RAP80 RECRUITMENT TO DNA DOUBLE STRAND BREAKS REQUIRES BINDING TO BOTH SUMO- AND UBIQUITIN-CONJUGATES

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Running head: Rap80 contain motifs binding to both SUMO- and Ubiquitin- conjugates

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Background: Ubiquitin and Small ubiquitin-related molecule (SUMO) conjugation occurs at DNA double strand breaks (DSBs).

Results: Rap80, a component of the BRCA1-A complex, binds to both SUMO and ubiquitin conjugates.

Conclusion: Rap80 binding to both SUMO and ubiquitin conjugates is required for proper cellular response to DSB.

Significance: It provides insights into how Rap80 and BRCA1 are recruited to DSBs for maintaining genome stability.

SUMMARY

Ubiquitin modifications at sites of DNA double-strand breaks (DSBs) play critical roles in the assembly of signaling and repair proteins. The UIM domain of Rap80, which is a component of the BRCA1-A complex, interacts with ubiquitin Lys63-linkage conjugates and mediates the recruitment of BRCA1 to DSBs. Small ubiquitin-related modifier (SUMO) conjugation also occurs at DSBs and promotes ubiquitin-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 ubiquitin Lys63-linkage and SUMO2 conjugates. Both the SIM and UIM domain 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 ubiquitin modification are coordinated to recruit Rap80 and BRCA1 to DNA damage sites.

Modification of proteins by the covalent attachment of ubiquitin (Ub) or small ubiquitin-related modifier (SUMO) to the lysine (Lys) residue of a target protein are 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 DNA damage response kinases ATM/ATR (5-7). ATM/ATR phosphorylation on Ser139 of histone H2AX directly recruits MDC1 through MDC1’s BRCT domains. The subsequent MDC1 phosphorylation-dependent recruitment of ubiquitin ligases RNF8 and RNF168, together with an ubiquitin E2 conjugase, Ubc13, generate ubiquitin Lys63 (K63) linkage chains on the damaged chromatin. In addition, a HECT domain-containing E3 ligase HERC2 is also involved in facilitating the formation of K63-linked ubiquitin conjugates. Ub K63-linkage polyubiquitin chain formation plays important roles in the recruitment of repair factors including 53BP1 and BRCA1 (5-7).

Breast and ovarian tumor suppressor BRCA1 plays critical roles in the DNA damage response (DDR) regulating multiple repair and checkpoint mechanisms for maintaining genome stability. Through its C-terminus BRCT domains, BRCA1 forms at least three different complexes, the BRCA1 A, B and C complexes by binding to Abraxas (Abraxas), Bach1 and CtIP respectively (5, 7-8). While all three complexes have been indicated in BRCA1’ role 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 ubiquitin modification. The BRCA1 A complex contains at least five different components, Abraxas,
NBA1/MERIT40, BRE, Rap80, and BRCC36 (8). Rap80 contains two UIM domains that display a binding specificity toward Ub K63-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 cell 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 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, by contrast, does not form chains efficiently (16), it might, however, serve as terminator of SUMO2/3 chains (17). SUMO1 and SUMO2/3 accumulate at DSB sites in mammalian cells (13,14). SUMO E3 ligases PIAS1/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 short conserved SUMO interaction 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 SIM and UIM domains play important roles in recruiting Rap80 to DNA damage sites and confer cellular sensitivity to ionizing irradiation (IR).

**Experimental Procedures**

Plasmids, siRNAs and antibodies - Retroviral expression constructs for GFP-Rap80 wild type and mutants were made using MSCV vectors containing GFP tag at the N terminus as described (9,20). Deletion mutants of Rap80 were either previously described (9,20) or generated by cloning the corresponding cDNA fragments into the above retroviral vector. Site-directed mutagenesis was performed with the QuickChangeII site-directed mutagenesis kit (Stratagene, CA) for generating various point mutants of Rap80. Gst-tagged or His-tagged Sumo2, wild type or mutants of Rap80 1-129 amino acids fragment were generated with pDEST15 (GST) or pDEST17 (His) vectors (Invitrogen) via gateway recombination system. siRNAs used for knocking down Rap80, Abraxas, BRE, and BRCC36 were previously described (9,20,21). Ub K63 2-7 and Sumo2 3-8 chain, as well as agarose-Sumo2 beads were purchased from Boston Biochem. Rabbit anti-NBA1, anti-BRE and anti-Abraxas antibodies were generated as previously described (9,12,21). Other antibodies used were BRCC36 antibodies (ProSci Incorporated); Rap80 antibodies (Bethyl Laboratories); BRCA1 antibodies (D9, Santa Cruz Biotechnologies); GFP antibodies (Invitrogen); Rabbit anti-myc antibodies (Sigma); mouse anti-HA antibodies (Covance); Ub antibodies (P4D1, Santa Cruz); Sumo2/3 antibodies (Cell Signaling); Gst (Cell Signaling); γH2AX (Upstate).

