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Rap80 Protein Recruitment to DNA Double-strand Breaks Requires Binding to Both Small Ubiquitin-like Modifier (SUMO) and Ubiquitin Conjugates*[S]

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Background: Ubiquitin (Ub) and small ubiquitin-like modifier (SUMO) conjugation occurs at DNA double-strand breaks (DSBs).
Results: Rap80, a component of the BRCA1-A complex, binds to both SUMO and Ub conjugates.
Conclusion: Rap80 binding to both SUMO and Ub conjugates is required for proper cellular response to DSBs.
Significance: This work provides insights into how Rap80 and BRCA1 are recruited to DSBs to maintain genome stability.

Ubiquitin (Ub) modifications at sites of DNA double-strand breaks (DSBs) play critical roles in the assembly of signaling and repair proteins. The Ub-interacting motif (UIM) domain of Rap80, which is a component of the BRCA1-A complex, interacts with Ub Lys-63 linkage conjugates and mediates the recruitment of BRCA1 to DSBs. Small ubiquitin-like modifier (SUMO) conjugation also occurs at DSBs and promotes Ub-dependent recruitment of BRCA1, but its molecular basis is not clear. In this study, we identified that Rap80 possesses a SUMO-interacting motif (SIM), capable of binding specifically to SUMO2/3 conjugates, and forms a tandem SIM-UIM-UIM motif at its N terminus. The SIM-UIM-UIM motif binds to both Ub Lys-63 linkage and SUMO2 conjugates. Both the SIM and UIM domains are required for efficient recruitment of Rap80 to DSBs immediately after damage and confer cellular resistance to ionizing radiation. These findings propose a model in which SUMO and Ub modification is coordinated to recruit Rap80 and BRCA1 to DNA damage sites.

Modification of proteins by the covalent attachment of ubiquitin (Ub)[3] or small ubiquitin-like modifier (SUMO) to the Lys residue of a target protein is involved in regulatory mechanisms of many cellular processes, including the DNA damage response (DDR) (1–4). Ub modification at DNA double-strand breaks (DSBs) occurs upon detection of DNA damage and activation of the DDR kinases ATM/ATR (5–7). ATM/ATR phosphorylation at Ser-139 of histone H2AX directly recruits MDC1 through the MDC1 BRCT (BRCA1 C terminus) domains. The subsequent MDC1 phosphorylation-dependent recruitment of ubiquitin ligases RNF8 and RNF168, together with a ubiquitin E2 conjugase (UBC13), generates Ub Lys-63 linkage chains on the damaged chromatin. In addition, a HECT domain-containing E3 ligase (HERC2) is also involved in facilitating the formation of Lys-63-linked Ub conjugates. Ub Lys-63 linkage polyubiquitin chain formation plays important roles in the recruitment of repair factors, including 53BP1 and BRCA1 (5–7).

The breast and ovarian tumor suppressor BRCA1 plays critical roles in the DDR, regulating multiple repair and checkpoint mechanisms for maintaining genome stability. Through its C-terminal BRCT domains, BRCA1 forms at least three different complexes, the BRCA1-A, BRCA1-B, and BRCA1-C complexes, by binding to Abraxas (Abra1), Bach1, and CtIP, respectively (5, 7, 8). Although all three complexes have been indicated in the role of BRCA1 in cell cycle checkpoint control and DNA repair, the BRCA1-A complex is known to target BRCA1 to DNA damage sites in response to DNA damage-induced Ub modification. The BRCA1-A complex contains at least five different components: Abraxas, NBA1/MERIT40, BRE, Rap80, and BRCC36 (8). Rap80 contains two ubiquitin-interacting motif (UIM) domains that display a binding specificity toward Ub Lys-63 linkage chains generated through RNF8/RNF168 E3 ligases at sites of damage. The BRCA1-A complex associates with BRCA1 through interaction of phosphorylated Abraxas with the BRCA1 C-terminal BRCT domains (8–11). It appears that the integrity of the BRCA1 complex is also important for the recruitment of BRCA1 (12). Down-regulation of each component of this complex compromises the recruitment of BRCA1 to DNA damage sites, leading to increased cellular sensitivity to ionizing radiation (IR) and inability of cells to arrest the cell cycle.

Recent findings demonstrate that SUMO ligases PIAS1 and PIAS4, as well as the SUMO-conjugating enzyme UBC9, are also recruited to DSBs at a relatively early step in the DDR (13, 14). Mammalian cells express three SUMO paralogs that can be

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[3] The abbreviations used are: Ub, ubiquitin; SUMO, small ubiquitin-like modifier; DDR, DNA damage response; DSB, double-strand break; UIM, ubiquitin-interacting motif; IR, ionizing radiation; SIM, SUMO-interacting motif; MEF, mouse embryonic fibroblast.

conjugated to target proteins: SUMO1, SUMO2, and SUMO3 (15). SUMO2 and SUMO3 are nearly identical and are assumed to be largely redundant in their functions. Similar to Ub, SUMO2/3 can be conjugated to substrates in chains (polysumoylation) (16). SUMO1 is 45% identical to SUMO2/3 but, by contrast, does not form chains efficiently (16); it might serve, however, as terminator of SUMO2/3 chains (17). SUMO1 and SUMO2/3 accumulate at DSB sites in mammalian cells (13, 14). The SUMO E3 ligases PIAS1 and PIAS4 modify DNA repair and signaling proteins such as 53BP1 and BRCA1 through SUMO1 or SUMO2/3 conjugation (13, 14). It has been shown that sumoylation facilitates the recruitment of 53BP1 and BRCA1 to DNA repair foci, but the molecular basis is not clear. SUMO recognition is mediated by a short conserved SUMO-interacting motif (SIM). SIMs are composed of short stretches of hydrophobic residues that directly engage the SUMO molecule (18, 19).

In this study, we identified that Rap80 possesses a SIM domain forming a tandem SIM-UIM-UIM motif at its N terminus. The Rap80 SIM domain binds specifically to SUMO2/3. Both the SIM and UIM domains play important roles in recruiting Rap80 to DNA damage sites and confer cellular sensitivity to IR.

EXPERIMENTAL PROCEDURES

Plasmids, siRNAs, and Antibodies—Retroviral expression constructs for GFP-tagged wild-type and mutant Rap80 were made using murine stem cell virus (MSCV) vectors containing a GFP tag at the N terminus as described (9, 20). Deletion mutants of Rap80 were either as described previously (9, 20) or generated by cloning the corresponding cDNA fragments into the above retroviral vector. Site-directed mutagenesis was performed with the QuikChange II site-directed mutagenesis kit (Stratagene) to generate various point mutants of Rap80. GST-tagged or His-tagged SUMO2 and wild-type or mutant Rap80(1–129) were generated with pDEST15 (GST) and pDEST17 (His) vectors (Invitrogen) via the Gateway recombination system. siRNAs used for knocking down Rap80, Abraxas, BRE, and BRCC36 were described previously (9, 20, 21). Ub Lys-63 2–7 and SUMO2 3–8 chains, as well as agarose-SUMO2 beads, were purchased from Boston Biochem. Rabbit anti-NBA1, anti-BRE, and anti-Abraxas antibodies were generated as described previously (9, 12, 21). Other antibodies used were anti-BRCC36 (ProSci Inc.), anti-Rap80 (Bethyl Laboratories), anti-BRCA1 (D9) and anti-Ub (P4D1) (Santa Cruz Biotechnology), anti-GFP (Invitrogen), rabbit anti-Myc (Sigma), mouse anti-HA (Covance), anti-SUMO2/3 and anti-GST (Cell Signaling), and anti-γH2AX (Upstate).

