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Modification of the base excision repair enzyme MBD4 by the small ubiquitin-like molecule SUMO1

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Abstract

The base excision repair DNA *N*-glycosylase MBD4 (also known as MED1), an interactor of the DNA mismatch repair protein MLH1, plays a central role in the maintenance of genomic stability of CpG sites by removing thymine and uracil from G:T and G:U mismatches, respectively. MBD4 is also involved in DNA damage response and transcriptional regulation. The interaction with other proteins is likely critical for understanding MBD4 functions. To identify novel proteins that interact with MBD4, we used tandem affinity purification (TAP) from HEK-293 cells. The MBD4-TAP fusion and its co-associated proteins were purified sequentially on IgG and calmodulin affinity columns; the final eluate was shown to contain MLH1 by western blotting, and MBD4-associated proteins were identified by mass spectrometry. Bands with molecular weight higher than that expected for MBD4 (~66kD) yielded peptides corresponding to MBD4 itself and the small ubiquitin-like molecule-1 (SUMO1), suggesting that MBD4 is sumoylated in vivo. MBD4 sumoylation was validated by co-immunoprecipitation in HEK-293 and MCF7 cells, and by an in vitro sumoylation assay. Sequence and mutation analysis identified three main sumoylation sites: MBD4 is sumoylated preferentially on K137, with additional sumoylation at K215 and K377. Patterns of MBD4 sumoylation were altered, in a DNA damage-specific way, by the anti-

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metabolite 5-fluorouracil, the alkylating agent N-Nitroso-N-methylurea and the crosslinking agent cisplatin. MCF7 extract expressing sumoylated MBD4 displays higher thymine glycosylase activity than the unmodified species. Of the 67 *MBD4* missense mutations reported in The Cancer Genome Atlas, 14 (20.9%) map near sumoylation sites. These results indicate that MBD4 is sumoylated in vivo in a DNA damage-specific manner, and suggest that sumoylation serves to regulate its repair activity and could be compromised in cancer. This study expands the role played by sumoylation in fine-tuning DNA damage response and repair.

Keywords

MBD4/MED1; SUMO1; sumoylation; DNA damage

INTRODUCTION

Sumoylation is a post-translational modification involving the covalent attachment of small ubiquitin-like molecules (SUMO) to target proteins. It is a reversible process akin to ubiquitination, as they share overall structural similarities and activation cycle [1–3]. Sumoylation is highly conserved in evolution; invertebrates have just one *SUMO* gene, whereas mammalian genomes contain four genes, *SUMO1–4* encoding SUMO proteins [4, 5].

All SUMO proteins are synthesized as inactive isoforms and then cleaved by isopeptidases SENPs to expose two Glycines in the C-terminus of the protein [6]. In an ATP-dependent reaction, SUMO is activated by activating enzyme E1, which is a heterodimer with two subunits, AOS1 and UBA2. SUMO is subsequently transferred onto a catalytic cysteine of the second cofactor of the sumoylation cycle, called E2, also known as UBC9, which moves SUMO to its target protein and for this reason it is called conjugating enzyme. The final step leads to the formation of a peptide bond between the glycine residue of SUMO and a lysine residue of the target protein. Sometimes, the sumoylation cycle requires the presence of another cofactor, the E3 ligase, whose main role is to enhance the identification of SUMO targets [7–9]. Most of SUMO target proteins show an acceptor lysine within the consensus site: ψKX(D/E), in which ψ is a large hydrophobic residue [10]. E3 can be omitted in *in vitro* experiments [7].

The molecular consequences of sumoylation are profoundly different from (poly)ubiquitination, which leads to protein degradation. In general, sumoylation has three main consequences: i) it may interfere with the interaction between a target and its partner, and in this case, the interaction is permissive in the absence of SUMO; ii) it may provide a non-covalent binding site for an interaction partner containing a SUMO-interacting motif (SIM); iii) it may promote a conformational change and modify the activity of the target. Sumoylation is involved in many cellular processes, including transcription, replication, chromosome segregation, cell cycle progression, DNA damage response and DNA repair [11–14].

The effects of sumoylation on base excision repair (BER) are substantial [15]. Sumoylation is particularly important for the regulation of the BER enzyme and epigenetic factor,

Thymidine DNA Glycosylase (TDG). Sumoylation induces a conformational change in TDG, which leads to decreased binding to G:T, G:U mismatches and G:AP (abasic) product site; since many DNA Glycosylases are product-inhibited [16–21], it was proposed that sumoylation increases the turnover of TDG [22, 23]. Single-turnover experiments have shown that sumoylated TDG exhibits lower catalytic activity on G:T mismatches, as well as on the epigenetically relevant substrates G:formylcytosine (fC) and G:carboxylcytosine (caC) [24], involved in active DNA demethylation [25]. However, sumoylated TDG retains substantial affinity for fC and caC, raising the possibility that sumoylation converts TDG into a reader of these epigenetic modifications [24]. TDG is also known to interact with the transcriptional coactivators CREB-binding protein (CBP) and p300; sumoylation abrogates the ability of TDG to interact with these co-activators [26]. More recently, it has been shown that sumoylation affects the assembly of a BERosome in DNA demethylation [27].

