the SUMO-enriched heterochromatin regions in C-33A cells compared with the two other members of the HP1 protein family examined. Although these results imply that the various HP1 members have only overlapping functions with respect to the regulation of SUMO-enriched heterochromatin, the physiological relevance of this phenomenon remains to be elucidated in future studies.

In conclusion, our results have provided direct evidence that modification of MBD1 by either SUMO-2/3, SUMO-1 or both, and facilitated anchorage of MCAF1 to SUMOylated MBD1 participate in the formation of heterochromatin in C-33A cells. Considering the increasing numbers of SUMO substrates involved in chromatin modification, remodeling and epigenetic control, our findings lay the foundation for future exploration of currently undiscovered SUMO signaling events that regulate gene silencing and heterochromatin formation in both physiological and pathological situations.

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FOOTNOTES

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**FIGURE LEGENDS**

Figure 1. MCAF1 is a SUMO-binding protein. (A) Schematic representation of human MCAF1 and summary of the GST pull-down assay. The MCAF1 deletion constructs used in the experiments are represented as thin black lines. The numbers on both sides of the lines correspond to the amino acid residue numbers in human MCAF1. The table on the right summarizes the interactions of each deletion mutant with (His)$_e$-SUMO-3 in GST pull-down assays. (+) indicates high affinity binding, while (-) indicates negligible binding. Two highly conserved domains of the MCAF family proteins with unknown functions are indicated as Domain 1 and Domain 2 (24). (B) Endogenous MCAF1 interacts with GST-SUMOs with a preference for GST-SUMO-2/3. Aliquots (500 μl) of a C-33A cell lysate (+: lanes 3, 5, 7 and 9) or PBS buffer (-: lanes 2, 4, 6 and 8) were incubated with beads bound to 3 μg of GST-SUMO-1 (lanes 2 and 3), GST-SUMO-2 (lanes 4 and 5), GST-SUMO-3 (lanes 6 and 7) or GST alone (lanes 8 and 9). Following the incubation, the proteins associated with the beads were analyzed by 6% SDS-PAGE followed by immunoblot analysis using an anti-MCAF1 antibody. The total C-33A lysate (5% of the input) was loaded in lane 1. The position of MCAF1 is indicated by the arrowhead. The positions of size markers are shown on the left. (C) Direct binding of MCAF1 to SUMO-2/3. Aliquots (500 μl) of a lysate from E. coli expressing recombinant full-length (His)$_e$-T7-MCAF1 (+) were incubated with beads bound to 3 μg of GST-SUMO-1, GST-SUMO-2, GST-SUMO-3 or GST (lanes 3, 5, 7 and 9, respectively). As a control, PBS buffer was mixed with the beads (-: lanes 2, 4, 6 and 8). Following the incubation, the proteins associated with the beads were analyzed by 6% SDS-PAGE. The total bacterial lysate (5% of the input) was loaded in lane 1. Proteins were visualized by immunoblot analysis using an anti-T7 antibody. The position of (His)$_e$-T7-MCAF1 is indicated by the arrowhead. (D) GST-MCAF1$_{965-975}$ binds to (His)$_e$-SUMOs. The indicated amounts of (His)$_e$-SUMO-1, (His)$_e$-SUMO-2 or (His)$_e$-SUMO-3 were incubated with a fixed amount of GST-MCAF1$_{965-975}$ (5 μg) bound to glutathione-Sepharose beads. Following extensive washing with binding buffer, the proteins associated with the beads were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (right panel). The band intensities of (His)$_e$-SUMO-1 (triangles), (His)$_e$-SUMO-2 (black circles) and (His)$_e$-SUMO-3 (open circles) were quantified using an image analysis system, and the results were plotted (left panel). a.u., arbitrary relative band density units. (E) Sequence comparison with previously identified SUMO-binding peptides. Dashes within the sequences indicate spaces inserted to create an optimal match. Black underlines indicate amino acid residues identical to MCAF1$_{965-975}$. Grey underlines represent amino acid residues similar to MCAF1$_{965-975}$.