Cell Lines and Cell Culture—U2OS cells were grown in McCoy's 5A medium supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin. 293T cells and Rap80$^{-/-}$ mouse embryonic fibroblast (MEF) cells were grown in DMEM supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin. Stable cells lines were generated by infecting U2OS, 293T, or Rap80$^{-/-}$ MEF cells with retrovirus containing various GFP-tagged proteins, followed by selection with puromycin.

Cell Lysis and Immunoprecipitation—Cells were lysed in NETN buffer (0.15 M NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 0.5% Nonidet P-40) with protease and protein phosphatase inhibitors, 1 mM NaF, 1 mM Na_3VO_4, and 10 mM *N*-ethylmaleimide. Immunoprecipitations were carried out in the same buffer with appropriate antibodies and protein A/G-Sepharose beads (Santa Cruz Biotechnology) overnight at 4 °C. FLAG immunoprecipitation was carried out using FLAG (M2) beads (Sigma).

GST-SUMO2 Pulldown and Mass Spectrometry Analysis of SUMO2-binding Proteins—GST-tagged SUMO2 fragments were expressed from the pDEST15 expression vector in *Escherichia coli* DE3 cells (Invitrogen) and purified using glutathione-Sepharose beads (Amersham Biosciences). 293T cells were treated or not treated with 10 grays of IR, followed by a 2-h incubation at 37 °C before harvesting, and cell lysates were prepared as described above. *In vitro* pulldown assay was performed with purified GST-SUMO2 (50 μg) incubated overnight at 4 °C with cell lysates (20 mg of total protein) prepared as described above. Associated proteins were eluted from the beads and separated on SDS-polyacrylamide gel. Proteins eluted from the gel slices were then analyzed by mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School).

Pulldown Assays—GST- or His-tagged proteins were expressed in *E. coli* DE3 cells and purified using glutathione-Sepharose or TALON metal affinity resin (Clontech) according to the manufacturer's instructions. For pulldown assay with cell lysate, purified protein fragments on beads were incubated with cell lysates overnight at 4 °C. Beads were then collected by centrifugation and washed five times with NETN buffer before suspension in 1× SDS loading buffer for gel separation and subsequent immunoblotting with various antibodies. For binding assay with the Ub Lys-63 or SUMO2 chain, purified protein fragments on beads were incubated overnight at 4 °C with the Ub Lys-63 or SUMO2 chain in a 0.5-ml total volume of NETN buffer. Beads were then collected and washed five times with NETN buffer before suspension in 1× SDS loading buffer for gel separation.

Agarose-SUMO2 Pulldown Assay of the GST-SIM-UIM-UIM Fragment and Ub Lys-63 2–7 Chain Conjugates—The wild-type or mutant GST-SIM-UIM-UIM fragment was expressed and purified using glutathione-Sepharose and eluted in elution buffer (100 mM Tris, 100 mM NaCl, 5% glycerol, and 40 mM glutathione). The agarose-SUMO2 beads (50 μg of SUMO2) were first blocked in NETN buffer with 0.5% BSA for 3 h and then incubated with 10 μg of purified GST-tagged Rap80(1–129) (GST-SIM-UIM-UIM) in a 0.4-ml total volume of NETN buffer for 1 h. Beads were collected and washed five times with NETN buffer. The beads were then incubated with 300 ng of Ub Lys-63 2–7 chain in 0.4 ml of NETN buffer for 1 h at 4 °C. After five washes with NETN buffer, the beads were suspended in 1× SDS loading buffer for gel separation and immunoblotting.

Colony Formation Assay—The assay was performed as described previously (12). Briefly, MEF Rap80$^{-/-}$ stable cell lines were seeded at low density and irradiated with 5 or 10 grays of IR using a ^{137}Cs radiation source. The cells were then incubated at 37 °C for 14 days to allow colonies to form. Colo-

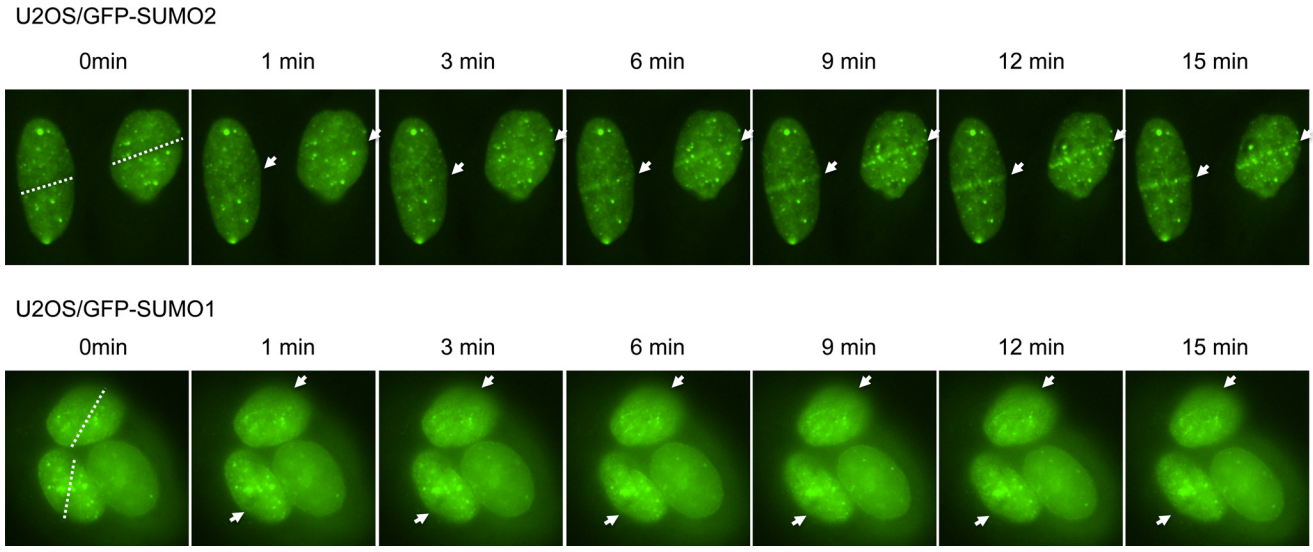

FIGURE 1. **GFP-SUMO2 is recruited to DNA damage sites immediately after damage.** U2OS cells stably expressing GFP-SUMO1 or GFP-SUMO2 were treated with a UV laser. Live cell imaging was performed immediately after laser treatment at room temperature. Images were taken at 30-s intervals for 15 min. Images from various time points are shown. Live cell imaging videos are provided in supplemental Videos 1 and 2.

nies were stained with 2% methylene blue and 50% ethanol. Colonies containing 50 or more cells were counted, and statistical data were analyzed by Student's *t* test.

Laser-induced DNA Damage and Live Cell Imaging—Cells were treated with 10 μM BrdU (BD Biosciences) for 24 h prior to laser irradiation on a Nikon TE2000 inverted microscope integrated with a MicroPoint laser system. Nuclei were irradiated with a UV laser (364 nm) with five pulses (total of 335 ms). A 60× water lens was used for the operation. The laser energy output was set to 23%. Cells were either fixed for immunostaining at the indicated times or monitored by live cell imaging. For live cell imaging, images were captured immediately after laser microirradiation at 30-s intervals. The total time course lasted for 15 or 30 min.