Cell lines and cell culture - U2OS cells were grown in McCoy’s 5A medium supplemented with 10% FBS, 100 μg/ml penicillin/streptomycin. 293T cells and Rap80-/- MEF cells were grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% FBS, 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 (50mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40) with protease inhibitors and protein phosphatase inhibitors, 1 mM NaF, 1 mM Na3VO4 and 10mM N-ethylmaleimide (NEM). Immunoprecipitations (IP) were carried out in the same buffer with appropriate antibodies and protein A/G-Sepharose beads (Santa Cruz Biotechnology) overnight at 4
FLAG IP 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 pDEST15 expression vector in E.coli DE3 cells (Invitrogen) and purified using glutathione-sepharose beads (Amersham Biosciences). 293T cells were treated or not treated with 10 Gy IR followed by 2 hour incubation at 37°C before harvested and cell lysates were prepared as previously described. In vitro pull-down assay was performed with purified Gst-SUMO2 (50 ug) incubated with cell lysates (20 mg total protein) prepared above at 4°C overnight. Associated proteins from gel slices were then analyzed by mass spectrometry (Taplin Mass Spectrometry Facility/Harvard Medical School).

**Pull-down Assays** - Gst- or His-tagged proteins were expressed in E.coli DE3 cells (Invitrogen) and purified using glutathione-sepharose (Amersham Biosciences) 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, washed five times with NETN buffer before suspended in 1x SDS loading buffer for gel separation and subsequent immunoblotting with various antibodies. For binding assay with Ub K63- or SUMO2-chain, purified protein fragments on beads were incubated with Ub K63- or SUMO2-chain (Boston Biochem) in a total 0.5-ml volume of NETN buffer at 4°C overnight. Beads were then collected and washed five times with NETN buffer before suspended in 1x SDS loading buffer for gel separation.

**Agarose-SUMO2 pull down of Gst-SIM-UIM-UIM fragment and Ub K63 2-7 conjugates** - Gst-SIM-UIM-UIM fragment wild type or mutants were expressed and purified using glutathione-sepharose (Amersham) and eluted by elution buffer (100mM Tris, 100mM NaCl, 5% glycerol, 40 mM glutathione). The SUMO2 agarose beads (50ug SUMO2, Boston Biochem) were first blocked in NETN buffer with 0.5% BSA for 3 hours, then incubated with 10ug purified Gst-tagged Rap80 1-129aa (Gst-SIM-UIM-UIM) fragment in a 0.4-ml total volume of NETN buffer for 1 hour. Beads were then collected, washed 5 times with NETN w/o BSA buffer. The beads were then incubated with 300 ng Ub K63 2-7 chain (Boston Biochem) in 0.4 ml NETN for 1 hour at 4°C. After 5x wash with NETN buffer, beads were then suspended in 1xSDS loading buffer for gel separation and immunoblotting.

**Colony formation assay** - The assay was performed as previously described (12). Briefly, MEF Rap80 -/- stable cell lines were seeded at low density and irradiated with 5 or 10 Gy ionizing irradiation using $^{137}$Cs radiation source. The cells were then incubated at 37°C for 14 day to allow colonies to form. Colonies were stained with 2% methylene blue / 50% ethanol. Colonies containing 50 or more cells were counted and statistical data was analyzed by the t-test.

