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Human MMS21/NSE2 Is a SUMO Ligase Required for DNA Repair†

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DNA repair is required for the genomic stability and well-being of an organism. In yeasts, a multisubunit complex consisting of SMC5, SMC6, MMS21/NSE2, and other non-SMC proteins is required for DNA repair through homologous recombination. The yeast MMS21 protein is a SUMO ligase. Here we show that the human homolog of MMS21 is also a SUMO ligase. hMMS21 stimulates sumoylation of hSMC6 and the DNA repair protein TRAX. Depletion of hMMS21 by RNA interference (RNAi) sensitizes HeLa cells toward DNA damage-induced apoptosis. Ectopic expression of wild-type hMMS21, but not its ligase-inactive mutant, rescues this hypersensitivity of hMMS21-RNAi cells. ATM/ATR are hyperactivated in hMMS21-RNAi cells upon DNA damage. Consistently, hMMS21-RNAi cells show an increased number of phospho-CHK2 foci. Finally, we show that hMMS21-RNAi cells show a decreased capacity to repair DNA lesions as measured by the comet assay. Our findings suggest that the human SMC5/6 complex and the SUMO ligase activity of hMMS21 are required for the prevention of DNA damage-induced apoptosis by facilitating DNA repair in human cells.

Accurate replication and segregation of a cell's genome to its daughter cells are essential for the survival of an organism (3, 33, 40). To maintain genomic stability, cells must also be able to cope with DNA damage that occurs both intentionally and unintentionally, including double-strand breaks (DSBs), single-strand breaks, and other types of lesions (20, 51). In addition to being caused by exogenous factors, certain types of DNA damage, such as DSBs, are also crucial intermediates during meiotic homologous recombination (HR), V(D)J recombination, and immunoglobulin class switch recombination in immune cells (21, 49). To prevent DNA damage from compromising the genetic integrity of an organism, cells employ surveillance mechanisms to sense damaged DNA and to elicit coordinated cellular responses, such as DNA repair, cell cycle arrest, and apoptosis (20, 41, 51). Mutations of genes involved in DNA damage response pathways can lead to cancer, immune deficiencies, and other human diseases (20, 21, 51).

Two related protein kinases, ATM and ATR, are key proximal signal transducers of the DNA damage response (20, 41, 51). They phosphorylate distinct, yet overlapping, sets of downstream substrates, including CHK1, CHK2, p53, BRCA1, and NBS1 (20, 41, 51). CHK1 and CHK2 are effector kinases and in turn phosphorylate the CDC25 family of phosphatases and p53, leading to cell cycle arrest or apoptosis (20, 41, 51). Upon DNA damage, many proteins involved in DNA damage sensing and repair are enriched in nuclear foci, which are thought to represent active cellular centers for DNA repair (2, 25, 26, 49).

The structural maintenance of chromosomes (SMC) family of proteins is essential for chromosomal architecture and organization (11, 14, 36). There are six known eukaryotic SMC proteins, SMC1 to -6, which form three types of heterodimers (14). The SMC1/3 heterodimer is a part of the cohesin complex that maintains sister chromatid cohesion (11, 14, 23, 36). SMC2/4 are components of the condensin complex that mediates chromosome condensation during mitosis (14, 44). A third SMC complex, containing SMC5/6, is mainly involved in the cellular response to DNA damage (7, 11, 14, 24, 34).

Most studies of the SMC5/6 complex have been conducted in fission and budding yeasts. The yeast SMC5/6 complex contains several non-SMC elements (NSE), including NSE1, MMS21/NSE2 (hereafter referred to as MMS21 for simplicity), NSE3, and NSE4 (8, 17, 29, 32, 35, 42, 50). SMC5, SMC6, NSE1, and MMS21 are all essential genes in yeasts (7, 8, 24, 29, 34, 35, 39). Cells harboring hypomorphic alleles of these genes show an increased sensitivity to a broad spectrum of DNA damage agents, including ionizing radiation, UV, and methyl methanesulfonate (MMS) (7, 8, 17, 24, 29, 34, 35, 38, 39). Genetic analysis has shown that SMC5/6, NSE1, and MMS21 function together with RAD51 in the repair of DNA DSBs through HR in yeasts (13, 29, 31, 34, 35). Recently, the SMC5/6 complex has also been shown to play a role in the segregation of repetitive sequences during mitosis in budding yeast (47). A similar SMC5/6 complex exists in human cells, although the non-SMC components of this complex have not been characterized (46).

Small ubiquitin-like modifier (SUMO) is a ubiquitin-like protein that can be covalently conjugated to target proteins (9, 18). Similar to ubiquitination, sumoylation of substrates is catalyzed by a cascade of enzymes: the E1 SUMO-activating enzyme (AOS1-UBA2), the E2 conjugating enzyme (UBC9), and the E3 SUMO ligases (9, 18). Several classes of SUMO ligases have been identified (9, 18). Among them, the PIAS class of SUMO ligases contains an SP-RING domain that is similar to the RING finger domain found in many ubiquitin ligases (9, 18). Unlike ubiquitination, sumoylation of substrates does not strictly require a SUMO ligase. UBC9 is sufficient to mediate sumoylation due to its ability to directly recognize ΦKXE (Φ,

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hydrophobic residues; X, any residue) motifs on substrates. SUMO ligases merely enhance the rate of sumoylation. Furthermore, sumoylation of substrate proteins does not generally lead to their degradation (9, 18). Instead, it appears to regulate the functions of target proteins by multiple, context-dependent mechanisms, such as altering their subcellular localization, increasing their stability, or mediating their binding to other proteins (9, 18).

Interestingly, NSE1 contains a RING finger domain similar to ubiquitin ligases whereas MMS21 contains an SP-RING domain that is related to the PIAS family of SUMO ligases (29). It has recently been reported that both budding and fission yeast MMS21 proteins function as SUMO ligases (1, 50). Therefore, the SMC5/6 complex is a conserved chromatin-associated DNA repair complex that potentially has both ubiquitin ligase and SUMO ligase activities. Numerous studies have established multiple roles of sumoylation in regulating cellular responses for coping with DNA damage in both yeasts and mammals (12, 15, 16, 27, 28, 43, 45). Recently, using an in vitro expression cloning strategy, we have identified a large panel of human SUMO substrates (10). Several of these substrates are involved in DNA repair, including Ku80, XRCC1, and translin-associated factor X protein (TRAX) (10).

Our interest in sister chromatid cohesion and ubiquitin-like protein ligases prompted us to investigate the function of the SMC5/6 complex in human cells. We show that, similarly to the yeast MMS21, human MMS21 is a SUMO ligase that stimulates the sumoylation of hSMC6 and TRAX. RNA interference (RNAi)-mediated knockdown of hMMS21 increases the propensity of HeLa cells to undergo apoptosis in response to various DNA damage agents. Importantly, ectopic expression of the wild-type hMMS21, but not a ligase-inactive mutant of hMMS21, in hMMS21-RNAi cells rescues their hypersensitivity to DNA damage. This indicates that the SUMO ligase activity of hMMS21 is required for proper cellular response to DNA damage. Upon DNA damage, hMMS21-RNAi cells contained higher ATM/ATR activity and an increased number of phospho-CHK2 nuclear foci. Consistently, we observed an increased amount of unrepaired DNA lesions in hMMS21-RNAi cells as measured by the comet assay. These results suggest that hMMS21 and the hSMC5/6 complex play an important role in the repair of damaged cellular DNA.

MATERIALS AND METHODS

Plasmids and antibodies. The coding regions of human NSE1 and MMS21 were amplified by PCR from a human fetal thymus cDNA library (Clontech) and cloned into appropriate vectors. The coding region of human hSMC6 was amplified by PCR from a human testes cDNA library (Clontech) and cloned into appropriate vectors. The amplified product from the testes library was an alternatively spliced form of hSMC6 as reported previously (46). This form of SMC6 encodes an N-terminally truncated protein of 95 kDa. The hSMC5 plasmid and an hSMC6 plasmid encoding the full-length hSMC6 protein were obtained from A. R. Lehmann (University of Sussex, United Kingdom). The ligase-inactive mutants of hMMS21 were constructed with the QuikChange site-directed mutagenesis kit (Stratagene). The full length and an N-terminal fragment of hMMS21 (hMMS21N; residues 1 to 165) and the N-terminal fragment of hSMC5 (hSMC5N; residues 1 to 233) were expressed in bacteria and purified as glutathione *S*-transferase (GST) fusion proteins using glutathione-agarose beads (Amersham). GST-hMMS21N and GST-hSMC5N were used for antibody production (Zymed Laboratories). Crude antibody sera were first precleared with GST-coupled Affi-Gel beads (Bio-Rad) and subsequently affinity-purified using beads coupled to GST-hMMS21N or GST-hSMC5N. An hSMC5 antibody for Western blotting was obtained from A. R. Lehmann (University of Sussex, United Kingdom). The commercial antibodies used in this study are as follows: anti-myc and antihemagglutinin (anti-HA) antibodies (Roche; 1 μg/ml), rabbit anti-ATM/ATR phosphosubstrate antibody (Cell Signaling Technology; 1:100), rabbit anti-phospho-CHK2 (T68) antibody (Cell Signaling Technology; 1:100), mouse anti-Asp214 cleaved poly(ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology; 1:1000), and mouse anti-GMP1 (SUMO1) antibody (Zymed Laboratories; 1:500).