Immunofluorescence—Cells grown on coverslips were fixed with 3.6% formaldehyde for 15 min, permeabilized with 0.5% Triton X-100 solution, and incubated with primary antibodies at 37 °C for 2 h, followed by appropriate Alexa 488-conjugated (green; Invitrogen) and Cy3-conjugated (red; Amersham Biosciences) secondary antibodies. All images were obtained with a Nikon TE2000 inverted microscope with a Photometrics CoolSNAP *HQ* camera.

RESULTS

SUMO2/3 Modification Occurs in Response to DNA Damage—Involvement of the SUMO pathway in the DDR has been reported previously (2, 3, 13, 14). It has been demonstrated that SUMO1 and SUMO2/3 conjugates accumulate at DSBs (13, 14). To compare the accumulation of SUMO1 and SUMO2/3 conjugates at DSBs, we employed laser microirradiation to induce DNA damage in living cells stably expressing GFP-SUMO1 or GFP-SUMO2. Live cells were monitored for GFP-tagged SUMO1 or SUMO2 accumulation at the laser track. We observed that although GFP-SUMO1 accumulation at the laser track was not apparent up to 15 min after DNA damage, GFP-tagged SUMO2 appeared to accumulate at the laser track immediately after laser treatment. The fluorescence intensity reached a maximum level after a few minutes and persisted for the duration of monitoring (up to 15 min) (Fig. 1 and supplemental Videos 1 and 2).

Rap80 Mediates Binding of the BRCA1-A Complex to SUMO2/3 Conjugates—We then decided to identify proteins that are recruited to DNA damage sites through binding to SUMO2/3 conjugates. Previously, it was indicated that conjugation of SUMO2/3, but not that of SUMO1, is stimulated by cellular stresses such as exposure to heat shock (22). In a proteomic analysis of proteins that associate with GST-SUMO2 in response to DNA damage using mass spectrometry analysis of GST-SUMO2 pulldown proteins, we found that components of the BRCA1-A complex, as well as BRCA1, were among the proteins that associated with GST-SUMO2 (Fig. 2*A* and supplemental Table 1). The BRCA1-A complex contains at least five different components: Abraxas, Rap80, BRE, BRCC36, and NBA1. We then confirmed the interaction of these proteins with purified GST-SUMO2 or His-SUMO2 in *in vitro* pulldown assays with cell lysates (Fig. 2, *B* and *C*).

Interestingly, we found that binding of the BRCA1-A complex with SUMO2 was likely to be mediated by Rap80 (Fig. 2*D*). In an *in vitro* pulldown assay, the binding of Abraxas to purified His-SUMO2 was significantly decreased using lysates of cells treated with Rap80 siRNAs compared with that of cells treated with control siRNA. When any of the other components of the BRCA1-A complex was depleted by siRNAs, the binding of Rap80 to His-SUMO2 was not affected in the *in vitro* pulldown assay (supplemental Fig. 1).

Rap80 Contains a SIM—Because it appears that Rap80 mediates the association of the BRCA1-A complex with SUMO2/3 conjugates, this promoted us to investigate which region of Rap80 binds to SUMO2/3. We generated deletion mutants of Rap80 and tested the binding of these mutants to purified GST-SUMO2. GFP-tagged Rap80 or deletion mutants were transiently expressed in cells. Lysates from these cells were then incubated with purified GST-SUMO2 beads for pulldown

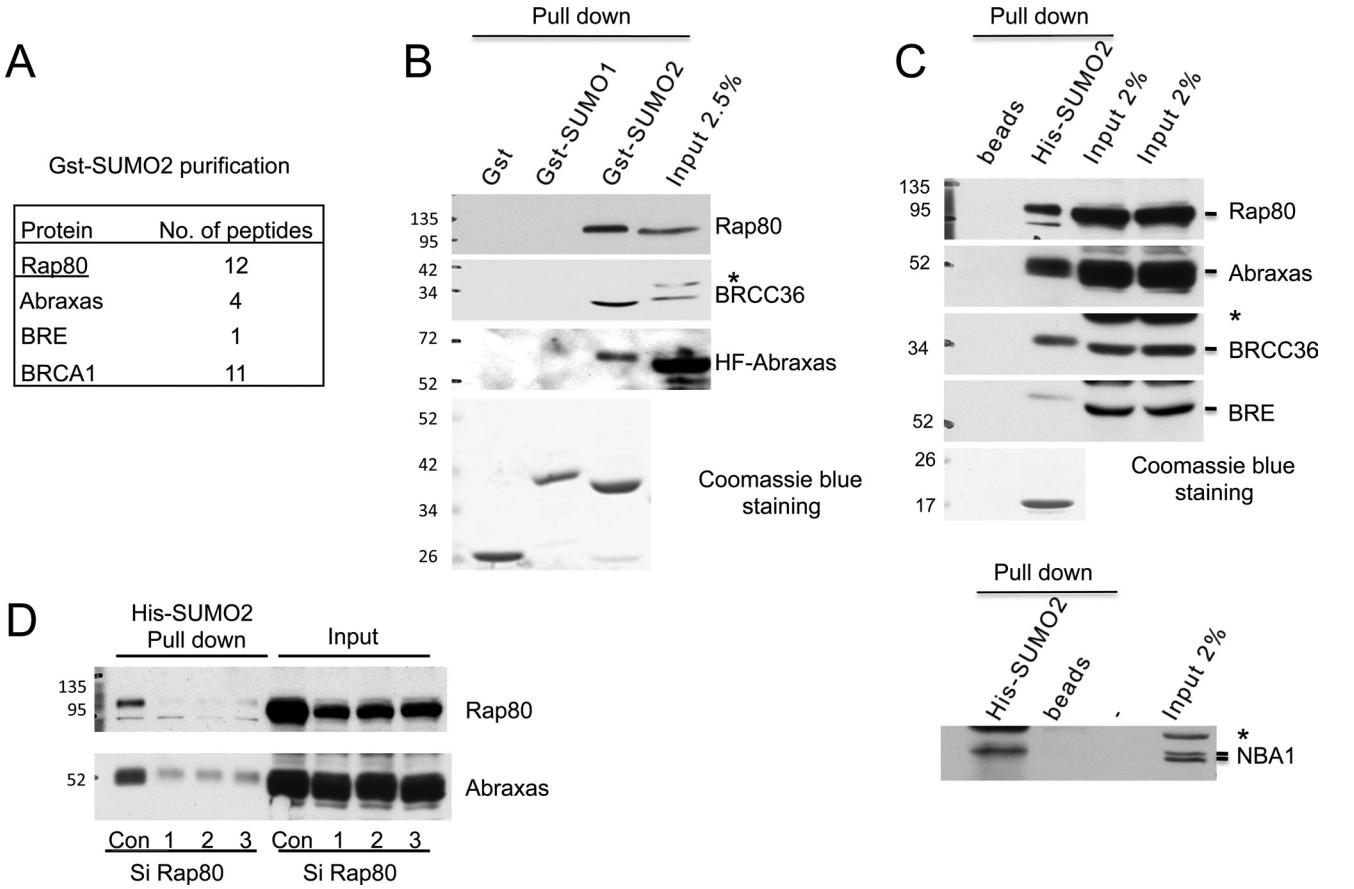

FIGURE 2. **Rap80 mediates binding of the BRCA1-A complex to SUMO2.** *A*, mass spectrometry analysis of GST-SUMO2-binding proteins identified Rap80 and other components of the BRCA1-A complex. The number of peptides identified is listed. *B*, GST-SUMO2 binds to BRCA1-A complex components Rap80, Abraxas, and BRCC36. Protein fragments of GST, GST-SUMO1, and GST-SUMO2 were purified from bacteria. The purified GST and GST-tagged proteins (10 μg) on beads were incubated with lysates of 293T cells or 293T cells expressing HA-FLAG (*HF*)-tagged Abraxas for pulldown assay. Western blotting was carried out with various antibodies. The *asterisk* indicates a nonspecific band. *C*, purified His-SUMO2 binds to the BRCA1-A complex component proteins. His-SUMO2 was purified from bacteria expressing His-SUMO2. Purified His-SUMO2 (10 μg) on beads was used. Empty beads were used as a control. 2% input was included in the blot. The *asterisk* indicates a nonspecific band. *D*, Rap80 mediates binding of the BRCA1-A complex to GST-SUMO2. 293T cells were transfected with siRNAs against Rap80 (*Si Rap80*). 48 h after transfection, cells were lysed, and total lysates were used for incubation with purified GST-SUMO2 (10 μg) on beads for pulldown assay.