MBD4 (also known as MED1) is a G:T and G:U mismatch glycosylase whose function is to maintain the integrity of CpG sites in the genome and prevent the mutagenic consequences of deamination of 5-methylcytosine and cytosine to thymine and uracil, respectively, i.e. prevent CpG to CpA/TpG transition mutations [17, 28]. MBD4 was identified independently as an interactor of the mismatch repair protein MLH1 [29] and as a protein containing a MethylCpG binding domain (MBD)[30]. Indeed, MBD4 has a tripartite structure with an N-terminal MBD and a C-terminal glycosylase domain, separated by a central region of low sequence complexity that appears to act as a spacer [31]. Recent studies have shown also its involvement in Immunoglobulin Class Switch Recombination [32, 33]. MBD4 has additional functions, including regulation of apoptosis of cells exposed to DNA-damaging agents [34, 35], modulation of the expression levels of core mismatch repair proteins [34], and transcriptional regulation [36]. MBD4 also shows a prominent activity in repairing halogenated pyrimidines and is essential for the cytotoxicity of the radiosensitizing agent, 5-iododeoxyuridine [37, 38]. Inactivation of MBD4 plays a role in tumorigenesis. In fact, secondary inactivating mutations of MBD4, causing frameshifts at A_6 and A_{10} polyadenine tracks in its coding region, have been described in colorectal, endometrial, pancreatic and stomach cancers characterized by microsatellite instability [39–41]; and its promoter is silenced by hypermethylation in ovarian cancer silencing [42, 43]. More recently, biallelic inactivation of MBD4 has been described in hypermutable juvenile acute myelogenous leukemia [44] and was associated with outlier response of an uveal melanoma patient to immunotherapy [45].

We reasoned that identification of other interacting partners of MBD4 beside MLH1 may help clarify its multiple functions. For this reason, we performed tandem affinity purification, and identified several associated polypeptides, including prominently SUMO1, which led us to analyze in detail MBD4 sumoylation.

MATERIAL AND METHODS

Cell Culture –

HEK-293 and MCF7 cells were cultured and expanded in DMEM, supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 0.1 mM non-essential amino acids, 1mM Sodium

Pyruvate, 0.01mg/ml Bovine Insulin, 10 units/ml penicillin and 10 μg/ml streptomycin. Cells were grown at 37°C in 5% CO_2.

Expression Plasmids –

For TAP experiments, human MBD4 cDNA was cloned in the EcoRI site of the vector pcDNA4 TO/TAP [46], to create an N-terminal tagged version of MBD4 (TAP-MBD4). Plasmids for HA-tagged MBD4 [29] and T7-tagged SUMO1 [47, 48] have been previously described. Site-directed mutants were generated using overlap extension PCR [49].

Transfections –

Cells were plated in 10 cm Petri dishes to obtain 80% of confluence (2.4×10^5 cells). Transfections were carried out using a total of 10 μg of plasmid DNA, 20 μL of Lipofectamine 2000 (Invitrogen) and Optimem (GIBCO).

Tandem affinity purification –

Tandem affinity purification was conducted as described in mammalian cells [46], with few modifications. Briefly, HEK-293 cells were transfected with TAP-MBD4 plasmid and, as negative control, empty pCDNA4/TO/TAP vector. After approximately 48 hours, the cells were lysed on ice in buffer containing 0.2% Nonidet P-40, 40 mM Tris, pH8, 150 mM NaCl, 10% glycerol, 1 mM ZnCl2, 10 mM NaF, and supplemented with Complete proteinase inhibitor (Roche), as previously described [29]. Lysates were loaded onto an IgG column and washed with lysis buffer without inhibitors. Subsequently, the protein A domain was cleaved off using TEV protease (New England Biolabs) during a 3-hour incubation with the enzyme in washing buffer supplemented with 0.5mM EDTA and 1mM DTT. Next, the TEV eluate was supplemented with 2 mM $CaCl_2$ and loaded onto the calmodulin column, washed in calmodulin binding buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% Nonidet P-40, 10 mM beta-mercaptoethanol) and eluted with calmodulin elution buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 10 mM EGTA, 0.1% Nonidet P-40, 10 mM beta-mercaptoethanol) in fractions. All volumes loaded onto the IgG and calmodulin columns and eluates were analyzed by Western blot to assess the purification process.

Mass spectrometry –

The affinity purified MBD4 complexes were resolved by SDS-PAGE and visualized by staining with colloidal Coomassie brilliant blue R250 (BioRad). Tryptic peptide mass mapping was used to identify the excised spots shown in Figure 1B. Bands excised from the gel were destained, reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin [50, 51]. Aliquots of the digests were applied to Scout384 MALDI sample targets using alpha-cyano-4-hydroxy cinnamic acid as matrix. The Bruker Reflex IV mass spectrometer was programmed to collect mass spectra, perform internal mass calibration using trypsin autolysis products and write a peptide mass list for each sample. Peptide mass lists were used as input to the MASCOT sequence database search engine [52, 53].