In vitro sumoylation assay. Proteins were in vitro translated (IVT) from pCS2-myc plasmids in reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. IVT proteins were then subjected to in vitro sumoylation reactions with reaction mixtures that contained 2 μl of IVT product, 2 μg of AOS1-UBA2, 0.5 μg of UBC9, 1 μg of SUMO1, and 1 μl of energy mix (150 mM phosphocreatine, 20 mM ATP, 2 mM EGTA, 20 mM $MgCl_2$, pH 7.7). Reaction mixtures were adjusted to 10 μl with XB buffer (10 mM HEPES, pH 7.7, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl, and 50 mM sucrose). After 2 h at 30°C, reactions were stopped with 10 μl of 2× sodium dodecyl sulfate (SDS) sample buffer, and reaction mixtures were boiled and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. Reactions with reaction mixtures containing GST-hMMS21 were performed as described above except that 0.5 μg recombinant GST-hMMS21 purified from bacteria was used. Detection of GST-hMMS21 sumoylation was performed by incubating the SUMO reaction mixtures with glutathione-agarose beads for 1 h at 4°C. After washing, proteins bound to beads were eluted with 2× SDS sample buffer, boiled, and subjected to SDS-PAGE followed by immunoblotting with anti-SUMO1.

Human AOS1-UBA2, UBC9, and SUMO1 proteins were expressed and purified in bacteria as described previously (10). Briefly, pET28b-SUMO1 and pET28b-UBC9 were expressed as His_6-tagged proteins in BL21(DE3) and purified using Ni^{2+}-nitrilotriacetic acid beads per the manufacturer's protocols (QIAGEN). For the expression of AOS1-UBA2, pET11c-AOS1 and pET28b-UBA2 were cotransformed into BL21(DE3). The resulting AOS1-UBA2 complex was purified with Ni^{2+}-nitrilotriacetic acid beads followed by a Superdex 200 gel filtration column (Amersham Biosciences) to remove the excess amount of AOS1. hMMS21 was expressed as a GST-tagged protein in BL21(DE3) and purified using glutathione-agarose beads (Amersham Biosciences).

Cell culture, transfections, and treatments. HeLa cells were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 100 μg/ml penicillin and streptomycin (Invitrogen). At 40 to 50% confluence, plasmid or small interfering RNA (siRNA) transfection was performed using the Effectene reagent (QIAGEN) and the Oligofectamine reagent (Invitrogen), respectively. The siRNA oligonucleotides against hMMS21 (5′-CUCUGGUAUGGACACAGCUTT-3′) and hSMC5 (5′-GCAGUGGAUUCAGGGUUGATT-3′) were chemically synthesized at an in-house facility. The annealing and transfection of the siRNAs were performed as previously described (6). After 48 h, cells were treated with either 0.015% MMS or 40 μg/ml etoposide for the desired duration.

Immunoblotting and immunoprecipitation. At 24 h post-transfection of the desired plasmids, HeLa cells were lysed in SDS sample buffer, sonicated, boiled, separated by SDS-PAGE, and blotted with the indicated antibodies. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (Amersham Biosciences) was used as secondary antibody, and immunoblots were developed using the ECL reagent (Amersham Biosciences) per the manufacturer's protocols. For immunoprecipitation of overexpressed proteins, whole-cell lysate was made by lysing cells in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, and 1× protease inhibitor cocktail) on ice for 15 min and passing them through a 25-gauge needle 10 times, followed by centrifugation at 16,000 × *g* for 15 min at 4°C. Antibodies against myc and HA were covalently coupled to Affi-Prep Protein A beads (Bio-Rad) and incubated with the supernatants for 2 h at 4°C. The beads were then washed three times with the NP-40 lysis buffer. The proteins bound to the beads were dissolved in SDS sample buffer, boiled, separated by SDS-PAGE, and blotted with the indicated antibodies.

For immunoprecipitation of endogenous proteins, whole-cell lysate was made by lysing cells in buffer A (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 5 mM $MgCl_2$, 50 mM NaF, 80 mM β-glycerophosphate, 1 mM dithiothreitol, and 1× protease inhibitor cocktail) on ice for 10 min and then sonicated for 30 s. The lysate was then treated with 150 U/ml DNase I at 25°C for 1 h to help solubilize chromatin-bound proteins (46). The lysate was cleared by centrifugation at 16,000 × *g* for 15 min at 4°C. Antibodies against hSMC5, hBUBR1, and GST were covalently coupled to Affi-Prep Protein A beads (Bio-Rad) and incubated with the supernatants for 2 h at 4°C. After being washed with buffer A, the proteins bound to the beads were dissolved in SDS sample buffer, boiled, separated by SDS-PAGE, and blotted with the indicated antibodies.

Cell death quantification. Cells were treated with the appropriate conditions, and cell death was quantified in the following ways. Cells were stained with Hoechst 33258 (1:100; Molecular Probes) for 30 min at 37°C. All cells (floating and adherent) were collected and washed in phosphate-buffered saline (PBS). A portion of the cells was stained with trypan blue for 5 min. The number of blue cells (dead cells) was counted with a hemacytometer. At least 100 cells were counted for each condition in each experiment. The percentage of dead cells was then plotted. The stained nuclei were also imaged for nuclear condensation. The rest of the cells were lysed in sample buffer, sonicated, boiled, and separated by SDS-PAGE. The samples were immunoblotted with anti-Asp214 cleaved PARP.

Immunofluorescence. Cells were plated in four-well chambered slides (Lab-Tek) and treated according to experimental conditions. Cells were washed with PBS followed by fixation with 4% paraformaldehyde at 4°C for 30 min. The fixed cells were washed with PBS and blocked and permeabilized in Tris-buffered saline (TBS) containing 5% normal donkey serum and 0.03% Triton X-100 for 30 min at room temperature. After blocking, the cells were incubated in the appropriate primary antibodies overnight at 4°C in TBS containing 1% normal donkey serum and 0.03% Triton X-100. The cells were then washed with TBS and incubated with fluorescent secondary antibodies (Molecular Probes) in TBS containing 1% normal donkey serum and 0.03% Triton X-100 for 2 to 6 h at 4°C. After incubation, cells were washed with TBS and nuclei were stained with Hoechst 33258 (1:10,000) for 20 min at 4°C. Cells were again washed with TBS and mounted. Cells were viewed with a 63× objective on a Zeiss Axiovert 200M fluorescence microscope. Images were acquired with a charge-coupled device camera using Slidebook imaging software (Intelligent Imaging Innovations). Images examining the number of phospho-CHK2 (T68) foci were taken at 0.2-μm intervals, deconvolved using the nearest neighbor algorithm, and stacked to better resolve the number of foci. ATM/ATR phosphosubstrate antibody intensities were measured using the Slidebook imaging software. For both measurements, at least 100 cells were counted for each condition in each experiment.

Comet assay. Comet assays were performed according to the manufacturer's protocol (Trevigen). Briefly, cells were collected after the indicated treatments, mixed with low-melting-point agarose, and spread onto slides. The cell/agarose pad was then allowed to harden at 4°C for 30 min. Cells were then lysed by immersing slides in cold lysis solution for 30 min at 4°C. After lysis, slides were immersed in alkaline solution (200 mM EDTA, pH 13) for 20 min at room temperature to unwind and denature the DNA and hydrolyze sites of damage. Slides were then washed two times with Tris-buffered EDTA (TBE) for 5 min. Slides were then placed in a horizontal electrophoresis apparatus, equidistant from the electrodes in TBE. Voltage was applied for 10 min at 12 V (1 V per cm measured electrode to electrode). Slides were then immersed in 70% ethanol for 5 min and allowed to air dry at room temperature overnight. Slides were stained with SYBR Green I (Molecular Probes) for 10 min and then washed once in TBE. All incubations were performed in the dark to prevent unintended DNA damage. Slides were viewed using a 20× objective as described above. The percentage of cellular DNA in the comet tails was calculated by subtracting the nuclear florescence intensity from that of the entire cell. At least 100 cells were scored in each condition over three separate experiments.