assays. We found that a fragment of Rap80 corresponding to amino 1–129 acids and containing two UIM domains appeared to be sufficient for binding to GST-SUMO2 in pulldown assays, whereas other regions of Rap80 were not required (Fig. 3). In addition, although UIM domains were not required for binding, a region corresponding to amino acids 1–50 of Rap80 was required (Fig. 3). We analyzed the protein sequence of this region and found that Rap80 contains a SIM domain that is next to the two UIM domains (Fig. 3). SIMs are composed of short stretches of hydrophobic residues that directly engage the SUMO molecule (18, 19). We aligned the SIM domain sequence of Rap80 across various species and with two well known SIM domain sequences from human DAXX and PML proteins (Fig. 3*C*). We then mutated the first two hydrophobic residues (F40A/I41A), as well as the two conserved serine residues (S44A/S46A), to alanine. Phosphorylation of these two conserved serine residues in the DAXX SIM motif appears to increase the SIM binding affinity for SUMO (23). GFP-tagged mutants of Rap80 were then transiently expressed in cells for GST-SUMO2 pulldown assay. We found that mutation of the hydrophobic and serine residues abolished the binding of Rap80 to GST-SUMO2 (Fig. 3*D*). This indicates that the SIM domain of Rap80 is required for Rap80 binding to SUMO2/3 conjugates.

Rap80 N-terminal Fragment Containing a Tandem SIM-UIM-UIM Domain Binds to Ub Lys-63 and SUMO2 Chains—Because the N-terminal fragment (amino acids 1–129) of Rap80 contains SIM and UIM domains, we examined whether this fragment binds to both SUMO2 and Ub Lys-63 conjugates. We first tested whether this region binds to SUMO2 chains. We purified GST-tagged Rap80(1–129) fragments containing the tandem SIM-UIM-UIM motif, as well as mutants of this fragment, including the SIM domain mutants F40A/I41A (FI/AA) and S44A/S46A (SS/AA), a more complete SIM domain mutant with all four residues mutated (F40A/I41A/S44A/S46A, called SIM*), and a UIM domain mutant with mutations of the conserved residues in both UIM domains (A88S/S92A/A113S/S117A, called UIM*) that we previously have shown failed to bind to Ub Lys-63 chains (9, 20, 21). We then tested the binding of these mutants to SUMO2 3–8 chains *in vitro*. We found that the Rap80 SIM-UIM-UIM fragment associated with SUMO2 3–8 chains with a preference for polyconjugates with higher molecular weight. The SIM domain mutants F40A/I41A and SIM* abolished binding to SUMO2 chains, whereas mutant

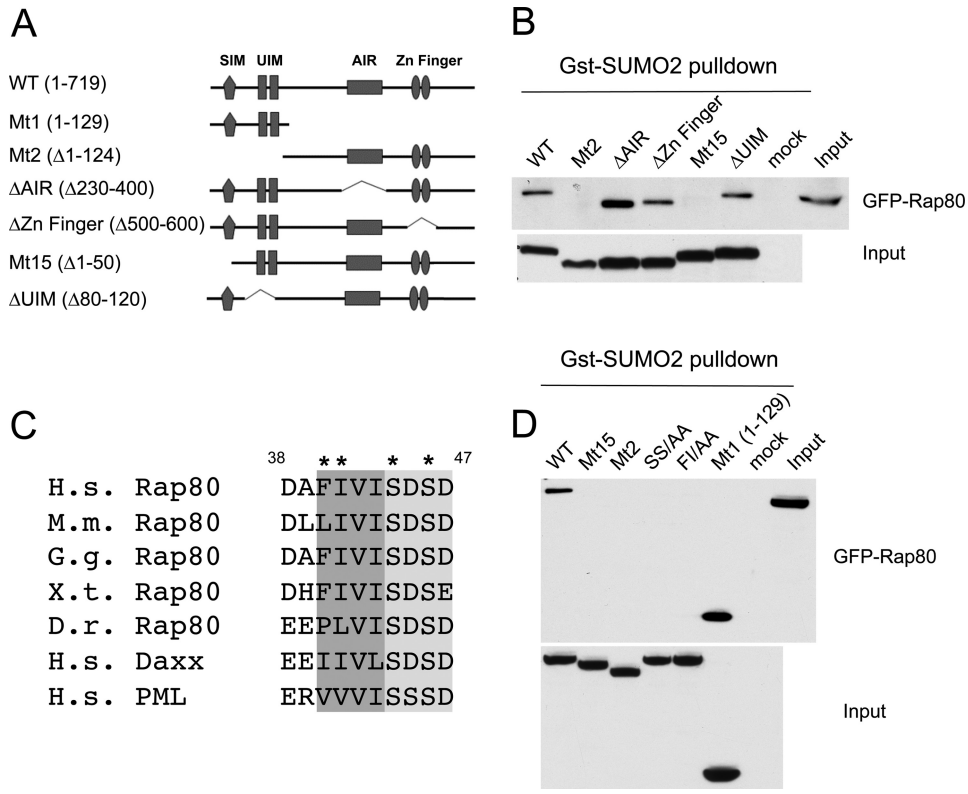

FIGURE 3. **Rap80 contains a SUMO-binding domain (SIM) at the N terminus.** *A*, diagram of various mutants (*Mt*) generated for Rap80. *AIR*, Abraxas interacting region (9). *B*, the N terminus of Rap80 is required for binding to GST-SUMO2. GFP-tagged wild-type or mutant Rap80 was transiently expressed in 293T cells. Purified GST-SUMO2 (10 μg) on beads was incubated with lysates from cells expressing GFP-tagged wild-type or mutant Rap80 for the pulldown assay. After extensive washing, proteins associated with GST-SUMO2 beads were loaded onto SDS-polyacrylamide gel for separation. Western blotting was carried out with anti-GFP antibody. The input was 2.5%. *C*, alignment of the SIM domain of Rap80. *H.s.*, *Homo sapiens*; *M.m.*, *Mus musculus*; *G.g.*, *Gallus gallus*; *X.t.*, *Xenopus tropicalis*; *D.r.*, *Danio rerio*. *D*, the SIM domain is required for binding to GST-SUMO2. Purified GST-SUMO2 on beads was incubated with lysates from cells expressing GFP-tagged wild-type or mutant Rap80 for the pulldown assay. Western blotting was carried out with anti-GFP antibody. Input was 2.5%. *SS/AA*, S44A/S46A; *FI/AA*, F40A/I41A.

S44A/S46A decreased binding to SUMO2 chains (Fig. 4*A*). Mutation of the UIM domain (UIM*) did not affect the binding of this fragment to SUMO2 chains (Fig. 4*A*). Similarly, mutation of the SIM domain did not appear to affect the binding of the UIM domain to Ub Lys-63 chains (Fig. 4*B*). Mutation of both the SIM and UIM domains (SIM*/UIM*) led to complete abolishment of the binding of this fragment to both SUMO2 and Ub Lys-63 chains (Fig. 4).