Co-Immunoprecipitation and immunoblotting –

HEK-293 and MCF7 cells were washed three times with cold PBS (Phosphate buffered Saline) and lysed with cold RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 1 mM each of sodium pyrophosphate, sodium orthovanadate, dithiothreitol, and EDTA) plus protease inhibitors for 30 minutes on ice. Cells were scraped off the dish and sonicated for 30 seconds. After centrifugation for 30 minutes at max speed (14,000 rpm, in microcentrifuge), the supernatant was isolated and protein concentration was determined with Bradford assay (Bio-Rad) and BCA assay (Thermo Scientific Pierce), using bovine serum albumin as a standard. Immunoprecipitations were performed in 400 μL of RIPA buffer using 500 μg of protein lysate. For each protein lysate, 3 μL of anti-T7-tag antibody (Novagene) were added, and the lysates were incubated for 3 hours at 4°C on a rotating platform. Immune complexes were collected on protein A/G beads (Thermo Scientific) for 2 hours at 4°C. At the end of the incubation, the mix was gently spun, and the supernatant was discarded. The beads were washed three times with RIPA buffer, resuspended in 40 μL of Laemmli buffer (Novex) and boiled for 10 minutes. Immune complexes were resolved by 4–12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% nonfat dry milk in PBS and incubated with primary antibodies (Anti-T7 tag, mouse, 1:2500 by Novagene; Anti-HA tag, rabbit, 1:5000 by Cell Signaling; anti-MBD4, rabbit, 1:500 in-house) overnight at 4°C, and then with secondary antibody (goat anti-mouse and goat anti-rabbit by Santa Cruz, 1:4000). Detection was performed using enhanced chemiluminescence (Pierce).

In vitro sumoylation –

We have previously described purification of recombinant activating E1 (Aos1-Uba2) and conjugating E2 (Ubc9) enzymes [47]; Yellow Fluorescent Protein-SUMO1 fusion was purified as described [47], using pET11d-YFP-SUMO (kind gift of Dr. Frauke Melchior). Purification of recombinant MBD4 [17] and TDG [54] has also been described. In vitro sumoylation reactions were carried out, as previously described [47], for 2hrs at 30°C in 50 mM Tris-Cl (pH 7.5) and 5 mM $MgCl_2$, in the presence of an ATP regeneration system (2 mM ATP, 10 mM creatine phosphate disodium salt, 3.5 U/ml creatine kinase and 0.6 U/ml inorganic pyrophosphatase), with 500 ng Uba2/Aos1, 2 μg Ubc9, 2 μg YFP-SUMO1, 1 μg of recombinant MBD4 or recombinant TDG. Reactions were separated by SDS-PAGE and analyzed by immunoblotting with anti-SUMO1 antibody (Invitrogen).

Subcellular fractionation –

Subcellular fractionation was conducted with Nucbuster Protein Extraction Kit (Millipore), following the manufacturer's recommendations.

Drug Treatments –

N-methyl-N-nitrosourea (MNU, Sigma-Aldrich) was dissolved in DMSO at the final concentration of 10 mg/mL; cisplatin (GensiaSicor Pharmaceuticals, 1 mg/ml) and 5-fluorouracil (American Pharmaceutical Partners, Inc., 50 mg/ml) were used as supplied by the manufacturer. Drug treatments were performed 24 hours after transfection by removing

the medium from cultures of logarithmically growing cells and adding fresh medium containing drugs (2 μM). After 24 hours of drug exposure, cells were rinsed with PBS and harvested for analysis.

MBD4 repair activity –

MBD4/AP-Endonuclease activity of nuclear extracts was measured using a G:T mismatch-containing molecular beacon hairpin with quenching upon folding and fluorescence release upon base removal/cleavage at the lesion site [55]. The substrate 5'dT(FAM)-CCACT**T**GTGAATTGACAGCCCATGTGCATCAATTCAC**G**AGTGG-T (Dabsyl)3' (Biosearch Technologies, Novato, CA) was annealed by heating and slow-cooling in 4X BER buffer (1X: Hepes-KOH 25mM, KCl 150mM, Glycerol 1%, DTT 0.5mM)[55]. Substrate (0.56 μM) and nuclear extracts (50 μg) were mixed in 1X BER buffer supplemented with $MgCl_2$ (1.2 mM) and DTT (10 mM). The reaction was run at 37°C in a real-time PCR machine (Applied Biosystems 7900 HT) and FAM fluorescence was recorded for 2 hrs at 0.5sec intervals.

Bioinformatics –

Prediction of sumoylation sites was conducted with SUMOplot (http://www.abgent.com/sumoplot).

RESULTS

Tandem affinity purification identifies sumoylation of MBD4

To purify the complex of MBD4-associated proteins, we conducted a tandem affinity purification tag (TAP-tag) strategy [56]. In this approach, cells are transfected with a plasmid expressing the protein of interest fused C-terminally to two IgG binding domains from *S. aureus* protein A, the cleavage site for the protease TEV, and the calmodulin binding peptide (Figure 1A). The presence of two high affinity tags in tandem separated by a protease site allows a two-step purification procedure of remarkable effectiveness not only in yeast but also in mammalian systems [46, 57]. We expressed in HEK-293 cells an N-terminal tagged MBD4 construct (MBD4-TAP), fused to protein A and calmodulin binding peptide (Suppl. Fig. 1). Total cellular lysate was first applied to an IgG beads affinity column; after washing, the MBD4 fusion protein and its interacting partners were released by cleavage with the TEV protease; the eluate was then applied onto calmodulin beads for a second affinity step; finally, the MBD4 complex was eluted with EGTA [46, 56], and fractions containing MBD4 were acetone-precipitated and separated by SDS-PAGE (Figure 1B). Western blotting demonstrated isolation of the MBD4 interactor, MLH1, thus further confirming that the interaction between these two proteins occurs in vivo in mammalian cells [29](Fig. 1C). Furthermore, mass spectrometry (MS) analysis of bands purified from the denaturing SDS-PAGE revealed that several bands with molecular weight higher than that predicted for MBD4 (~66kD) contained peptides corresponding to MBD4 itself and SUMO, suggesting that MBD4 is sumoylated in vivo (Fig. 1D). A list of additional interactors identified by MS and examples of protein ID determination are shown in Suppl. Table 1 and Suppl. Fig. 2, respectively.