Figure 2. SUMO-MCAF1 interaction is impaired by a mutation in MCAF1$_{965-975}$. (A) (His)$_e$-T7-MCAF1-D968A and -L969A mutants abolish SUMO-2/3-binding in vitro. Bacterial lysates expressing recombinant full-length (His)$_e$-T7-MCAF1-wild type (lanes 5 and 9), -D968A (lanes 6 and 10), and -L969A (lanes 7 and 11) mutants were incubated with beads containing 3 μg of GST-SUMO-2 (lanes 5 and 7) and GST-SUMO-3 (lanes 9-11), respectively. For control experiments, PBS buffer was mixed with GST-SUMO-2- or GST-SUMO-3-conjugated beads (lanes 4 and 8, respectively). Following incubation, GST pull-down assays were carried out, and the proteins associated with the beads were subjected to immunoblot analysis using an anti-T7 antibody. A total of 5% of the input of each lysate was loaded in lanes 1-3, respectively. The arrowhead indicates the position of (His)$_e$-T7-MCAF1. (B) Myc-SUMO-3 is enriched in EGFP-MCAF1-wt, but not in the EGFP-MCAF1-D968A mutant, in vivo. EGFP-MCAF1-wt (upper) and EGFP-MCAF1-D968A (lower) were co-expressed with a conjugation-deficient Myc-SUMO-3G mutant. Following culture for 60 h, the cells were fixed and subjected to indirect immunofluorescence analysis using an anti-Myc antibody. The preferences for colocalization of the EGFP and Myc signals were detected using a line scan plot program (Lumina Vision; Mitani Co.). White arrows indicate the direction and distance of the track of the line scan. The line scans of the fluorescence intensities of the EGFP (green) and Myc (red) signals are shown on the right. a.u., arbitrary relative fluorescence intensity units. * and ** indicate the positions of the peaks detected in each line scan.
Figure 5. MCAF1 preferentially binds to SUMOylated forms of MBD1 373-605. (A) SUMO-2/3 and MCAF1 foci colocalize in C-33A cells. Exponentially growing C-33A cells were subjected to indirect immunofluorescence analysis using anti-SUMO-2/3 (left) and anti-MCAF1 (middle) antibodies. Merged images of the signals are shown on the right. Bar = 5 μm. (B) SUMO-2/3 colocalizes with SUMO-1 and is enriched in heterochromatin proteins. C-33A cells were subjected to indirect immunofluorescence analyses using an anti-SUMO-2/3 antibody (green) together with other antibodies, as indicated (red). Merged images are shown on the right. Bar = 5 μm.