We also tested whether the Rap80 N-terminal SIM-UIM-UIM fragment binds to SUMO2 conjugates *in vivo*. We coexpressed the HA- and FLAG-tagged wild-type or mutant Rap80 SIM-UIM-UIM fragment and Myc-SUMO2 in 293T cells. We then carried out immunoprecipitation with anti-FLAG beads. We found that the SIM-UIM-UIM fragment associated with Myc-SUMO2 conjugates that can be recognized by anti-Myc antibodies (Fig. 4*D*, *lane 2*). Mutation of the SIM domain (SIM*) (Fig. 4*D*, *lane 6*) largely abolished the binding of this fragment to SUMO2 conjugates, whereas partial SIM domain mutants (F40A/I41A and S44A/S46A) decreased the binding to SUMO2 conjugates (Fig. 4*D*, *lanes 4* and *5*). Interestingly, mutation of the UIM domain also decreased the level of binding to SUMO2 conjugates (Fig. 4*D*, *lane 3*), suggesting that some ubiquitinated targets to which UIM binds are also sumoylated. Mutation of both the SIM and UIM domains of this fragment completely abolished the binding to SUMO2 conjugates (Fig. 4*D*, *lane 7*). This indicates that some of the target proteins might be both ubiquitinated and sumoylated.

Rap80 SIM-UIM-UIM Region Binds to Both Ub Lys-63 and SUMO2 Conjugates Simultaneously through Its UIM and SIM Domains—Because the SIM domain is right next to the UIM domains at the N terminus of Rap80 and because the tandem SIM-UIM-UIM region binds to both Ub Lys-63 and SUMO2 conjugates, we then examined whether SIM binding to SUMO conjugates affects UIM domain binding to Ub Lys-63 conjugates and vice versa. In an *in vitro* binding assay, we first tested whether addition of the Ub Lys-63 chains affects the binding of the Rap80 SIM-UIM-UIM fragment to SUMO2 chains. We found that the amount of SUMO2 chain binding to the GST-tagged SIM-UIM-UIM fragment was not significantly changed in the presence of increased amounts of Ub Lys-63 chains (Fig. 5*A*). This indicates that the binding of Ub chains to UIMs does not affect the SIM binding to SUMO2 chains. Similarly, we observed that increased binding of SUMO2 chains to the SIM-UIM-UIM fragment did not appear to affect the binding to Ub Lys-63 chains through UIMs (Fig. 5*B*). It also appeared to us that the SIM-UIM-UIM fragment had much more affinity for binding to Ub Lys-63 chains than to SUMO2 chains (supplemental Fig. 2). These results suggest that Rap80 binds to SUMO2 and Ub chains independently.

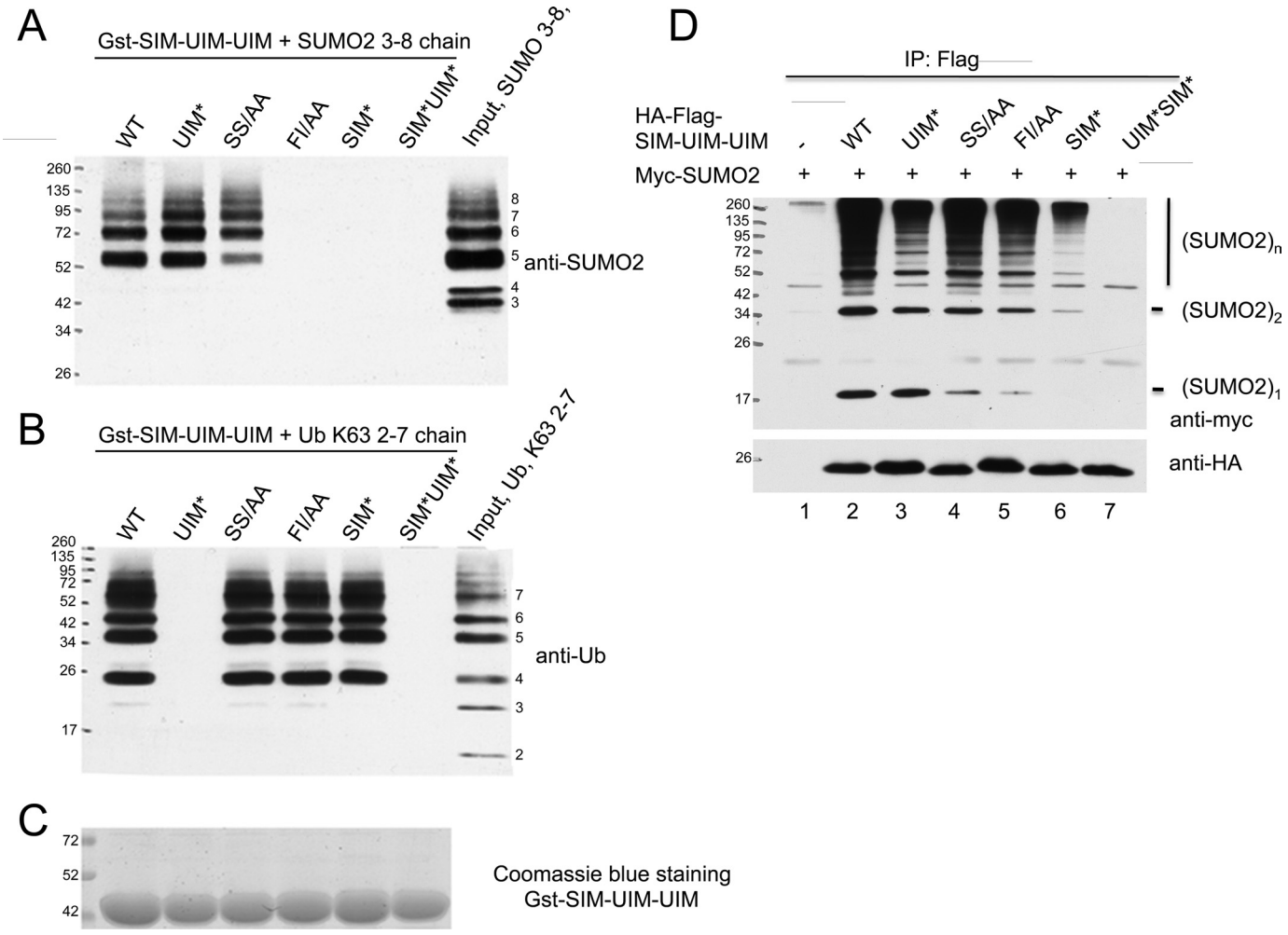

FIGURE 4. **Rap80 N-terminal fragment (amino acids 1–129), containing a tandem SIM-UIM-UIM motif, binds to SUMO2 and Ub chains.** *A*, the SIM domain is required for SIM-UIM-UIM fragment binding to the SUMO2 chain. The SIM-UIM-UIM fragments with mutations of the SIM domain (SIM* (F40A/I41A/S44A/S46A), F40A/I41A (*FI/AA*), and S44A/S46A (*SS/AA*)) or the UIM domain (UIM* (A88S/S92A/A113S/S117A)) were purified from bacterial cells. The purified GST-tagged proteins (20 μg) on beads were incubated with 150 ng of SUMO2 3–8 chains in NETN buffer. The beads were then washed extensively before loading onto SDS-polyacrylamide gel. The *Input lane* shows the amount of 100 ng SUMO2 chains. *B*, the SIM domain is not required for SIM-UIM-UIM fragment binding to Ub Lys-63 chains. The experiment was performed as described above, but the purified GST-tagged fragments (20 μg) on beads were incubated with 150 ng of Ub Lys-63 2–7 chains. *C*, Coomassie Blue staining of purified GST-tagged proteins used in the pulldown assay. *D*, the Rap80 N-terminal SIM-UIM-UIM fragment binds to SUMO2 conjugates *in vivo*. 293T cells were transiently transfected with expression constructs carrying HA- and FLAG-tagged Rap80(1–129) or various mutants and Myc-SUMO2 as indicated. 48 h after transfection, immunoprecipitations (*IP*) were carried out with anti-FLAG antibody using total cell lysates. Immunoprecipitates were then analyzed by Western blotting with anti-Myc antibody to detect SUMO2 conjugates.