**Laser induced DNA damage and live cell imaging** - Cells were treated with 10 uM BrdU (BD) for 24 h prior to laser irradiation on a TE2000 inverted microscope (Nikon) integrated with the MicroPoint laser system. Nuclei were irradiated with a UV laser (364 nm) with 5 times pulse (total 335ms). A 60x water lens is used for the operation. The laser energy output was set to 23%. Cells were either fixed for immunostaining at indicated times or monitored with live cell imaging. For live cell imaging, images were captured immediately after laser microirradiation at 30 sec intervals. The total time course lasted for 15 min 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 previously reported (2,3,13,14). Previously it was reported that SUMO1, SUMO2/3 conjugates accumulate at DSBs.
To compare the accumulation of SUMO1 and SUMO2/3 conjugates at DSBs, we employed laser micro-irradiation 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 to the laser-tracks. We observed that, while GFP-SUMO1 accumulation at 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 maximum level after a few minutes and persisted for the duration of monitoring (up to 15 minutes) (Fig. 1 and Supplemental video 1-2).

Rap80 mediates the binding of the BRCA1-A complex to SUMO2/3 conjugates

We then decided to identify proteins that were recruited to DNA damage sites through binding to SUMO2/3 conjugates. Previously it has been 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 components of the BRCA1 A complex as well as BRCA1 were among the proteins that associated with GST-SUMO2 (Fig. 2A 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 pull-down assays with cell lysates (Fig. 2B and C).

Interestingly, we found the binding of the BRCA1 A complex with SUMO2 was likely to be mediated by Rap80 (Fig. 2D). In an in vitro pull-down assay, the binding of Abraxas to purified His-SUMO2 was significantly decreased using lysates of cells treated with siRNAs against Rap80 compared to that of cells treated with control siRNA. Yet, when either of the other components of the BRCA1 A complex was depleted by siRNAs, binding of Rap80 to His-SUMO2 was not affected in the in vitro pull-down assay (Supplemental Fig. S1).

Rap80 contains a SUMO interacting motif (SIM). Since it appears that Rap80 mediates association of the BRCA1 A complex to SUMO2/3 conjugates, it 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 pull-down assays. We found a fragment of Rap80 corresponding to 1-129 amino acids containing two UIM domains appears to be sufficient for binding to Gst-SUMO2 in pull-down assays, while other regions of Rap80 are not required (Fig. 3). In addition, while UIM domains were not required for binding, a region corresponding to 1-50 amino acids of Rap80 was required (Fig. 3). We analyzed the protein sequence of this region and found that Rap80 contains a SUMO interacting motif (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. 3C).

We then mutated the first two hydrophobic residues (FI/AA mutant, F40A,I41A), as well as the two conserved serine residues to alanine, (SS/AA mutant, S44A,S46A). Phosphorylation of these two conserved serine residues in DAXX SIM motif appears to increase the SIM binding affinity to 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 residues as well as the serine residues abolished the binding of Rap80 to Gst-SUMO2 (Fig 3D). This indicates that the SIM domain of Rap80 is required for Rap80 binding to SUMO2/3 conjugates.

Rap80 N-terminus fragment containing tandem SIM-UIM-UIM domain binds to Ub K63 chains and SUMO2 chains

Since the N-terminus fragment (1-129 amino acids) of Rap80 contains SIM and UIM domains, we examined whether this fragment binds to both SUMO2 and Ub K63 conjugates. We first tested whether this region binds to SUMO2 chains. We purified Gst-tagged Rap80 (1-129aa)
fragments containing the tandem SIM-UIM-UIM motif, as well as mutants of this fragment including the SIM domain mutants, FI/AA, SS/AA and a more complete SIM domain mutant with all four residues mutated (SIM*, F40A,I41A,S44A,S46A), and a UIM domain mutant with mutations of conserved residues in both UIM domains, (UIM*, A88S,S92A,A113S,S117A) that we previously have shown failed to bind to Ub K63- 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 associates with SUMO2 3-8 chains with a preference for poly-conjugates with higher molecular weight. The SIM domain mutants FI/AA and SIM* abolished binding to SUMO2 chains while another mutant SS/AA decreased the binding to SUMO2 chains (Fig. 4A). Mutation of the UIM domain (UIM*) does not affect the binding of this fragment to SUMO2 chains (Fig. 4A). Similarly, mutation of the SIM domain did not appear to affect the binding of UIM domain to Ub K63 chains (Fig. 4B). Mutation of both the SIM and UIM domains (SIM*UIM*) led to complete abolishment of the binding of this fragment to both SUMO2 chains and Ub K63-chains (Fig. 4).