Figure 4. MBD1 is modified by both SUMO-2/3 and SUMO-1 in vivo. (A) Schematic diagram of the domain structure of MBD1 and summary of the bacterial SUMOylation assays. MBD, methyl-CpG-binding domain; C1, C2 and C3, CXXC-domain 1, 2 and 3, domains containing the repeat unit of cysteine-X-X-cysteine residues; TRD, transcriptional repression domain (26,33). The table on the right summarizes the bacterial SUMOylation assays. (+) indicates efficient SUMOylation, while (-) represents inefficient SUMOylation. (B) The C-terminal region of MBD1 containing the TRD is efficiently SUMOylated in the bacterial SUMOylation system. GST-MBD1 (lanes 1 and 2), GST-MBD11,175 (lanes 3 and 4), GST-MBD1166-386 (lanes 5 and 6), GST-MBD1373-605 (lanes 7 and 8), GST-MBD1521-605 (lanes 9 and 10) and GST (lanes 11 and 12) proteins were expressed in the absence (-) or presence (+) of pT-E1E2S3, followed by precipitation with glutathione-Sepharose beads. The proteins associated with the beads were separated by 5-20% SDS-PAGE and subjected to immunoblot analysis using anti-GST (left) and anti-SUMO-2/3 (right) antibodies. Arrows indicate the positions of the non-modified forms of the recombinant proteins. Circles represent the positions of the SUMOylated forms. (C) Exogenously expressed MBD1 efficiently conjugates with SUMO-2/3. HeLa cells were transfected with FLAG-MBD1 (lanes 2 and 5) or FLAG-MBD1 plus Myc-SUMO-3GG (lanes 3 and 6). As a control, the vector plasmid only was transfected (lanes 1 and 4). Nuclear extracts prepared from the transfected cells were subjected to immunoprecipitation using an anti-FLAG M2 antibody, followed by immunoblotting with anti-FLAG M5 (lanes 1-3) or anti-SUMO-2/3 (lanes 4-6) antibodies. Immunoprecipitation was carried out with RIPA buffer using highly stringent conditions that do not allow the precipitation of non-covalently associated proteins. Arrows indicate the position of the expressed FLAG-MBD1 (non-modified form). Circles represent the positions of the expressed FLAG-MBD1 modified with endogenous SUMO-2/3. Arrowheads show the positions of the expressed FLAG-MBD1 modified with Myc-SUMO-3. (D) Endogenous MBD1 is SUMOylated in C-33A cells. Nuclear extracts were prepared from exponentially growing C-33A cells, followed by immunoprecipitation analysis using an anti-MBD1 antibody (lanes 3, 6 and 9) or IgG as a control (lanes 2, 5 and 8). Immunoprecipitation was carried out with RIPA buffer using highly stringent conditions. Immunoblot analysis of the immunoprecipitated proteins was carried out using anti-MBD1 (lanes 1-3), anti-SUMO-2/3 (lanes 4-6) or anti-SUMO-1 (lanes 7-9) antibodies. A total of 1% of each nuclear extract (input) was applied in lanes 1, 4 and 7, respectively. Arrows indicate the positions of the 85-kDa and 90-kDa proteins that may represent the two major splicing variants of MDB1 in C-33A cells. Circles indicate several bands reactive to anti-MBD1, anti-SUMO-2/3 and anti-SUMO-1 antibodies. Black circles represent non-specific bands.

Figure 3. SUMO-2/3 and SUMO-1 overlap with MCAF1 foci and several heterochromatin proteins in C-33A cells. (A) SUMO-2/3 and MCAF1 foci colocalize in C-33A cells. Exponentially growing C-33A cells were subjected to indirect immunofluorescence analysis using anti-SUMO-2/3 (left) and anti-MCAF1 (middle) antibodies. Merged images of the signals are shown on the right. Bar = 5 μm. (B) SUMO-2/3 colocalizes with SUMO-1 and is enriched in heterochromatin proteins. C-33A cells were subjected to indirect immunofluorescence analyses using an anti-SUMO-2/3 antibody (green) together with other antibodies, as indicated (red). Merged images are shown on the right. Bar = 5 μm.
either 3 μg of GST-MCAF1 (lanes 9 and 10), GST-MCAF1965-975 (lanes 11 and 12) or GST alone (lanes 13 and 14), followed by precipitation with glutathione-Sepharose beads. Following extensive washing of the precipitated beads, the associated proteins were separated by SDS-PAGE and subjected to immunoblotting analysis with an anti-MBP antibody. Arrows indicate the position of the 80-kDa non-modified form of MBP-MBD1373-605 (p80). Black arrowheads represent the position of the 120-kDa form of modified MBP-MBD1373-605 (p120). White arrowheads represent the position of the 160-kDa form of modified MBP-MBD1373-605 (p160). (B) Comparison of the relative binding affinities of full-length MCAF1 and MCAF1965-975 for SUMOylated MBD1373-605. The intensities of the p80, p120, and p160 bands detected in (A) were quantified by scanning densitometry. The X-axis shows the relative enrichment of the p120 (black arrowheads) and p160 (white arrowheads) bands when the concentration level of the p80 band of the non-modified form of MBP-MBD1373-605 (arrows) is set to 1.0, while the Y-axis indicates the bands measured in the assay.