To further test whether the SIM-UIM-UIM fragment binds simultaneously to Ub Lys-63 and SUMO2 conjugates, we designed an *in vitro* binding assay as illustrated in Fig. 6*A*. We first incubated Rap80 SIM-UIM-UIM fragments with agarose-SUMO2 beads, and after extensive washing, we incubated the beads with Ub Lys-63 chains. We reasoned that if the SIM-UIM-UIM fragment binds to both SUMO2 conjugates and Ub Lys-63 chains simultaneously, we would be able to detect Ub chains by pulling down the SIM-UIM-UIM fragment with agarose bead-anchored SUMO2. We found that agarose-SUMO2 bound to wild-type Rap80 SIM-UIM-UIM fragments and brought down Ub Lys-63 chains (Fig. 6), indicating that the SIM-UIM-UIM fragment associates with SUMO and Ub Lys-63 conjugates at the same time. Agarose-SUMO2 failed to bind SIM-UIM-UIM fragments with mutation of the SIM domain (FI/AA, SIM*, and SIM*UIM*) and thus lacked the ability to pull down Ub Lys-63 chains. In addition, agarose-SUMO2 bound to SIM-UIM-UIM fragments with mutation of the UIM domain (UIM*), yet it failed to bring down Ub Lys-63 chains due to the inability of UIM* to bind to Ub Lys-63 chains (Fig. 6).

Both the SIM and UIM Domains Are Required for Efficient Rap80 Recruitment to DSBs—Rap80 is recruited to DSBs immediately after damage (9, 24, 25). Previously, it was shown that the recruitment of Rap80 to DNA damage sites depends on its ability to bind to Ub conjugates (9, 24, 25). To examine the role of the SIM domain in Rap80 recruitment to DSBs, we stably expressed GFP-tagged human wild-type, SIM mutant (F40A/I41A/S44A/S46A), or UIM mutant Rap80 in Rap80-deficient ($Rap80^{-/-}$) MEF cells (26) and monitored the recruitment of Rap80 and its mutants to laser-induced DNA damage tracks. We found that in the early period (up to 30 min after damage) of Rap80 recruitment, mutation of the SIM or UIM domain decreased the efficiency of Rap80 recruitment to DNA damage sites (Fig. 7*A* and supplemental Videos 3–6). We also examined

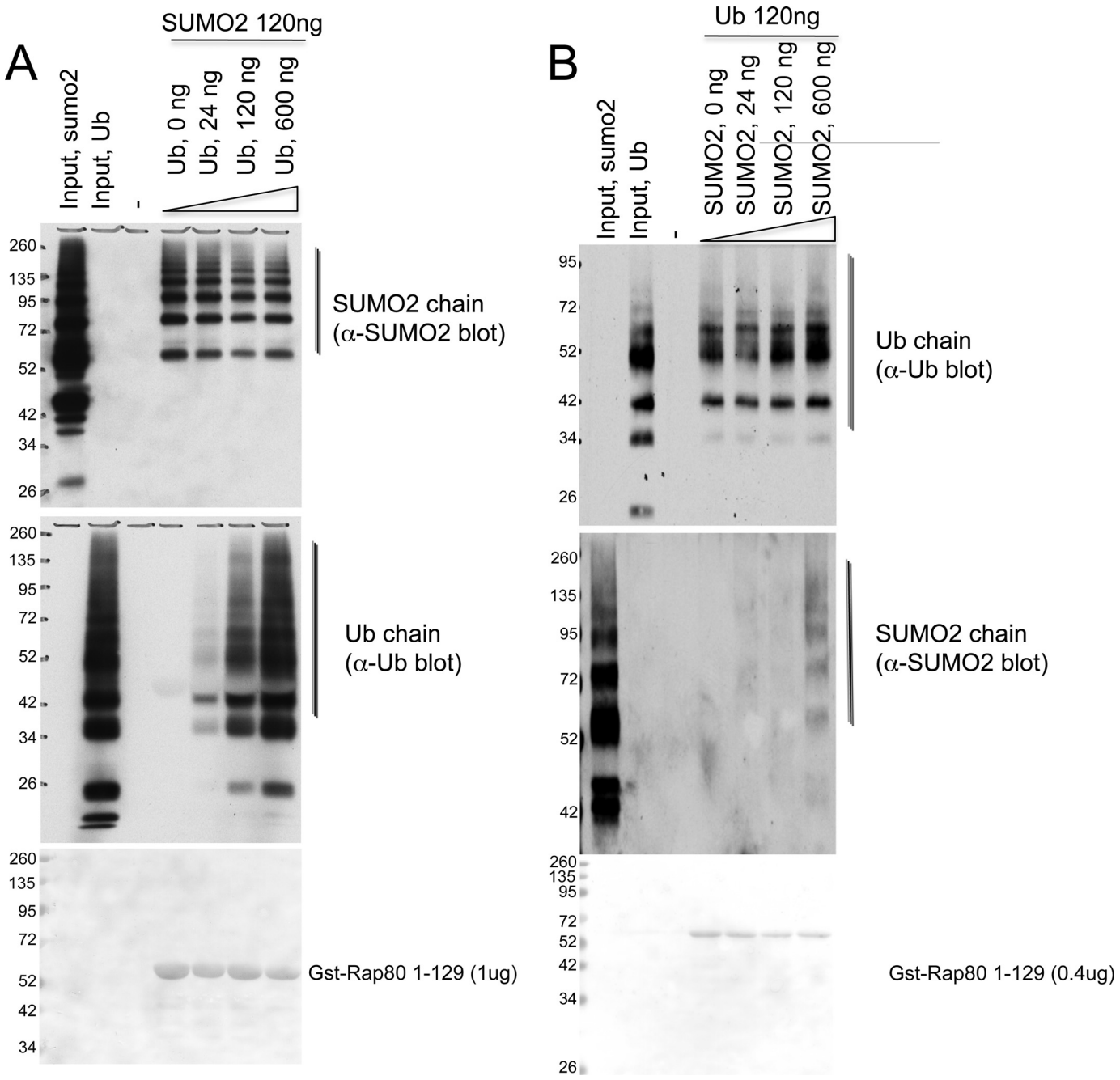

FIGURE 5. **Rap80(1–129), containing tandem SIM-UIM-UIM motif, binds to SUMO2 and Ub chains independently.** *A*, binding to SUMO2 chains is not affected by binding to Ub Lys-63 chains. Purified GST-Rap80(1–129) (1 μg) on beads was incubated with 120 ng of the SUMO2 3–8 chain in 400 μl of NETN buffer overnight in the absence of the Ub chain or in the presence of increased amounts of the Ub Lys-63 2–7 chain for a pulldown assay. Western blotting was carried out with anti-SUMO2 or anti-Ub antibody. *B*, binding to the Ub chain is not affected by binding to the SUMO2 chain. Purified GST-Rap80(1–129) (0.4 μg) on beads was incubated with 120 ng of Ub 2–7 chain in 400 μl of NETN buffer overnight in the absence of the SUMO2 chain or in the presence of increased amounts of the SUMO 3–8 chain for a pulldown assay.