RESULTS

Human MMS21 is a subunit of the hSMC5/6 complex. We identified the human homologs of yeast NSE1 and MMS21 by searching the human expressed sequence tag database with the amino acid sequences of their RING finger and SP-RING domains, respectively (Fig. 1A). We cloned the full-length cDNAs of hNSE1 and hMMS21 from a human fetal thymus cDNA library. The sequences of the hNSE1 and hMMS21 genes that we isolated were identical to those reported previously (29). Both human and yeast MMS21 proteins contain a SP-RING domain that is present in human PIAS1, a known SUMO ligase (Fig. 1B). SP-RING domains are putative Zn^{2+}-binding motifs that contain five cysteine/histidine residues as Zn^{2+}-coordinating ligands (18). Sequence alignment between human PIAS1 and MMS21 proteins from various species reveals the five conserved cysteine/histidine residues thought to be responsible for Zn^{2+} coordination in the SP-RING domain (Fig. 1B).

We next tested their interactions with hSMC5. Both hNSE1 and hMMS21 interacted with hSMC5 in HeLa cells (Fig. 1C). To examine the endogenous hSMC5/6 complex, we raised antibodies against hMMS21 and hSMC5. The antibodies were shown to be specific using RNAi knockdown experiments of the endogenous proteins in HeLa cells (Fig. 1D and E). Endogenous hMMS21 protein was pulled down specifically by immunoprecipitation with anti-hSMC5, but not with control anti-GST or anti-BUBR1 (Fig. 1F). Therefore, hMMS21 is likely the human ortholog of the yeast MMS21 and is a subunit of the hSMC5/6 complex.

hMMS21 is a SUMO ligase in vitro and in vivo. We then tested whether hMMS21 has SUMO ligase activity in vitro. Because several known SUMO ligases, such as PIAS1 and RanBP2, undergo efficient autosumoylation (19, 37), we first tested whether hMMS21 was sumoylated in an in vitro sumoylation assay. Briefly, the ^{35}S-labeled hMMS21 protein translated in rabbit reticulocyte lysate was incubated with purified, bacterially expressed AOS1-UBA2, UBC9, and SUMO1 proteins in the presence of ATP. hMMS21 efficiently formed high-molecular-weight conjugates on SDS-PAGE (Fig. 2A). The appearance of these high-molecular-weight conjugates was dependent on the presence of ATP, AOS1-UBA2, UBC9, and SUMO1, since the removal of each of these components abrogated the laddering (Fig. 2A). This suggested that hMMS21 was sumoylated in this assay. Addition of recombinant GST-PIAS1 or the SUMO ligase domain of RanBP2, two known E3 SUMO ligases, did not enhance the sumoylation of hMMS21 (see Fig. S1 in the supplemental material). In fact, addition of PIAS1 partially suppressed hMMS21 sumoylation (see Fig. S1 in the supplemental material), presumably due to competition between hMMS21 and PIAS1 for certain components in the SUMO reaction.

To verify that hMMS21 is a SUMO ligase and not simply an efficient SUMO substrate for UBC9, we mutated each of the five conserved cysteine/histidine residues in the SP-RING domain of hMMS21 and tested the ability of these mutants to be sumoylated in vitro. Mutations of cysteine/histidine residues will not remove any potential SUMO conjugation sites in hMMS21, as sumoylation occurs on lysines. Consistent with hMMS21 itself being a SUMO ligase, mutation of any of the five conserved cysteines/histidines significantly decreased the sumoylation of hMMS21 (Fig. 2B). Although mutation of a single cysteine/histidine in the SP-RING domain of hMMS21 did not completely prevent SUMO conjugation, it dramatically reduced the formation of high-molecular-weight hMMS21-SUMO1 conjugates (Fig. 2B). Therefore, this indicates that sumoylation of hMMS21 is dependent on its SP-RING domain.

We next performed in vitro sumoylation reactions using bacterially purified GST-hMMS21. GST-hMMS21 formed a high-molecular-weight ladder on SDS-PAGE in reactions with reaction mixtures containing ATP, AOS1-UBA2, UBC9, SUMO1, and GST-hMMS21 (Fig. 2C). These high-molecular-weight species of GST-hMMS21 were absent in reaction mixtures lacking any given one component (Fig. 2C). To confirm that these high-molecular-weight GST-hMMS21 species were indeed GST-hMMS21-SUMO1 conjugates, the same reaction mixtures were blotted with anti-SUMO1. A similar high-molecular-weight ladder of SUMO1 was observed when all com-