Figure 6. SUMO-2/3 and SUMO-1 are critical for proper assembly of MCAF1 in MBD1-containing heterochromatin regions in vivo. (A-E) C-33A cells were transfected with SUMO-2/3#1 or control siRNA duplexes. At 60 h post-transfection, the cells were triple-stained with the indicated antibodies. Merged images are shown on the right. (F) The results were quantified by scoring at least 50 cells from two independent experiments. The bar chart depicts the ratios of cells in which MCAF1 (white), trimethyl-H3-K9 (gray) or HP1β (black) were delocalized from MBD1 foci.

Figure 7. Overexpression of monomeric DsRed-MCAF1965-975 perturbs the SUMO-2/3 pathway, resulting in delocalization of endogenous MCAF1 and HP1β from MBD1-containing heterochromatin regions. (A-C) C-33A cells were transfected with monomeric DsRed-MCAF1965-975 or monomeric DsRed as a control. At 48 h post-transfection, the cells were double-stained with the indicated antibodies. Merged images are indicated on the right.
**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure 1. MCAF1 contains conserved amino acid residues responsible for SUMO-2/3 binding.** (A) MCAF1_965-975 is conserved among vertebrate species. A database search for MCAF1_965-975 using the basic local alignment search tool algorithm BLAST detected several proteins with very high scores, including mouse (CAB77024), rat (XP_232488), dog (XP_543801) and chicken (NP_001012831) orthologs of human MCAF1. (B) Single-point-mutants of MCAF1_965-975 and their interactions with SUMO-3. Single-point-mutants of MCAF1_965-975 were generated by individually changing appropriate amino acids to alanine (A), asparagine (N) or glutamine (Q). A GST fusion protein with each mutated peptide (5 μg) was mixed with a fixed amount of (His)_6-SUMO-3 (5 μg) and analyzed by a GST pull-down assay. The proteins associated with GST-beads were separated by 15% SDS-PAGE, and visualized by Coomassie Brilliant Blue staining. The intensity of each (His)_6-SUMO-3 band was measured, and the relative intensity to that of the SUMO-3 binding of the wild-type peptide was calculated (black bars). a.u., arbitrary relative band density units.

**Supplemental Figure 2. Failure to detect endogenous SUMOylated MCAF1.** A C-33A cell lysate was subjected to immunoprecipitation assays using a rabbit anti-MCAF1 antibody (lanes 2, 4 and 6) or rabbit IgG (lanes 1, 3 and 5) under highly stringent conditions (RIPA buffer). The immunoprecipitated fractions were subjected to immunoblot assays using an anti-MCAF1 antibody. The arrowhead indicates the position of ~240 kDa where the full-length recombinant MCAF1 migrates in SDS-PAGE, as shown in Figs. 1C and 2A. Asterisks show the position of the immunoglobulin heavy chain (IgG-H). IP, immunoprecipitation; WB, western blot (immunoblotting).

**Supplemental Figure 3. MCAF1 shows no significant SUMO E3 ligase activity toward MBD1 in an in vitro SUMOylation assay.** (A) The in vitro interaction between MCAF1 and Ubc9 is negligible. Aliquots (3 μg) of recombinant full-length GST-MCAF1 (lanes 1 and 2), GST-RanBP2-IR (lanes 3 and 4) or GST alone (lanes 5 and 6) were incubated with either PBS (lanes 1, 3 and 5) or 3 μg of (His)_6-Ubc9 (lanes 2, 4 and 6), respectively. Following the incubation, GST pull-down assays were carried out, and the proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Arrowheads indicate the position of each protein. (B) SUMO E3 activity of MCAF1 towards MBD1 is not detected in an in vitro SUMOylation reaction. The indicated amounts of recombinant full-length GST-MCAF1 were incubated with 0.1 μg of MBP-MBD1_373-605 (p80) in the presence of SUMO-3, E1, E2 and ATP (27). No increases in the signal intensities of the 100-kDa and 120-kDa bands of the SUMOylated forms of MBP-MBD1_373-605, referred to as p100 and p120, respectively, are detected under any of the conditions.
Figure 1

A.