MBD4 is sumoylated in vivo and in vitro

To verify the covalent interaction between MBD4 and SUMO1, stringent and reciprocal coimmunoprecipitation experiments, using RIPA lysates and SDS-PAGE, were performed in HEK-293 cells transfected with hemagglutinin-tagged (HA) human MBD4 [29] and T7-tagged SUMO1 [47, 48]. These experiments revealed detection of a band corresponding to T7-sumoylated HA-MBD4 (approximately 90kD) (Fig. 2A–B). This result was validated in a different cell line, MCF-7, in which coimmunoprecipitation experiments were conducted after transfecting cells with T7-tagged SUMO1 in combination with HA-tagged MBD4 and, as positive controls for sumoylation, TDG and the oncoprotein AKT1 [58, 59](Fig. 2C). To further confirm MBD4 sumoylation, we conducted an in vitro sumoylation assay, employing recombinant activating E1 (Aos1-Uba2) and conjugating E2 (Ubc9) enzymes, along with recombinant YFP-SUMO1 and MBD4 or, as positive control for sumoylation, TDG (Fig. 2D). These results indicate that MBD4 is sumoylated in vivo and in vitro.

Mapping of the main MBD4 sumoylation sites

To map the MBD4 sumoylation sites, we first turned to sequence analysis. The sumoylation prediction software SUMOplot identified three high-probability and ten low-probability sites (Fig. 3A). The first of the three putative sumoylation sites, K137, maps to the MBD4 MBD, whereas the other two high-probability sites, K215 and K377, map to the linker region between the MBD and the glycosylase domain (Fig. 3B). These three sites were mutated by substituting lysine with arginine, alone or in combination, creating five different mutants (K137R, K215R, K377R, K137R/K215R and K137R/K215R/K377R). Co-immunoprecipitation and western blot analysis in transfected MCF-7 cells were then performed to determine if the mutated sites in MBD4 would prevent sumoylation. The results indicated that MBD4 is sumoylated preferentially at K137, as sumoylation was dramatically decreased in the K137R mutant; and additional sumoylation sites are located at K215R and K377R, as sumoylation was almost completely abrogated in the triple mutant (Figure 3C, top). However, it is likely that sumoylation occurs at least at one more lysine in MBD4, because a longer exposure revealed a band corresponding to residual sumoylation even in the triple mutant (Figure 3C, bottom).

The sumoylation pattern of MBD4 is altered in response to DNA damage

In addition to its repair function, MBD4 plays a role in the apoptotic response to DNA damage induced by alkylating agents, cisplatin and 5-fluorouracil (5-FU)[34, 35]. We then sought to determine if treatment with these agents affected sumoylation patterns. 24 hours after transfection with HA-MBD4 + SUMO1-T7 and HA-MBD4 K137R/K215R/K377R + SUMO1-T7, MCF7 cells were treated with cisplatin, N-Nitroso-N-methylurea (NMU) and 5-FU at a 2μM concentration for 24 hours, after which co-IPs were performed (IP anti-T7, WB anti-HA). The results revealed that 5FU increases the extent of sumoylation at the three main sites, whereas NMU and cisplatin increased sumoylation at additional site(s) when the three main sites are mutated, thus confirming the existence of at least one more site of sumoylation (Fig. 4).

Sumoylation increases the repair activity of MBD4

Since sumoylation may alter the biochemical activity of the modified protein, using a fluorescence-based, molecular beacon assay for G:T mismatch repair [60, 61], we assessed the repair activity of nuclear extracts from untransfected MCF7 cells, and from MCF7 cells transfected with HA-MBD4 or HA-MBD4 + SUMO1-T7. The results revealed robust G:T repair activity of nuclear extracts of untransfected MCF7 cells, which was slightly increased by overexpression of HA-MBD4, and further boosted by HA-MBD4 + SUMO1-T7 (Fig. 5). The results suggest that sumoylation of MBD4 increases its glycosylase activity on G:T mismatches.

MBD4 mutations near sumoylation sites in cancer

Inactivating mutations of the *MBD4* gene in human cancer have been described by us and others [39–41]. The consequence of inactivating mutations of *MBD4* is to increase the mutation rate at CpG sites [44, 62, 63] and provide an advantage to cancer cells, even in the context of inactivating MMR mutations [64]. Since sumoylation boosts MBD4 repair activity, we reasoned that it may be compromised in cancer. Examination of The Cancer Genome Atlas (TCGA), revealed a total of 67 *MBD4* missense mutations reported to date (http://www.cbioportal.org/). Of these missense mutations, 14 (20.9%) map within 5 amino acids of sumoylation sites, and 6 are reported in endometrial cancer (Table 1). These results suggest that cancer-associated *MBD4* mutations may affect sumoylation of its gene product.