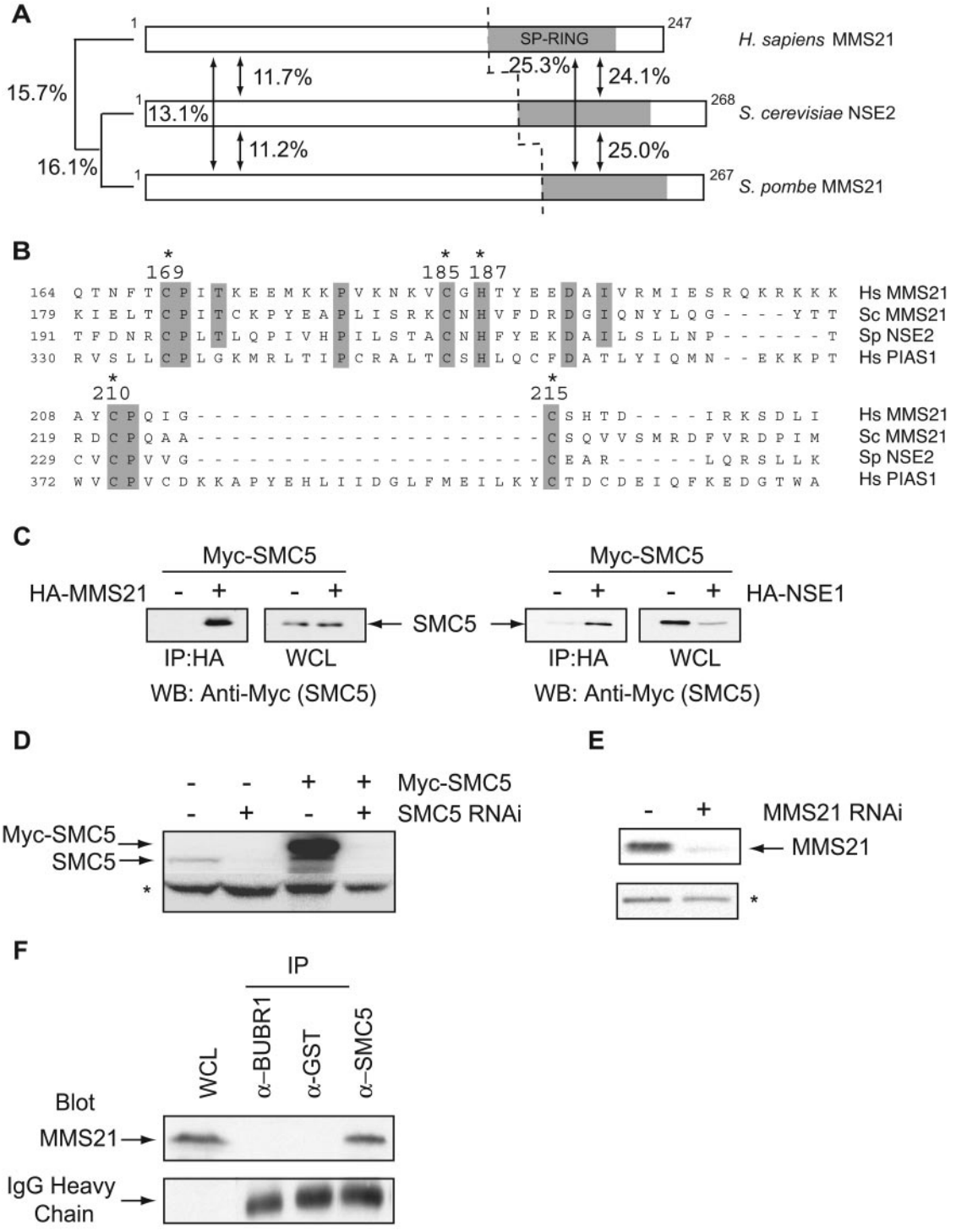

FIG. 1. hMMS21 is a subunit of the hSMC5/6 complex. (A) Sequence identities among MMS21 proteins of *H. sapiens*, *S. cerevisiae*, and *S. pombe*. The identities of the full-length hMMS21, the N-terminal domain, and the C-terminal SP-RING domain (indicated by dashed lines) are shown. (B) Sequence alignment of the SP-RING domains of PIAS1 and the MMS21 proteins from human (Hs), *S. cerevisiae* (Sc), and *S. pombe* (Sp). The conserved residues are shaded. The putative Zn^{2+}-coordinating cysteines and histidines are indicated by asterisks. Alignment was performed using ClustalW. (C) hMMS21 and hNSE1 interact with hSMC5. The pCS2-myc-hSMC5 plasmid was transfected into HeLa cells with or without cotransfection of plasmids encoding HA-tagged hMMS21 or hNSE1. Lysates of these cells were immunoprecipitated with anti-HA and blotted with anti-myc. The whole-cell lysates (WCL) were also blotted with anti-myc. IP, immunoprecipitation; WB, Western blot. (D) The hSMC5 antibody recognizes endogenous hSMC5 in HeLa cells. HeLa cells were transfected with mock siRNA or hSMC5 siRNA in the presence or absence of pCS2-myc-hSMC5. Lysates of these cells were blotted with anti-hSMC5 antibody. The asterisk indicates a nonspecific cross-reacting band that serves as a loading control. (E) The hMMS21 antibody recognizes endogenous hMMS21 in HeLa cells. HeLa cells were transfected with mock siRNA or hMMS21 siRNA for 48 h. Lysates of these cells were blotted with anti-hMMS21. The asterisk indicates a nonspecific cross-reacting band that serves as a loading control. (F) Endogenous hMMS21 interacts with hSMC5 in HeLa cells. HeLa cell lysates were immunoprecipitated with anti-GST, anti-BUBR1, or anti-hSMC5 and blotted with anti-hMMS21. Immunoglobulin G (IgG) heavy chain was used as a loading control. IP, immunoprecipitation; WCL, whole-cell lysate.

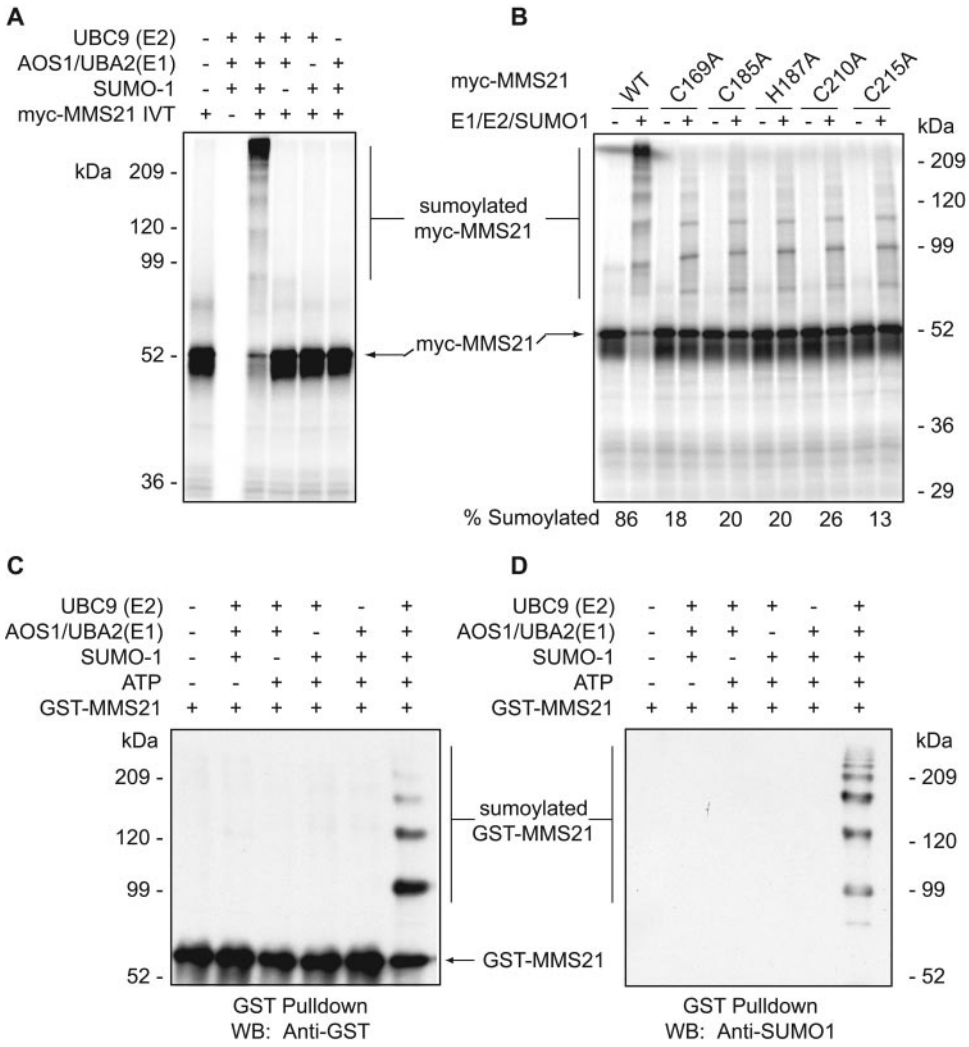

FIG. 2. hMMS21 is sumoylated in vitro. (A) hMMS21 is sumoylated in vitro. myc-hMMS21 was in vitro translated in the presence of [^{35}S]methionine and incubated with SUMO reaction mixtures containing the indicated components and analyzed by SDS-PAGE followed by autoradiography. (B) Sumoylation of hMMS21 in vitro requires an intact SP-RING domain. SUMO reactions were performed as in panel A using either wild type (WT) or the indicated mutants of hMMS21. The percentage of hMMS21 sumoylated was quantitated and is shown below the appropriate lanes. (C) hMMS21 is conjugated to SUMO1 in vitro. In vitro SUMO reactions were performed using GST-hMMS21 and the indicated components. GST-hMMS21 was pulled down using glutathione-agarose beads followed by SDS-PAGE and immunoblotting with anti-GST. (D) The reaction mixtures described in panel C were immunoblotted with anti-SUMO1. WB, Western blot.

ponents of the SUMO reactions were included (Fig. 2D). These results establish that hMMS21 is sumoylated in vitro and this sumoylation is dependent on an intact SP-RING domain.

We next tested whether hMMS21 was sumoylated in HeLa cells. myc-tagged hMMS21 was modified in the presence of untagged or green fluorescent protein (GFP)-tagged SUMO1 overexpression (Fig. 3A). As reported previously (10), GFP-SUMO1 was expressed at much higher levels and was thus conjugated more efficiently to substrates than untagged or endogenous SUMO1 (Fig. 3A). Modification of myc-hMMS21 was significantly reduced by expression of SENP2, a SUMO isopeptidase (Fig. 3A and B). In addition, modification of myc-hMMS21 was not observed with the overexpression of SUMO1 ΔGG, a SUMO mutant incapable of conjugation (18) (Fig. 3B). Finally, we performed immunoprecipitations of myc-hMMS21 in HeLa cells followed by immunoblotting with anti-SUMO1. Expectedly, the major modified form of hMMS21 contained SUMO1, indicating that hMMS21 was indeed sumoylated in HeLa cells (Fig. 3C). Furthermore, the myc-hMMS21 C215A mutant was sumoylated to a much lesser extent in HeLa cells, as was observed in vitro (Fig. 3B and C). These findings establish that hMMS21 is sumoylated in vivo and sumoylation of hMMS21 requires an intact SP-RING domain, consistent with hMMS21 being an SP-RING-type SUMO ligase.

hMMS21 enhances sumoylation of hSMC6 and TRAX. To formally prove that hMMS21 is a SUMO ligase, we need to show that hMMS21 can stimulate the sumoylation of target proteins other than itself. To do so, we tested whether purified recombinant GST-hMMS21 stimulated the sumoylation of the human SUMO substrates discovered in our in vitro expression cloning screen in an in vitro sumoylation assay (10). Among