MCAF1

| Domain 1 | Domain 2 |
|----------|----------|
| SUMO interaction | SUMO interaction |

pAS2-1#17

Input

B.

GST-SUMO-1 GST-SUMO-2 GST-SUMO-3 GST

MCAF1

C.

GST-SUMO-1 GST-SUMO-2 GST-SUMO-3 GST

(His)_6-MCAF1
### D.

![Graph showing band intensity vs. input (µg) with legend indicating CBB staining.]

### E.

| Protein     | Peptide Sequence | MCF1 (965-975) | PIAS1 (456-470) | PIASx (436-450) | SAE2 (586-596) | PML IV (555-566) | RanBP2/Nup358 (2631-2638) | TDG (307-314) |
|-------------|------------------|----------------|----------------|----------------|----------------|-------------------|---------------------------|---------------|
| MCAF1       | G V I D L T M D - - - D E E |               |                |                |                |                   |                           |               |
| PIAS1       | V E V I D L T I D S S S D E E |               |                |                |                |                   |                           |               |
| PIASx       | V D V I D L T I E S S S D E E |               |                |                |                |                   |                           |               |
| SAE2        | D V L I V D S D - - - E E D |               |                |                |                |                   |                           |               |
| PML IV      | R V V V I S S S E - - - D S D |               |                |                |                |                   |                           |               |
| RanBP2/Nup358 | D V L I V Y E L |               |                |                |                |                   |                           |               |
| TDG         | D V Q E V Q Y T |               |                |                |                |                   |                           |               |
### Figure 2

#### A.

|  | Input | GST-SUMO-2 | GST-SUMO-3 |
|---|-------|------------|------------|
| 1 | Wt    | Wt         | Wt         |
| 2 | D968A | D968A      | D968A      |
| 3 | L969A | L969A      | L969A      |

Fluorescence (a.u.) vs Distance (μm)

#### B.

- **EGFP-MCAF1**
  - WT
  - D968A

- **Myc-SUMO-3G**
  - WT
  - D968A

Fluorescence (a.u.) vs Distance (μm)
A.

SUMO-2/3  MCAF1  merge
Figure 3

B.

SUMO-1

MBD1

H3-K9 triMe

HP1β

HP1γ

HP1α

H3-K27 triMe

H3-K4 triMe

SC35
Figure 4

A.

| MBD1 | MBD | CXXC1 | CXXC2 | CXXC3 | TRD |
|------|-----|-------|-------|-------|-----|
| 1    | 1   | 166   | 386   | 373   | 521 | 605 a.a. |
| 1    | 175 | 605   |       |       |     | SUMO-3 conjugation |
|      |     |       | ++    |       |     | ++ |
|      |     |       | -     |       |     | - |
|      |     |       |       |       |     | + |

B.

GST-MBD1:

| 1-605 | 1-175 | 166-386 | 373-605 | 521-605 | GST |
|-------|-------|---------|---------|---------|-----|
| -     | +     | -       | +       | -       | +   |
| 1     | 2     | 3       | 4       | 5       | 6   |

WB: anti-GST

(kDa)

160-150-140-130-120-110-100-90-80-70-60-50-40-30-20-10-0

1  2  3  4  5  6  7  8  9  10  11  12

WB: anti-SUMO-2/3
Figure 4

C.