DISCUSSION

Sumoylation plays an important role in cellular functions, and particularly in DNA repair, affecting activity, localization and stability of target proteins. Despite the fact that sumoylation was known to affect the function of base excision repair proteins, identification of MBD4 sumoylation was unexpected, as the MBD4 fusion protein expressed from the TAP-tag construct became sumoylated in transfected HEK-293 cells. Sumoylation of MBD4 was later confirmed by co-IP experiments in both HEK-293 and MCF7 cells and reconstituted in vitro, using recombinant proteins.

Sumoylation of MBD4 is complex. In addition to the three main sites predicted by software on the basis of the consensus sumoylation sequence ψKX(D/E), in which ψ is a large hydrophobic residue [10], additional sites with a lower probability score are present in MBD4. In fact, the triple mutant K137R/K215R/K377R (arginine substitutions at the three main acceptor lysine residues) shows evidence of being sumoylated in vivo. Several bands of increasing molecular weight were detected by mass spectrometry and by co-IP, suggesting the different, but not mutually exclusive possibilities, of addition of monomeric SUMO1 molecules at multiple sites or of poly-SUMO1 chains (polysumoylation)[65]. The possibility that (some of) these sites can be targeted by SUMO molecules other than SUMO1, with potentially different functional consequences, should be considered.

While K215 and K377 are located in the low-complexity linker region, whose function is unknown, K137 is part of the MBD; it is likely that sumoylation at this site will affect binding affinity of the MBD for 5-methylcytosine. Putative site(s) of sumoylation are located

in the catalytic domain and may affect catalysis, as well as interaction with MLH1, which maps to this region [29]. In fact, only a small amount of MLH1 was identified in the TAP-MBD4 eluate (Fig. 1C), containing sumoylated MBD4.

Interestingly, sumoylation stimulated repair activity on a G:T mismatched substrate, a finding that is opposite to TDG sumoylation, which is known to decrease enzymatic activity [24]. This finding for MBD4 should be confirmed with recombinant proteins and with a precise measurement of the velocity of the repair reaction in the presence of unsumoylated or differentially sumoylated MBD4 molecules.

The pattern of MBD4 sumoylation changes in response to different kinds of DNA damage and, in particular, sumoylation at site(s) other than the three main sites is stimulated by cisplatin and MNU, when the three main sites are mutated, but not 5-FU. Since MBD4 affects the apoptotic response to DNA damage, but does not directly repair cisplatin or MNU damage, it is possible that sumoylation at different sites may serve to separate the apoptotic function of MBD4 from its repair function. To this end, it is interesting to note that 5-FU, which can be directly processed by MBD4 in the form of 5-FU:G mismatches [37], does not stimulate sumoylation at sites other than the three main sites. Again, it is possible that different SUMOs (i.e., SUMO1 vs. SUMO2/3 vs. SUMO4) may be involved in the apoptotic vs. repair role of MBD4).

Compromising both apoptotic and repair functions of MBD4 is likely to be important in tumorigenesis. We reported that a large fraction of *MBD4* missense mutations (20.9%) occur near sumoylation sites, and therefore may impact apoptotic and/or repair activity of the enzyme. Future studies will clarify these possibilities. Mutations abrogating sumoylation do not strictly have to be at the modified lysine, but can be nearby as well. For instance, the E318K mutation in *MITF,* that impairs sumoylation and predisposes to melanoma and renal carcinoma, occurs at the glutamic acid at position +2 of the sumoylation consensus site, ψKX(D/E) [66, 67]. Also, in a recent pan-cancer analysis of lysine modifications, including sumoylation, a window similar to ours in Table 1 was used, i.e. from position −7 to +7 around the lysine [68].

In summary, to our knowledge, this is the first description of MBD4 sumoylation. Understanding the consequences of this modification for MBD4 functions may be important not only from the basic science standpoint, but also for translational research, as it may help devising novel diagnostic and/or therapeutic strategies for cancer. This study expands the role played by sumoylation in fine-tuning DNA damage response and repair, and constitutes the first step towards possible translational opportunities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MBD4 is sumoylated at three main sites: K137, K215 and K377
- MBD4 sumoylation is altered, in a DNA damage-specific way (5FU, NMU & cisplatin)
- Sumoylation increases the G:T repair activity of MBD4 in cell extracts
- Of the 67 *MBD4* missense mutations in TCGA, 14 (20.9%) map near sumoylation sites