We also tested whether the Rap80 N-terminus SIM-UIM-UIM fragment binds to SUMO2 conjugates in vivo. We co-expressed HA- and Flag-tagged wild type or mutants of the 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. 4D, lane 2). Mutation of the SIM domain (SIM*) (Fig. 4D, lane 6) largely abolished the binding of this fragment to SUMO2 conjugates while other partially mutated SIM domain mutants, FI/AA and SS/AA mutants, partially decreased the binding to SUMO2 conjugates (Fig. 4D, lane 4 and 5). Interestingly, mutation of the UIM domain also decreased the level of binding to SUMO2 conjugates (Fig. 4D, lane 3) suggesting that some ubiquitinated targets that UIM binds to are also sumoylated. Mutation of both the SIM and UIM domain of this fragment completely abolished the binding to SUMO2 conjugates (Fig. 4D, lane 7). It indicates that some of the target proteins might be both ubiquitinated and sumoylated.

**Rap80 SIM-UIM-UIM region binds to both Ub K63 and SUMO2 conjugates simultaneously through its UIM domain and SIM domain**

Since the SIM domain is right next to the UIM domains at the N-terminus of Rap80 and the tandem SIM-UIM-UIM region binds to both Ub K63 and SUMO2 conjugates, we then examined whether SIM binding to SUMO conjugates affects the UIM domain binding to Ub K63 conjugates and vice versa. In an in vitro binding assay, we first tested whether addition of the Ub K63 chains affects the binding of the Rap80 SIM-UIM-UIM fragment to SUMO2 chains. We found that the amount of SUMO2 chains binding to the GST-tagged SIM-UIM-UIM fragment was not significantly changed in the presence of increased amount of Ub K63 chains (Fig. 5A). It indicates that the binding of Ub chains to UIMs does not affect the SIM binding to SUMO2 chains. Similarly we found that increased binding of SUMO2 chains to the SIM-UIM-UIM fragment does not appear to affect the binding to Ub K63 chains through UIMs (Fig. 5B). It also appeared to us that the SIM-UIM-UIM fragment has much more affinity towards binding to Ub K63 chains than to SUMO2 chains (Supplemental Fig. S2). These results suggested that Rap80 binds to SUMO2 chains and Ub chains independently.

To further test whether the SIM-UIM-UIM fragment binds simultaneously with Ub K63 conjugates and SUMO2 conjugates, we designed an in vitro binding assay as illustrated in Figure 6A. We first incubated Rap80 SIM-UIM-UIM fragments with agarose-SUMO2 beads, after extensive wash, we then incubated the beads with Ub K63- chains. We reasoned that, if SIM-UIM-UIM fragment binds to both SUMO2 conjugates and Ub K63 chains simultaneously, we would be able to detect Ub chains by pulling down the SIM-UIM-UIM fragment with agarose beads anchored SUMO2. We found that, indeed, agarose-SUMO2 bound to wild type Rap80 SIM-UIM-UIM fragments and brought down Ub K63 chains indicating that the SIM-UIM-UIM fragment associates with SUMO and Ub K63 conjugates at the same time (Fig. 6). Agarose-SUMO2 failed to bind SIM-UIM-UIM fragments with mutation of the SIM domain (FI/AA, SIM*, SIM*UIM*), and...
Thus lacked the ability to pull down Ub K63 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 K63 chains due to the inability of UIM* to bind to Ub K63 chains (Fig. 6).

Both SIM domain and UIM domain 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 SIM domain in Rap80 recruitment to DSBs, we stably expressed GFP-tagged human Rap80 wildtype, SIM mutant (F40A,I41A,S44A,S46A) or UIM mutant of 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 SIM domain or UIM domain decreased the efficiency of Rap80 recruitment to DNA damage sites (Fig. 7A and Supplemental Video 3-6). We also examined Rap80 IR induced foci (IRIF) formation in Rap80-deficient MEF cells complemented with wild type or mutants of GFP-tagged Rap80. Consistently, we found both SIM mutant and UIM mutant of Rap80 displayed decreased formation of Rap80 IRIF at early time points after IR, however, at later time points (90 minutes), the defects of the SIM domain mutant became minimal (Supplemental Fig. S3). 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 led to compromised DNA repair and increased cellular sensitivity to IR (9,24,25). To investigate whether the SIM domain is important for Rap80’s role in a proper DNA damage response, 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 can be rescued by expression of wild-type human GFP-tagged Rap80. However, expression of a UIM mutant of Rap80 or a SIM domain mutant of Rap80 failed to efficiently rescue the increased cellular sensitivity to IR displayed by the Rap80 null MEF cells (Fig. 7B and Supplemental Fig. S4).