Figure 4C shows immunoprecipitation (IP) experiments with FLAG-MBD1 and Myc-SUMO-3 GG. Columns 1, 2, and 3 were IP with anti-FLAG antibody, while 4, 5, and 6 were IP with anti-SUMO-2/3 antibody. Lanes 1 and 4 are input with anti-FLAG, lanes 2 and 5 are IP with anti-FLAG, and lanes 3 and 6 are IP with anti-SUMO-2/3. The left panel shows SUMOylated forms, and the right panel shows FLAG-MBD1 (non-modified).

D.

Figure 4D illustrates western blot (WB) analysis with FLAG-MBD1 and SUMOylation status. Lanes 1, 4, and 7 are WB with anti-MBD1, while lanes 2, 5, and 8 are WB with anti-SUMO-2/3, and lanes 3, 6, and 9 are WB with anti-SUMO-1. The kDa markers are indicated at the left side of the figure.
A. Figure 5

B. Table showing relative binding fold for different protein complexes:

- GST-MCAF1 + MBD1-SUMO-3:
  - p80: ▲
  - p120: ▲
  - p160: ▲

- GST-MCAF1965-975 + MBD1-SUMO-3:
  - p80: ▲
  - p120: ▲
  - p160: ▲

- GST-MCAF1 + MBD1-SUMO-1:
  - p80: ▲
  - p120: ▲
  - p160: ▲

- GST-MCAF1965-975 + MBD1-SUMO-1:
  - p80: ▲
  - p120: ▲
  - p160: ▲
Figure 6

A. SUMO-2/3 MCAF1 MBD1 merge
   control
   SUMO-2/3 KD

B. SUMO-2/3 H3-K9 triMe MBD1 merge
   control
   SUMO-2/3 KD
Figure 6

F.

![Graph showing % of cells with delocalized pattern for control, SUMO-2/3 KD, control (SUMO-1 positive), and SUMO-1 KD conditions. The graph displays data for MCAF1, H3-K9 triMe, and HP1β.]
Figure 7

A.

SUMO-2/3  MBD1  merge

control

MCAF1 965-975

B.

MCAF1  MBD1  merge

control

MCAF1 965-975

C.

HP1β  MBD1  merge

control

MCAF1 965-975
A.

human (AAO91864) (966-975) V I D L T M D D E E
mouse (CAB77024) (1002-1011) V I D L T M D D E E
rat (XP_232488) (1022-1031) V I D L T M D D E D
dog (XP_543801) (961-970) V I D L T M D D E E
chicken (NP_001012831) (780-789) V I D L T L D D E D

B.

![Relative SUMO-3 binding affinity (a.u.)]

| Residue | Wt | GVIDLTMDDDE |
|---------|----|-------------|
| G965    | A  |             |
| V966    | A  |             |
| I967    | A  |             |
| D968    | A  |             |
| L969    | A  |             |
| T970    | A  |             |
| M971    | A  |             |
| D972    | A  |             |
| D973    | A  |             |
| E974    | A  |             |
| E975    | A  |             |
| D972N   | N  |             |
| D973N   | N  |             |
| E974Q   | Q  |             |
| E975Q   | Q  |             |

Relative SUMO-3 binding affinity (a.u.)
Supplemental Figure 3

A.

[Image showing gel electrophoresis results with labeled bands for GST-MCAF1, GST-RanBP2-IR, and GST-Ubc9.]

B.

| MBP-MBD1373-605 | SUMO-3, E1, E2, ATP | GST-MCAF1 | (µg) |
|-----------------|---------------------|-----------|------|
| +               | +                   | +         | +    |
| +               | +                   | +         | +    |
| +               | +                   | +         | +    |
| +               | +                   | +         | +    |

[Image showing gel electrophoresis results with labeled bands for p120 (di-SUMO-3-modified), p100 (mono-SUMO-3-modified), p80 (non-modified), and GST-MCAF1.]
Involvement of SUMO modification in MBD1- and MCAF1-mediated heterochromatin formation

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