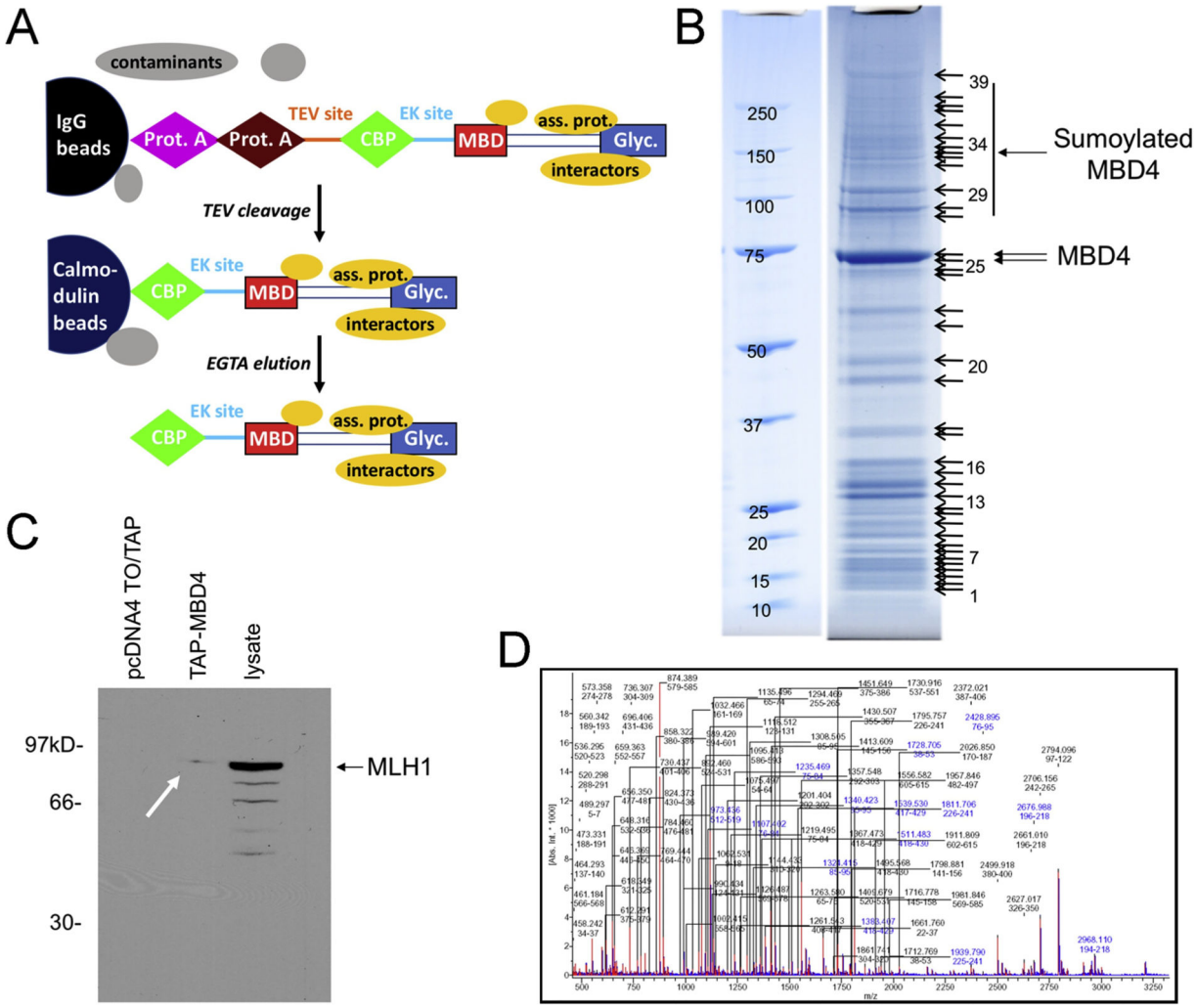

Figure 1 –. Tandem affinity purification of MBD4-associated proteins.

(A) Schematic of TAP-tag strategy (modified from [56]). The TAP-tag-MBD4 fusion protein and the two sequential affinity columns are indicated; elution from the IgG column occurs by cleavage with TEV protease; elution from the calmodulin column occurs by calcium chelation by EGTA. Both covalently associated proteins and interactors (yellow) are co-purified with MBD4, whereas contaminants (grey) are eliminated. (B) SDS-PAGE, stained with Coomassie brilliant blue of acetone-precipitated TAP-tag eluate from HEK-293 cells, with bands submitted to mass spectrometry identification. Bands 25 and 26 correspond to unmodified MBD4; several of the higher molecular weight bands contain peptides identified as SUMO1 and MBD4 by mass spectrometry. (C) Detection of the MBD4 interactor MLH1 by western blotting of lysates from TAP-MBD4-transfected cells but not from empty pCDNA4/TO/TAP-transfected cells. Total lysate from HEK-293 cells is a positive control. (D) Tryptic peptide mass fingerprint showing well-defined peaks allowing reliable identification of peptides corresponding to MBD4.

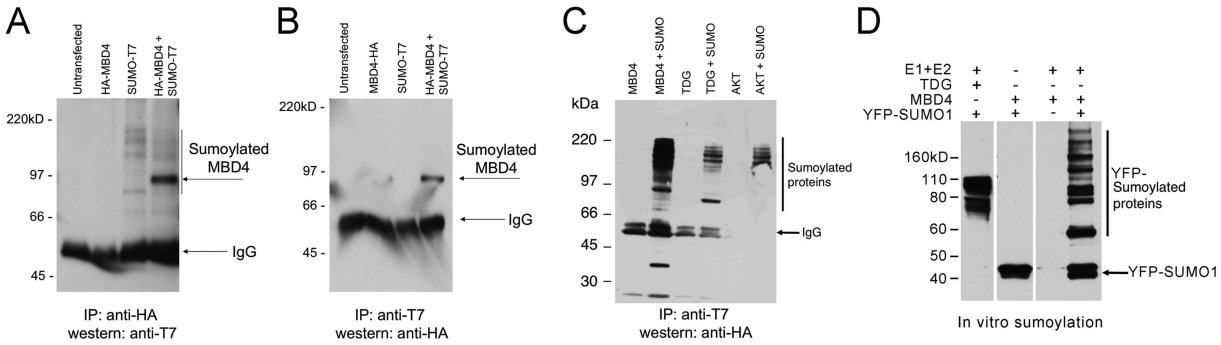

Figure 2 –. MBD4 is sumoylated in vivo and in vitro.