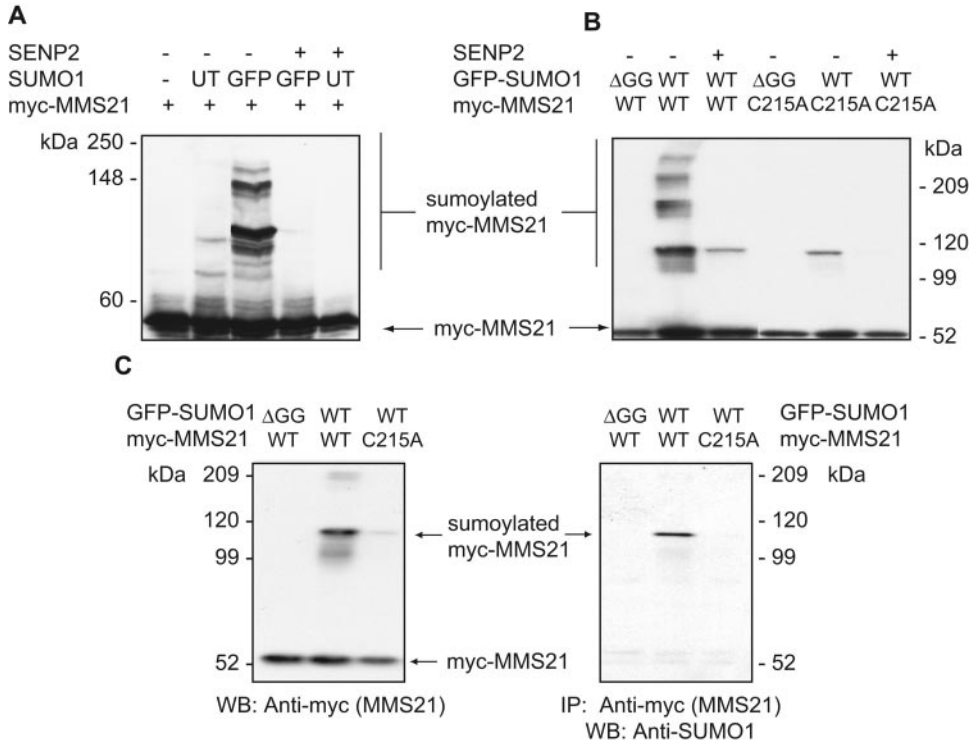

FIG. 3. hMMS21 is sumoylated in HeLa cells. (A) hMMS21 is sumoylated in vivo. myc-hMMS21 was expressed in HeLa cells with or without untagged (UT) or GFP-tagged (GFP) SUMO1 or the SUMO isopeptidase SENP2. Whole-cell lysates were blotted with anti-myc. (B) Sumoylation of hMMS21 in vivo requires an intact RING domain. The wild type (WT) or the C215A mutant of myc-hMMS21 was expressed in HeLa cells in the presence of WT or ΔGG mutant GFP-SUMO1 and with or without SENP2. Whole-cell lysates were blotted with anti-myc. (C) hMMS21 is conjugated to SUMO1 in HeLa cells. HeLa cells were transfected as described in panel B with either the wild type (WT) or the C215A mutant of myc-hMMS21 in the presence of WT or ΔGG mutant GFP-SUMO1. Whole-cell lysates were blotted with anti-myc (left panel) or immunoprecipitated with anti-myc followed by immunoblotting with anti-SUMO1 (right panel). IP, immunoprecipitation; WB, Western blot.

tens of substrates tested, only the sumoylation of TRAX was moderately (2.8- ± 0.2-fold over three separate experiments) enhanced by GST-hMMS21 (Fig. 4A and data not shown). As a control, GST-hMMS21 did not enhance the sumoylation of ETV1 (Fig. 4A). We next tested whether hMMS21 enhanced the sumoylation of TRAX and 10 candidate proteins involved in DNA damage/repair pathways that were potential targets of hMMS21 in vivo (Fig. 4B and data not shown). As expected, hMMS21 stimulated the sumoylation of TRAX in HeLa cells (Fig. 4B). Compared to the in vitro assay, sumoylation of TRAX was significantly enhanced by hMMS21 in HeLa cells. This could be due to the need for additional proteins from the SMC5/6 complex for the full activity of hMMS21 in vitro as was shown previously for *Saccharomyces cerevisiae* MMS21 (50). In addition, similar to the *Schizosaccharomyces pombe* NSE2, hMMS21 efficiently enhanced the sumoylation of both the full-length form (data not shown) and an alternatively spliced, N-terminally truncated hSMC6 in HeLa cells (Fig. 4B). Furthermore, the hMMS21 C215A mutant did not stimulate the sumoylation of TRAX or hSMC6 (Fig. 4B). To confirm that the high-molecular-weight species of hSMC6 and TRAX induced by the expression of hMMS21 were SUMO1 conjugates, we performed immunoprecipitation of myc-SMC6 (Fig. 4C) and myc-TRAX (data not shown), followed by immunoblotting using anti-SUMO1. We detected a high-molecular-weight ladder of SUMO1 conjugates when wild-type hMMS21, but not the hMMS21 C215A mutant, was coexpressed with hSMC6 and TRAX in HeLa cells (Fig. 4C and data not shown). These results confirm that hMMS21 enhances hSMC6 and TRAX sumoylation in HeLa cells. Therefore, hMMS21 is a SUMO ligase in vivo.

The SUMO ligase activity of hMMS21 is required for proper cellular DNA damage response. To examine the role of hMMS21 in DNA damage response, we transfected HeLa cells with siRNA against hMMS21 and then treated the cells with MMS. There was only a slightly higher incidence of cell death in hMMS21-RNAi cells in the absence of DNA damage (Fig. 5A), suggesting that a partial loss of hMMS21 did not cause cell lethality. In contrast, after exposure to a moderate dose of MMS (0.015%) for 6 h, about 80% of the hMMS21-RNAi cells had died while only 20% of the control cells were dead (Fig. 5A and B). Thus, compared to control cells, hMMS21-RNAi cells were much more sensitive to MMS. The hMMS21-RNAi cells were also more sensitive to etoposide (VP-16), a topoisomerase II inhibitor that induces DSBs (Fig. 5B). Therefore, consistent with studies in yeasts (1, 29, 50), hMMS21 is required for proper cellular responses to DNA damage induced by multiple agents.

We next attempted to rescue the phenotypes of hMMS21-RNAi cells with ectopic expression of either wild-type hMMS21 or its ligase-inactive mutant hMMS21 C215A. To avoid the knockdown of the ectopically expressed hMMS21