Rap80 IR-induced focus formation in Rap80-deficient MEF cells complemented with GFP-tagged wild-type or mutant Rap80. Consistently, we found that both the SIM and UIM mutants of Rap80 displayed decreased formation of Rap80 IR-induced foci at early time points after IR; however, at later time points (90 min), the defects of the SIM domain mutant became minimal (supplemental Fig. 3). These results indicate that the SIM domain plays important roles in recruiting Rap80 in the early period after DNA damage.

Rap80 SIM Domain Is Required for Cellular Resistance to IR—Rap80 deficiency in cells leads to compromised DNA repair and increased cellular sensitivity to IR (9, 24, 25). To investigate whether the SIM domain is important for the role of Rap80 in a proper DDR, we examined whether mutation of the SIM domain rescues Rap80 deficiency in cellular resistance to IR in a clonogenic survival assay. Rap80-null MEF cells were sensitive to IR, and this increased sensitivity could be rescued by expression of GFP-tagged human wild-type Rap80. How-

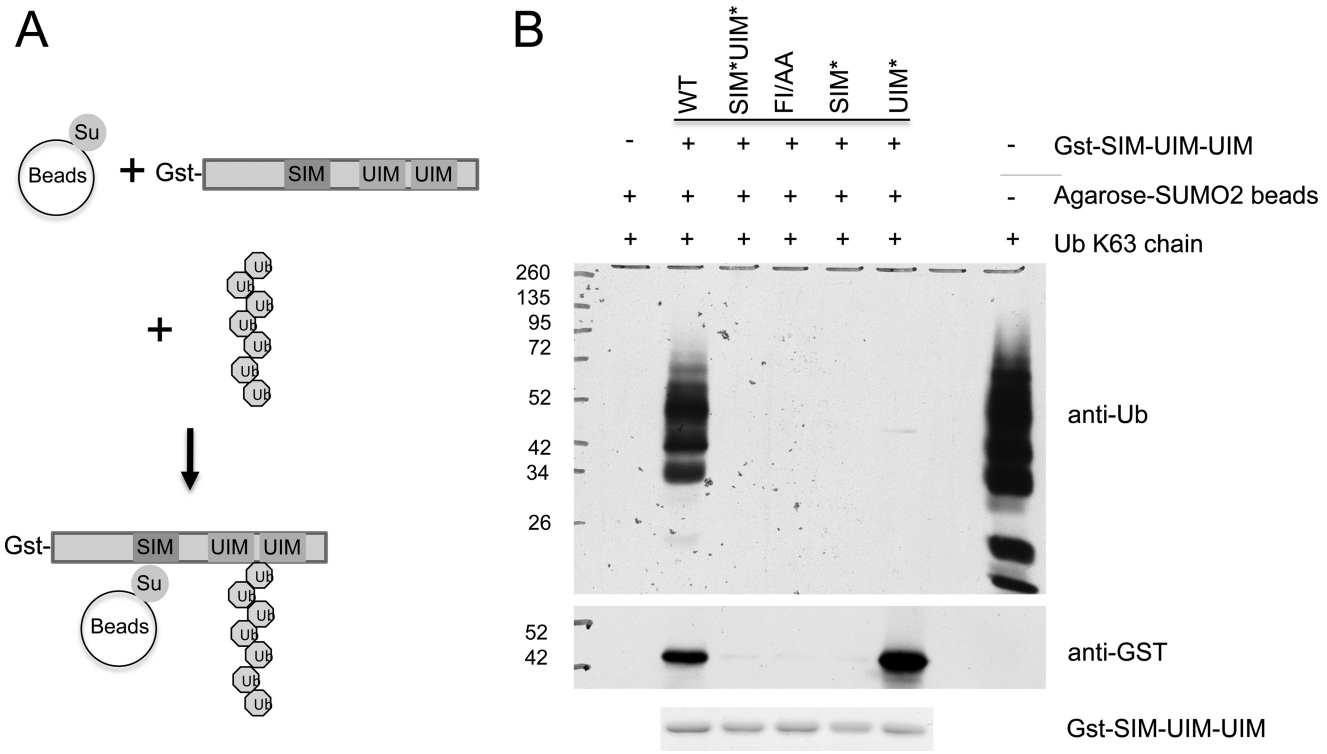

FIGURE 6. **Rap80 N-terminal tandem SIM-UIM-UIM fragment binds to SUMO2 and Ub Lys-63 conjugates simultaneously.** *A*, illustration of the *in vitro* pulldown assay used in this experiment. *Su*, SUMO2. *B*, the SIM-UIM-UIM fragment binds to both SUMO2 and Ub Lys-63 conjugates at the same time. The agarose-SUMO2 beads were first incubated with purified GST-SIM-UIM-UIM fragments, followed by addition of Ub Lys-63 2–7 chains. After extensive washing, bound proteins of the beads were loaded onto SDS-polyacrylamide gel for separation. Western blotting was carried out with anti-Ub or anti-GST antibody. *FI/AA*, F40A/I41A; *SIM**, F40A/I41A/S44A/S46A; *UIM**, A88S/S92A/A113S/S117A.

ever, expression of a UIM or SIM domain mutant of Rap80 failed to efficiently rescue the increased cellular sensitivity to IR displayed by the Rap80-null MEF cells (Fig. 7*B* and supplemental Fig. 4).

DISCUSSION

In this study, we identified a SUMO-binding motif, the SIM domain, which is present next to the two UIM domains at the N terminus of the Rap80 protein, forming a SIM-UIM-UIM motif. SUMO modification at DNA damage sites has been shown to be important for recruitment of 53BP1 and BRCA1 (13, 14). SUMO E3 ligases PIAS1 and PIAS4, as well as the SUMO-conjugating enzyme UBC9, accumulate at DNA damage sites, modulating SUMO modification that is required for Rap80 and BRCA1 recruitment downstream of RNF8. Three SUMO isoforms (SUMO1, SUMO2, and SUMO3) localize to DNA damage foci. Here, we have provided a mechanism by which Rap80 and thus the BRCA1-A complex are recruited to DNA damage sites through Rap80 binding to SUMO2/3 and Ub Lys-63 conjugates.

Our results indicate that the tandem SIM-UIM-UIM motif of Rap80 binds to SUMO2 and Ub Lys-63 conjugates simultaneously *in vitro*. It appears that this fragment of Rap80 also binds to SUMO2 and Ub conjugates *in vivo* when expressed in cells. Thus, it is possible that Rap80 binds to target proteins that are both ubiquitinated and sumoylated through the tandem SIM-UIM-UIM region. It is also possible that the tandem motif binds to different targets that are each sumoylated or ubiquitinated. Interestingly, although UIM mutants appeared to bind SUMO2 conjugates just as well as the wild-type fragment *in vitro* (Fig. 4*A*), mutation of the UIM domain affected the binding of this fragment to SUMO conjugates *in vivo* (Fig. 4*D*), indicating that at least some target proteins are likely to be modified by both Ub and SUMO conjugates. In addition, it has also been suggested that SUMO2/3 chains could be modified by Ub (27). The mixed SUMO2/3 and Ub Lys-63 conjugates may also be recognized by the SIM-UIM-UIM domain for binding.