(A, B) HEK-293 cells were transfected with the indicated plasmids or left untransfected. Co-IP conducted in both directions (IP: antihemagglutinin / western: anti-T7; and IP: anti-T7 / western: anti-hemagglutinin), as indicated, revealed detection of an approximately 90kD band corresponding to sumoylated MBD4. (C) MCF-7 cells were transfected with hemagglutinin-tagged MBD4, TDG and AKT, in combination with T7-tagged SUMO1. Co-IP reveals sumoylation of MBD4 and positive controls TDG and AKT1 (upper panel). Western blotting shows approximately equal expression of hemagglutinin-tagged MBD4, TDG and AKT (lower panel). (D) In vitro sumoylation assay, in which the indicated activating E1 (Aos1-Uba2) and conjugating E2 (Ubc9) enzymes were incubated with recombinant MBD4, TDG and YFP-SUMO1; detection of reaction products was done by western blotting with anti-SUMO1 antibody.

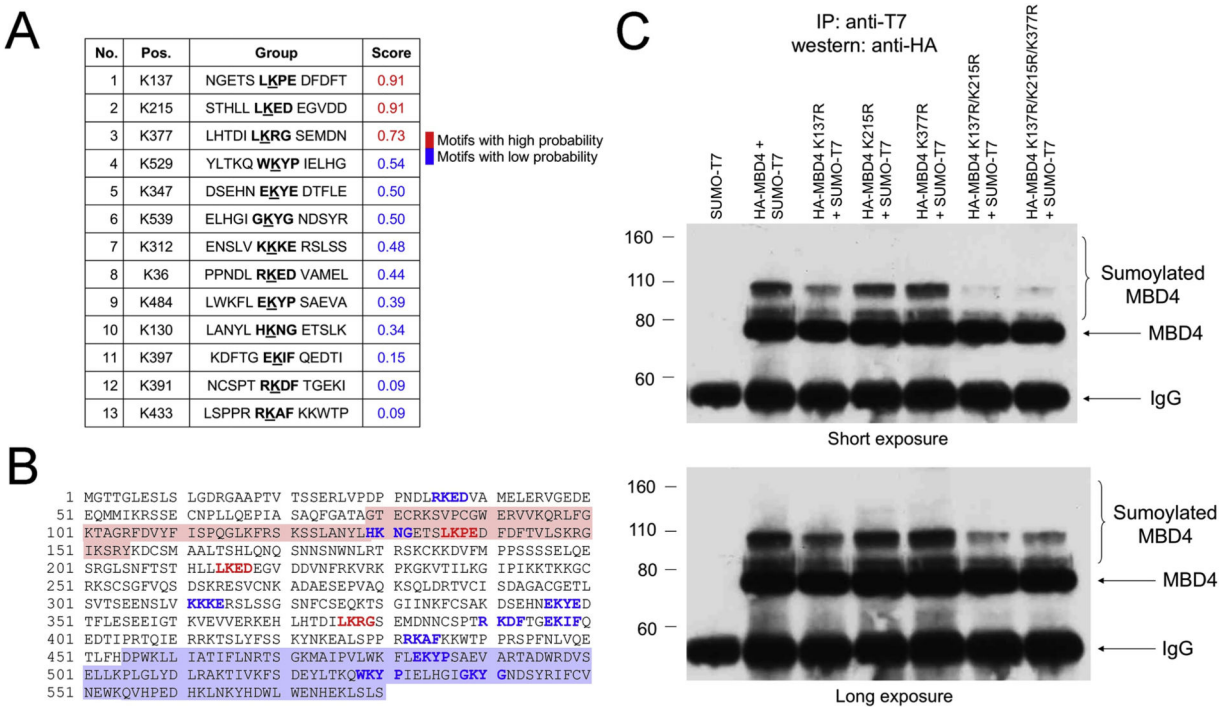

Figure 3 –. Mapping of the MBD4 sumoylation sites.

List (A) and location (B) of the high- (red) and low-probability (blue) sumoylation sites identified by the SUMOplot software. The MBD and glycosylase domain are shaded in light red and blue, respectively. (C) MCF-7 cells were transfected with hemagglutinin-tagged wild type MBD4 and indicated MBD4 mutants, in combination with T7-tagged SUMO1. Co-IP confirms that K137, K215 and K377 are the main sumoylation sites (upper panel, short exposure). However, long exposure (lower panel) shows residual sumoylation in the K137R/K215R/K377R, suggesting the existence of at least one more sumoylation site.