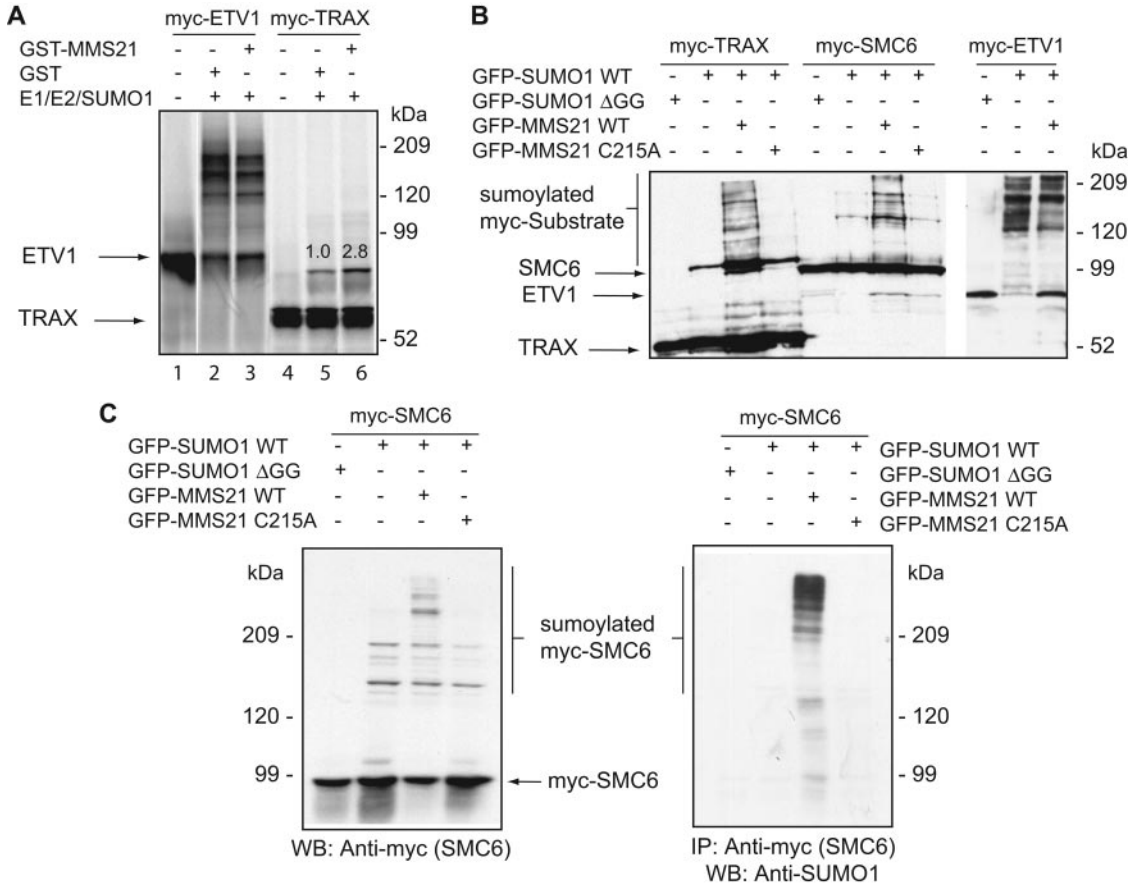

FIG. 4. hMMS21 is a SUMO ligase that stimulates sumoylation of hSMC6 and TRAX. (A) Recombinant GST-hMMS21 stimulates the sumoylation of TRAX, but not ETV1, in vitro. ETV1 and TRAX were in vitro translated in the presence of [^{35}S]methionine and subjected to SUMO reactions in the presence of GST or GST-hMMS21. The reactions were analyzed by SDS-PAGE followed by autoradiography. The fold increase of the monosumoylated TRAX in the presence of GST-hMMS21 is indicated. (B) Sumoylation of hSMC6 and TRAX, but not ETV1, is enhanced by hMMS21 in HeLa cells. myc-TRAX, myc-hSMC6, or myc-ETV1 was expressed with GFP-SUMO1 wild type (WT) or ΔGG in the presence or absence of wild-type or C215A mutant GFP-hMMS21 in HeLa cells. The whole-cell lysates were blotted with anti-myc. (C) hMMS21 stimulates SUMO1 conjugation to hSMC6 in HeLa cells. HeLa cells were transfected as described in panel B, and whole-cell lysates were either immunoblotted with anti-myc (left panel) or immunoprecipitated with anti-myc followed by immunoblotting with anti-SUMO1 (right panel). IP, immunoprecipitation; WB, Western blot.

proteins by RNAi, we first introduced silent mutations into the coding region of the hMMS21 gene that contains the sequence of the hMMS21 siRNA. Transfection of these hMMS21 plasmids restored the levels of hMMS21 protein in hMMS21-RNAi cells (Fig. 5D). Significantly, expression of the wild-type hMMS21 protein, but not hMMS21 C215A, largely rescued the cell death phenotype of hMMS21-RNAi cells following MMS treatment (Fig. 5C). This indicates that the ligase activity of hMMS21 is required for its function in DNA damage response.

To determine whether the increased cell death of hMMS21-RNAi cells following MMS treatment was due to apoptosis, we examined the nuclear morphology of these cells. As expected, after MMS treatment, hMMS21 RNAi caused an increased number of cells with hypercondensed nuclei (data not shown), a hallmark of apoptotic cells. The number of cells with condensed nuclei was greatly reduced by expression of the wild-type hMMS21 but not the ligase-inactive mutant hMMS21 C215A (data not shown). To further confirm that the observed cell death was indeed apoptosis, we examined the cleavage of a known caspase 3 substrate, PARP. PARP cleavage was observed in hMMS21-RNAi cells treated with MMS (Fig. 5D). Cleavage of PARP was prevented by expression of the wild-type hMMS21, but not hMMS21 C215A, in hMMS21-RNAi cells (Fig. 5D). These results indicate that hMMS21-RNAi cells are more prone to undergo apoptosis in the presence of DNA damage, and the SUMO ligase activity of hMMS21 is required for preventing DNA damage-induced apoptosis.

RNAi-mediated depletion of hMMS21 results in hyperactivation of ATM/ATR following DNA damage. ATM and ATR are related protein kinases that are activated upon DNA damage and phosphorylate many cellular targets at Ser/Thr-Gln sites (22). To determine whether hMMS21 is required for the DNA damage response through ATM/ATR, we stained control and hMMS21-RNAi cells with an antibody that specifically recognizes phosphorylated Ser/Thr-Gln motifs (5) (Fig. 6A). The intensity of the antibody staining was quantified and used as a measure for the activity of ATM/ATR (5) (Fig. 6B). As expected, the level of phosphorylated ATM/ATR substrates

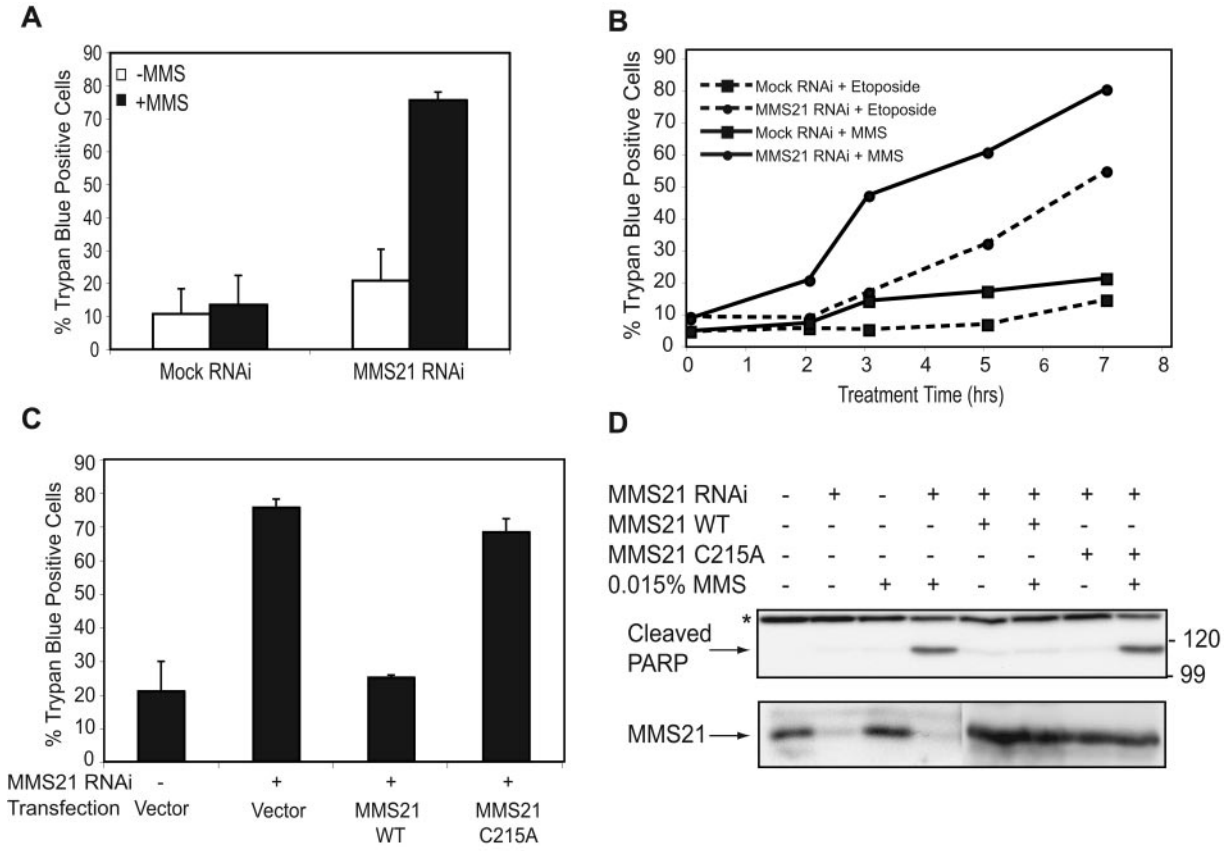

FIG. 5. The SUMO ligase activity of hMMS21 is required to block DNA damage-induced apoptosis. (A) hMMS21-RNAi cells are more sensitive to MMS. HeLa cells were transfected with mock or hMMS21 siRNA for 48 h and then either untreated or treated with 0.015% MMS for 6 h. The percentage of dead cells (trypan blue positive) is plotted. Results from three separate experiments are averaged with the standard deviation indicated. (B) Time course of cell death after MMS and etoposide treatment. Control (squares) or hMMS21-RNAi (circles) cells were treated with 0.015% MMS (solid lines) or 40 μg/ml etoposide (dashed lines) for the indicated times, and the percentage of dead cells was determined by trypan blue exclusion. (C) The wild-type (WT) hMMS21, but not its ligase-inactive C215A mutant, rescues the sensitivity of hMMS21-RNAi cells to MMS. Cells were transfected with mock or hMMS21 siRNA for 24 h and then transfected with either empty vector or plasmids encoding the wild type or the C215A mutant of myc-hMMS21. After 24 h, cells were treated with 0.015% MMS for 6 h. The percentage of dead cells (trypan blue positive) is plotted. Results from three separate experiments are averaged with the standard deviation indicated. (D) Whole-cell lysates of cells from panel C were blotted with antibodies against cleaved PARP or anti-hMMS21. The asterisk indicates a nonspecific cross-reacting band that serves as a loading control.

increased upon DNA damage (Fig. 6A and B). In the absence of MMS, the basal level of ATM/ATR-mediated phosphorylation was higher in hMMS21-RNAi cells. After DNA damage, the hMMS21-RNAi cells contained threefold-more phosphorylated ATM/ATR substrates than did control cells (Fig. 6A and B).