We have demonstrated that both the SIM and UIM domains are required for efficient recruitment of Rap80 to DNA damage sites immediately after damage. Mutation of either the SIM or UIM domain decreased the efficiency of Rap80 recruitment to laser-induced DNA damage tracks. Consistently, it appeared that the IR-induced focus formation of Rap80 at early time points was also affected by mutation of either the SIM or UIM domain (supplemental Fig. 3). Interestingly, however, at later time points after IR, the defects of IR-induced focus formation observed for the SIM domain mutant became less obvious (supplemental Fig. 3), indicating that the SIM domain and its ability to bind to SUMO2 conjugates are more important for the initial recruitment of Rap80 to DNA damage sites. More importantly, we found that both the SIM and UIM domains are required for cellular resistance to IR, as Rap80 SIM and UIM domain mutants failed to rescue the cellular sensitivity of Rap80$^{-/-}$ MEF cells. Although the SIM domain and Ub-binding domains such as the UIM have been identified in multiple proteins, the Rap80 SIM-UIM-UIM motif provides an example of a binding platform for simultaneous association with both SUMO and Ub

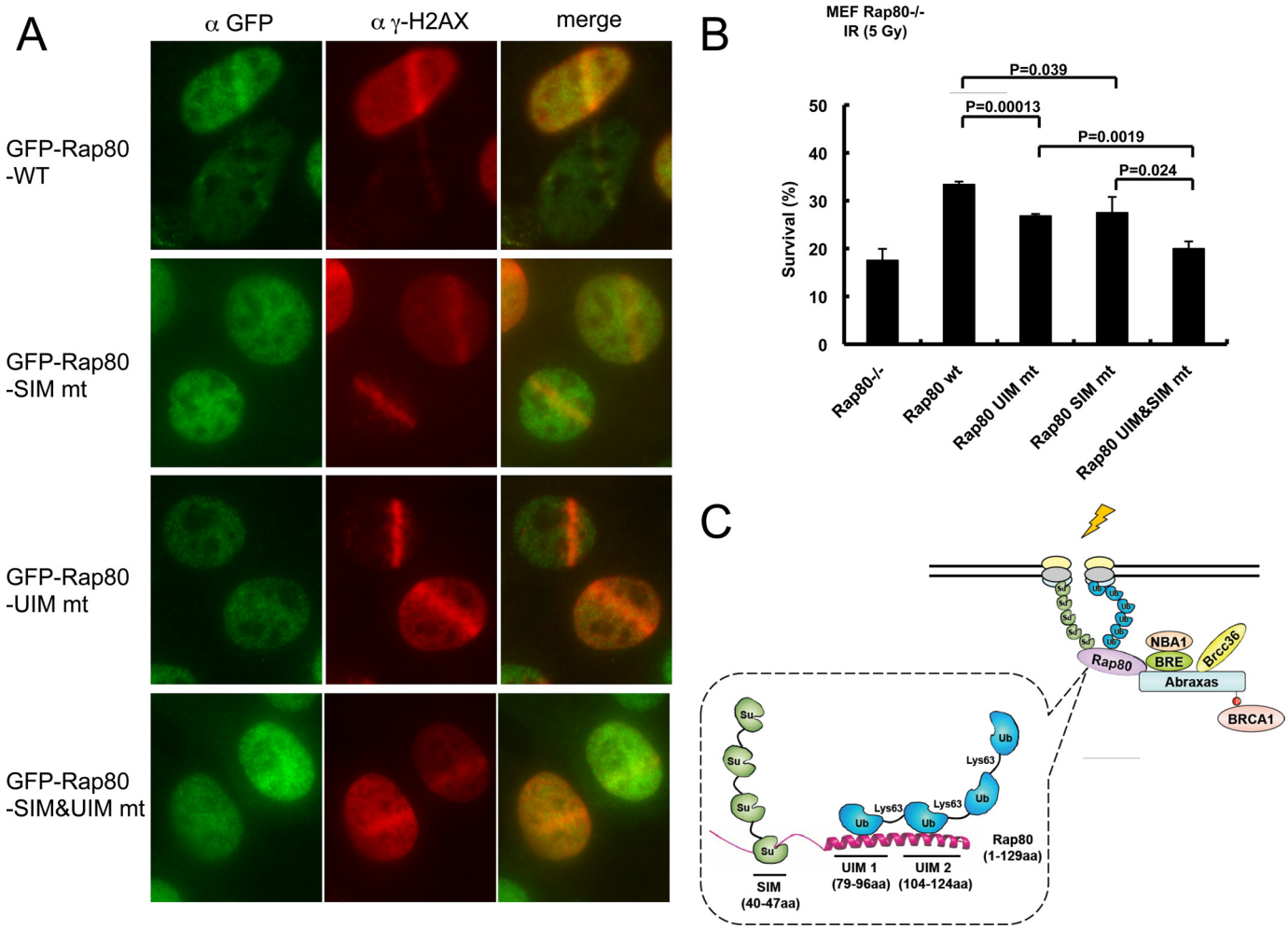

FIGURE 7. **Both the SIM and UIM domains of Rap80 are required for cellular response to DNA damage.** *A*, recruitment of the SIM and UIM mutants of Rap80 is decreased in response to laser-induced DNA damage within 30 min of DNA damage. Rap80$^{-/-}$ MEF cells stably expressing GFP-tagged human Rap80 or its SIM, UIM, or SIM&UIM mutant (*mt*) were treated with a UV laser. 30 min after DNA damage, cells were fixed and immunostained with anti-GFP and anti-γH2AX antibodies. *B*, both the SIM and UIM domains are required for cellular resistance to IR. Rap80$^{-/-}$ MEF cells stably expressing GFP-tagged wild-type, SIM mutant, or UIM mutant Rap80 were generated for the analyses of cellular resistance to IR using a colony-forming assay. *Gy*, grays. *C*, model for Rap80 recruitment to DNA damage sites through its tandem SIM-UIM-UIM motif binding to SUMO2 (*Su*) and Ub Lys-63 conjugates. *aa*, amino acids.

conjugates. What is the functional importance of the tandem SIM-UIM-UIM motif? It might provide specificity and affinity in binding to target proteins, *i.e.* target proteins that are both SUMO2- and Ub Lys-63-modified can be specifically bound by the SIM-UIM-UIM motif of Rap80 with increased affinity compared with target proteins that are modified only by Ub or SUMO2 alone. In addition, it might determine the location or timing of binding so that it occurs only at sites where target proteins are both sumoylated and ubiquitinated such as DNA damage sites.

In our live cell imaging system with cells expressing GFP-tagged SUMO1 or SUMO2, it appeared that SUMO2 conjugation occurred robustly and immediately after laser microirradiation. Previously, it was shown that, in contrast to SUMO1, SUMO2/3 cells respond to cellular stress such as heat shock by enhancing SUMO2/3 conjugation and poly-SUMO chain formation (22, 28). The SIM domain of Rap80 appears to bind specifically to SUMO2 conjugates, but not to SUMO1. Although BRCA1 is reported to be sumoylated and SUMO modification increases BRCA1 ubiquitin ligase activity (13, 14), we did not find that the Rap80 SIM-UIM-UIM motif binds to BRCA1 in pulldown assays (data not shown). The histone subfamilies were also identified as targets of SUMO (29, 30). In addition, Rap80 binds to UBC9, and itself is reported to be sumoylated at the N terminus (31). The actual target that the SIM domain of Rap80 binds to is still not clear and requires further studies. Nevertheless, it is apparent that dynamic Ub and SUMO modification at DNA damage sites plays critical roles in Rap80 and BRCA1 recruitment and efficient DNA repair.

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