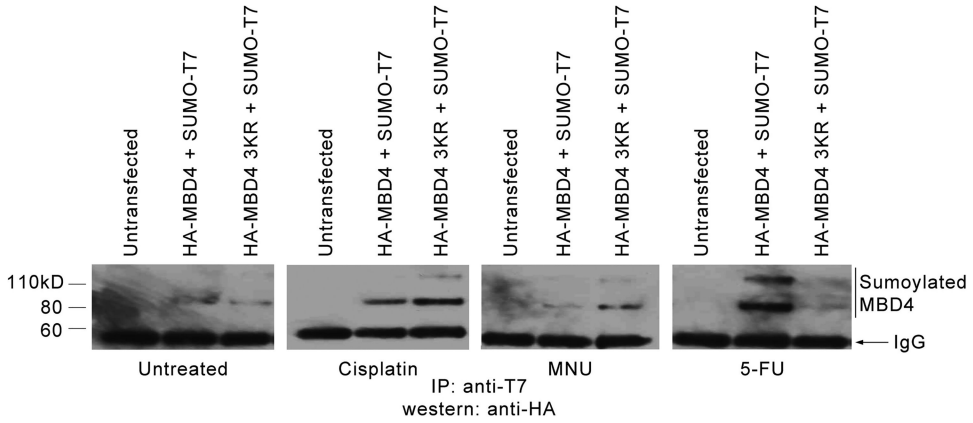

Figure 4 –. Sumoylation of MBD4 in response to DNA damage.

Co-immunoprecipitation analysis (IP: anti-T7 / western: anti-hemagglutinin) of MCF7 cells left untransfected and transfected with hemagglutinin-tagged wild type MBD4 or hemagglutinin-tagged K137R/K215R/K377R MBD4, as indicated, and treated with control DMSO or with cisplatin, NMU and 5-FU (2 μM for 24 hours).

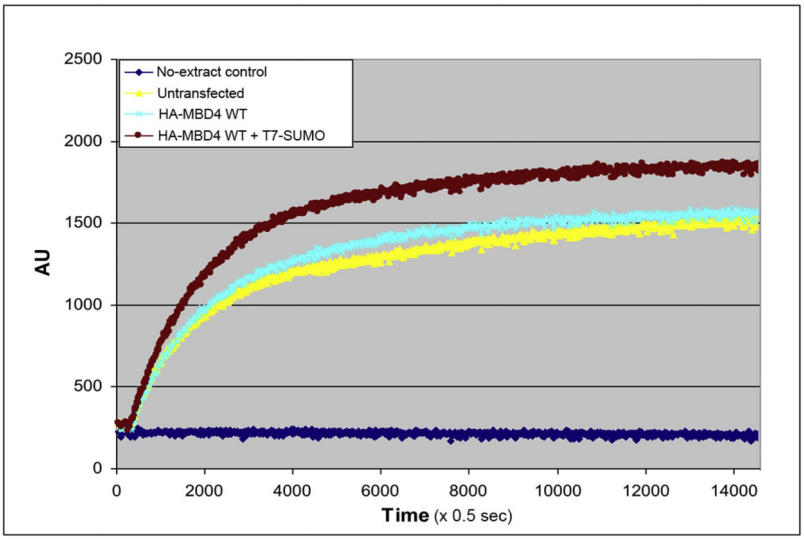

Figure 5 –. Sumoylation increases the repair activity of MBD4.

Molecular beacon assay for repair of G:T mismatches by extracts of untransfected MCF7 cells and MCF7 cells transfected with hemagglutinin-tagged MBD4 or hemagglutinin-tagged MBD4 plus SUMO-T7, showing time-dependent generation of fluorescence; fluorescence is in arbitrary units (AU); time is in 0.5-sec intervals.

Table 1.

Mutations near MBD4 sumoylation motifs

Mutation.	Cancer	Group	Pos.
N131S	Colon adenocarcinoma	**N**GETS **L**<u>**K**</u>**PE** DFDFT	K137
S135Y	Uterine endometrioid carcinoma	NGET**S L**<u>**K**</u>**PE** DFDFT	K137
S209Y	Lung squamous cell carcinoma	**S**THLL **L**<u>**K**</u>**ED** EGVDD	K215
H211Y	Uterine mixed endometrial carcinoma	ST**H**LL **L**<u>**K**</u>**ED** EGVDD	K215
D221H	Head and neck squamous cell carcinoma	STHLL **L**<u>**K**</u>**ED** EGV**D**D	K215
R378C	Uterine endometrioid carcinoma	LHTDI **L**<u>**K**</u>**RG** SEMDN	K377
R546Q	Uterine endometrioid carcinoma	ELHGI **G**<u>**K**</u>**YG** NDSY**R**	K539
R546Q	Breast invasive ductal carcinoma	ELHGI **G**<u>**K**</u>**YG** NDSY**R**	K539
S308N	Rectal adenocarcinoma	EN**S**LV **K**<u>**K**</u>**KE** RSLSS	K312
K311T	Cervical squamous cell carcinoma	ENSLV **K**<u>**K**</u>**KE** RSLSS	K312
S487L	Cervical squamous cell carcinoma	LWKFL **E**<u>**K**</u>**YP S**AEVA	K484
L124R	Papillary renal cell carcinoma	**L**ANYL **H**<u>**K**</u>**NG** ETSLK	K130
F399C	Uterine endometrioid carcinoma	KDFTG **E**<u>**K**</u>**IF** QEDTI	K397
T439A	Uterine endometrioid carcinoma	LSPPR **R**<u>**K**</u>**AF** KKW**T**P	K433

Mutations near motifs with high probability

Mutations near motifs with low probability