CHK2 is a key substrate of ATM and ATR (20). Phosphorylation of CHK2 at Thr 68 after DNA damage requires ATM and ATR (30). Phosphorylated T68 CHK2 forms nuclear foci, presumably at the sites of DNA damage (48). We thus stained control and hMMS21-RNAi cells with a phospho-CHK2 (T68) antibody (Fig. 6C). Compared to control cells, the hMMS21-RNAi cells contained an increased number of phospho-CHK2 nuclear foci after treatment with MMS (Fig. 6C). The mean number of foci in control cells was around eight or nine per cell while the mean number of foci in hMMS21-RNAi cells was about 15 or 16 per cell (Fig. 6D). Our data suggest that hMMS21 is not required for ATM/ATR-dependent DNA damage checkpoint response. Instead, consistent with hMMS21 playing a role in DNA repair, knockdown of hMMS21 might lead to defective DNA repair and an increased amount of unrepaired DNA lesions following DNA damage, thus increasing the amount of ATM/ATR activity and the number of phospho-CHK2 foci.

Knockdown of hMMS21 decreases repair of damaged DNA. To determine whether knockdown of hMMS21 resulted in an increased amount of unrepaired DNA lesions following DNA damage, we performed a single-cell gel electrophoresis assay (comet assay) (4). The comet assay is simple and widely used to evaluate the amount of DNA damage in cells. It is based on the ability of denatured, cleaved DNA fragments to migrate out of the cell when current is applied, whereas undamaged DNA migrates slower and remains within the confines of the nucleus (4).

We employed this assay to measure the amount of unrepaired DNA damage in control or hMMS21-RNAi cells after MMS treatment. As expected, control and hMMS21-RNAi cells had no comet tails without treatment of MMS (Fig. 7A).

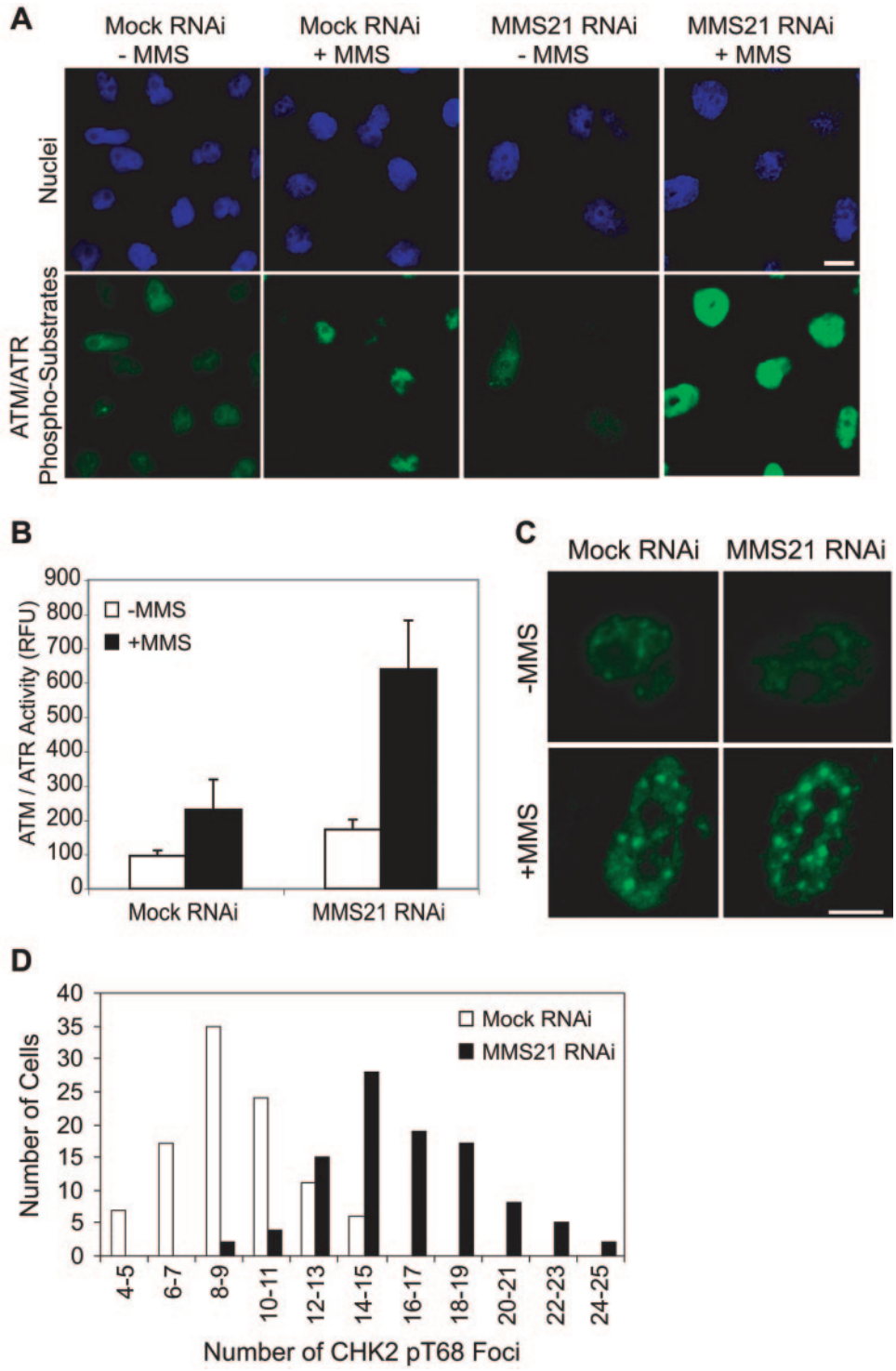

FIG. 6. ATM/ATR are hyperactivated in hMMS21-RNAi cells after DNA damage. (A) HeLa cells were transfected with either mock or hMMS21 siRNA for 48 h and were either untreated or treated with 0.015% MMS for 4 h. Cells were fixed and stained with Hoechst 33258 (blue) or a phosphospecific antibody against ATM/ATR phosphorylation sites on its substrates (green). Bar, 5 μm. (B) Quantitation of the fluorescence intensities of the ATM/ATR phosphosubstrate staining in panel A. Results from three separate experiments are averaged with the standard deviation indicated. (C) Cells treated as in panel A were stained with a phospho-T68 CHK2 antibody (green). Bar, 5 μm. (D) Histogram of cells in panel C with the indicated number of phospho-CHK2 foci.

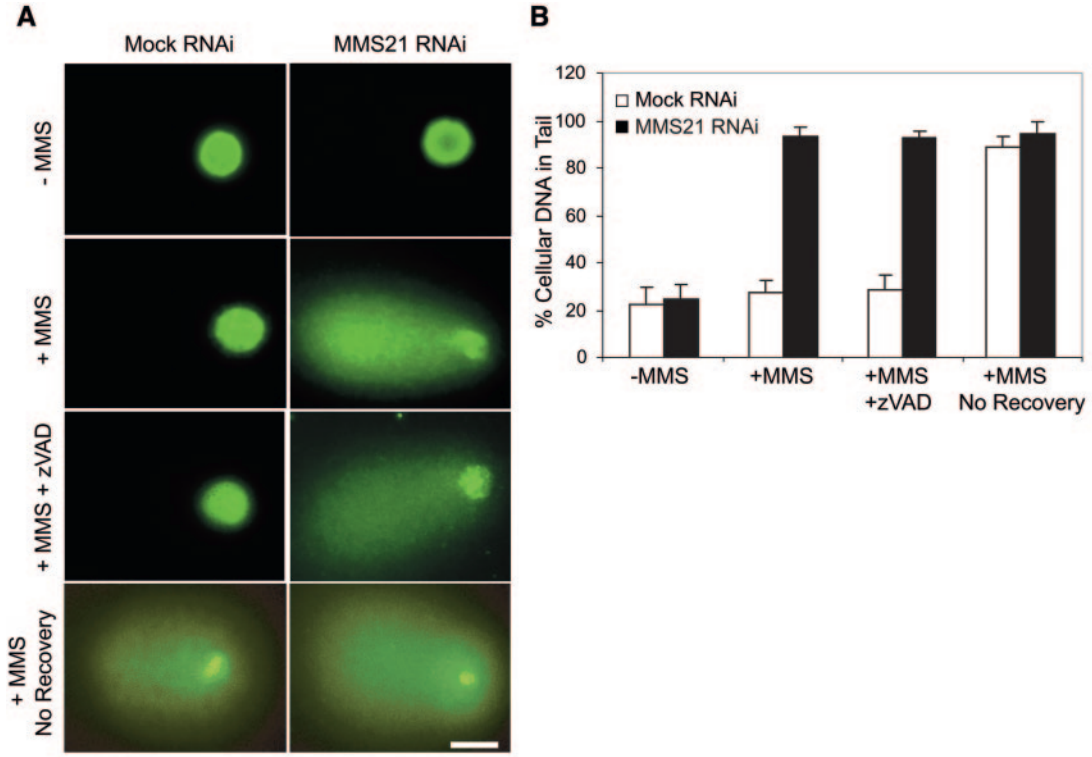

FIG. 7. hMMS21 is required for efficient repair of damaged DNA. (A) HeLa cells were transfected with mock or hMMS21 siRNA and treated with 0.015% MMS for 0 h (−MMS), with MMS for 1 h followed by a 3-h recovery period without MMS (+MMS), with MMS for 1 h with 3 h of recovery in the presence of the pan-caspase inhibitor zVAD-fmk (+MMS + zVAD), or with MMS for 1 h without recovery (+MMS No Recovery). Cells were collected and analyzed for unrepaired DNA lesions by the comet assay. SYBR green staining of DNA shows comet tails migrating out of the nucleus. Bar, 10 μm. (B) Quantitation of fluorescence intensities of cells in panel A to measure the percentage of DNA migrating out of the nucleus into tails. Results from three separate experiments are averaged with the standard deviation indicated.

To measure the ability of cells to repair damaged DNA, we treated cells with 0.015% MMS for 1 h and then washed out MMS and allowed cells to repair the damaged DNA for 3 h. The hMMS21-RNAi cells, unlike control cells, were incapable of repairing their DNA lesions (Fig. 7A). The amount of DNA that migrates out of the nucleus and forms a comet tail provides a quantitative measure of the amount of DNA damage in the cell (4). Quantitation of the amount of DNA in the comet tails showed a dramatic increase in the amount of unrepaired DNA lesions in the hMMS21-RNAi cells (95%) compared to control cells (25%) (Fig. 7B). To rule out the possibility that the increase in the amount of unrepaired DNA lesions was due simply to an increased number of apoptotic cells in the hMMS21-RNAi culture, we performed the experiment in the presence of a pan-caspase inhibitor, zVAD-fmk. Although zVAD-fmk could inhibit MMS-induced cell death in hMMS21-RNAi cells (see Fig. S2 in the supplemental material), addition of zVAD-fmk had no effect on the amount of unrepaired DNA present in hMMS21-RNAi cells (Fig. 7A and B). Finally, to rule out the possibility that hMMS21-RNAi cells were more susceptible to DNA damage than control cells, we treated cells with 0.015% MMS for 1 h without a period for repair. Treatment of both control and hMMS21-RNAi cells with MMS without recovery resulted in comet tails with similar, large amounts of DNA damage lesions (Fig. 7A and B). These results suggest that knockdown of hMMS21 causes a decreased ability of cells to repair DNA lesions.

DISCUSSION

Studies of yeasts have established a role for a chromatin-bound protein complex, containing SMC5, SMC6, MMS21, and other non-SMC proteins, in DNA repair. Here we show that, similarly to yeast MMS21, human MMS21 is a functional SUMO ligase. The SUMO ligase activity of hMMS21 is required for the prevention of apoptosis following DNA damage. We present evidence to further suggest that hMMS21 is required for efficient DNA damage repair.

The SUMO ligase activity of MMS21. Studies of both *S. cerevisiae* MMS21 and *S. pombe* NSE2 have identified MMS21 as a SUMO ligase that autosumoylates. Our results concur with these previous findings and show that hMMS21 is a SUMO ligase that undergoes autosumoylation in vitro and in vivo. The *S. cerevisiae* MMS21 has been shown to stimulate the sumoylation of two substrate proteins, SMC5 and Ku70 (50). The *S. pombe* NSE2 stimulates the sumoylation of SMC6 and NSE3, but not SMC5 or NSE1 (1). Consistent with the results in *S. pombe*, we show that hMMS21 enhances sumoylation of hSMC6. In addition, we also identify TRAX as a substrate of hMMS21. The differences with respect to MMS21-mediated

sumoylation of SMC5/6 in *S. pombe*, *S. cerevisiae*, and *Homo sapiens* could reflect interesting differences in the mechanism and regulation of the SMC5/6 complex in these organisms.

In fission yeast, SMC6 sumoylation is dependent on NSE2, indicating that SMC6 is a physiologically relevant substrate of NSE2 (1). Like many SUMO substrates in mammalian cells, we were unable to detect the sumoylation of SMC6 or TRAX in the absence of SUMO1 overexpression, presumably due to the low steady-state levels of SUMO conjugates of these proteins. Upon overexpression of SUMO1, the SUMO1 conjugates of SMC6 and TRAX were observed in HeLa cells. Overexpression of hMMS21 further enhanced the sumoylation of SMC6 and TRAX. However, we did not detect a decrease in the sumoylation of SMC6 or TRAX in hMMS21-RNAi cells. This could be due to the incomplete knockdown of hMMS21 by RNAi. Alternatively, sumoylation of SMC6 and TRAX in the presence of SUMO1 overexpression does not strictly require hMMS21. It is also possible that other SUMO ligases can mediate the sumoylation of hSMC6 and TRAX under these conditions. Therefore, our results indicate that hMMS21 is capable of stimulating the sumoylation of SMC6 and TRAX in living cells. It remains to be established whether and how sumoylation of SMC6 and TRAX is important for DNA repair in human cells.

Role of MMS21 and the SMC5/6 complex in DNA repair. Down-regulation of the SMC5/6 complex in yeasts and in mammalian cells (this study) renders cells more sensitive to a wide spectrum of DNA-damaging agents (7, 8, 17, 24, 29, 34, 35, 38, 39). It is possible that the SMC5/6 complex is directly involved in the repair of multiple types of DNA lesions. On the other hand, components of the SMC5/6 complex are epistatic with RAD51 and RAD52, suggesting that the SMC5/6 complex may function in the repair of DSB through the RAD51- and RAD52-dependent HR pathway (1, 29, 32, 34, 35). Because many types of DNA-damaging agents can indirectly lead to the generation of DSBs, it is also possible that a major function of the SMC5/6 complex is the repair of DSB through HR.

In addition to DNA repair, cohesin and condensin play roles in the DNA damage checkpoint that delays cell cycle progression to allow time for DNA repair (11). Though the SMC5/6 complex is not required for the initiation of the DNA damage checkpoint response in *S. pombe*, it is involved in the maintenance of the checkpoint (13). In this study, we show that ATM/ATR are activated to a greater extent in hMMS21-RNAi cells, which is more consistent with SMC5/6 playing a role in DNA repair. On the other hand, hMMS21 RNAi does not completely deplete the cellular pool of the hMMS21 protein. It is possible that the residual amount of hMMS21, though insufficient for DNA repair, is sufficient for DNA damage checkpoint signaling. In addition, hMMS21-RNAi cells might be defective in checkpoint pathways that do not involve ATM/ATR.

Role of sumoylation in DNA repair. Through its reversible, covalent modification of target proteins, SUMO regulates diverse cellular processes, including transcription, chromatin remodeling, and DNA repair (9). Several proteins involved in DNA repair are SUMO substrates, including PCNA, WRN, XRCC1, KU70, KU80, and TRAX (9, 10, 50). We show that a subunit of the hSMC5/6 complex, hMMS21, is a functional SUMO ligase. Furthermore, the SUMO ligase activity of hMMS21 is essential for its function in protecting cells from apoptosis induced by DNA damage, thus further establishing a role of sumoylation in DNA repair. At present, we do not know the mechanism by which the ligase activity of MMS21 regulates DNA repair. However, we envision two nonexclusive possibilities. First, hMMS21 might stimulate the sumoylation of other DNA damage checkpoint and repair proteins, such as TRAX, and mediate their recruitment to the sites of DNA damage. Consistent with this notion, sumoylation has been shown to alter the subcellular localization and/or kinetics of nucleocytoplasmic trafficking of certain target proteins (9, 18). However, we did not observe failure in the recruitment of DNA damage checkpoint and repair proteins, such as BRCA1, BRCA2, CHK1, CHK2, or RAD51, to nuclear foci after DNA damage in hMMS21-RNAi cells (Fig. 6C and data not shown). In a second possibility, sumoylation of SMC6 by MMS21 might regulate its ATPase activity, thus affecting the loading and unloading of the SMC5/6 complex onto chromatin. It will be interesting to examine the possibility that SMC5/6 loading and unloading are required for DNA repair and how the SUMO ligase activity of hMMS21 may regulate this process during the cellular DNA damage response.

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