DISCUSSION

In this study, we identified a SUMO binding motif, SIM domain, 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 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 provide a mechanism of through binding to SUMO2/3 and Ub K63 conjugates that Rap80 and thus the BRCA1 A complex is recruited to DNA damage sites.

Our results indicate that the tandem SIM-UIM-UIM motif of Rap80 binds to SUMO2- and Ub K63- 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. 4A), mutation of the UIM domain affected the binding of this fragment to SUMO conjugates in vivo (Fig. 4D), indicating that at least some target proteins are likely to be modified by both ubiquitin conjugates and SUMO conjugates. In addition, it also has been suggested that SUMO2/3 chains could be modified by Ub (27). The mixed SUMO2/3 and Ub K63 conjugates may also be recognized by the SIM-UIM-UIM for binding.

Our studies found that both the SIM and UIM domains are required for efficient recruitment of Rap80 to DNA damage sites.
immediately after damage. Mutation of either SIM or UIM domain decreased the efficiency of Rap80 recruitment to laser induced DNA damage tracks. Consistently, it appeared that the IRIF formation of Rap80 at early time points was also affected by mutation of either the SIM domain or UIM domain (Supplemental Fig. S3). Interestingly, however, at later time points after IR, the defects of IRIF formation observed for SIM domain mutant became less obvious (Supplemental Fig. S3) indicating that the SIM domain and its binding ability to SUMO2 conjugates are more important for the initial recruitment of Rap80 to DNA damage sites. More importantly, we found that both 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 SIM domain and ubiquitin binding domains such as UIM are identified in multiple proteins, the Rap80 SIM-UIM-UIM motif provides an example of a binding platform for simultaneously association with both SUMO and Ub conjugates. So what is the functional importance of tandem SIM-UIM-UIM motif? It might provide specificity and affinity in binding to target proteins, as in which target proteins that are both SUMO2 and Ub K63 modified can be specifically bound by the SIM-UIM-UIM motif of Rap80 with increased affinity than target proteins that are only modified by Ub or SUMO2 alone. In addition it might determine the location or timing of binding so that it only occurs 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 occurs robustly and immediately after laser microirradiation. Previously it has been shown that, in contrast to SUMO1, SUMO-2/3 cells respond to cellular stress such as heat shock by enhancing SUMO2/3 conjugation and polySUMO chain formation (22) (28). The SIM domain of Rap80 appears to bind specifically to SUMO2 conjugates but not SUMO1. Although BRCA1 is reported to be SUMOylated and SUMO modification increases BRCA1 ubiquitin ligase activity (13,14), we haven’t found that 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 ubiquitin and SUMO modification at DNA damage sites plays critical roles in Rap80 and BRCA1 recruitment and efficient DNA repair.

REFERENCES:


**FOOTNOTES**

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Abbreviations used in this paper: SIM, SUMO interacting motif; SUMO, small ubiquitin-like molecule; Ub, ubiquitin; K63-Ub, lysine 63- linkage ubiquitin conjugate; UIM, ubiquitin interacting motif; IIRIF, ionizing irradiation induced foci; BRCA1-A complex, BRCA1 associated A complex
Figure legends:

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 UV laser. Live cell imaging was performed immediately after laser treatment at room temperature. Images were taken at 30 seconds interval for 15 min. Images from various time point were shown. The live cell imaging video is in the supplemental movie 1-2.

Figure 2. Rap80 mediates the binding of 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 ug) on beads were incubated with lysates of 293T cells or 293T cells expressing HA-Flag tagged Abraxas for pulldown assay. Western blots were carried out with various antibodies. **” indicates a non-specific 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 ug) on beads were used. Empty beads were used as a control. 2% input was included in the blot. **” indicates a non-specific band. (D) Rap80 mediates the binding of BRCA1 A complex to Gst-SUMO2. 293T cells were transfected with siRNAs against Rap80. 48 hrs after transfection, cells were lysed and total lysates were used for incubation with purified Gst-SUMO2 on beads (10 ug) for pulldown assay.

Figure 3. Rap80 contains a SUMO binding domain SIM at the N-terminus. (A) A diagram for various mutants generated for Rap80. (B) The N-terminus of Rap80 is required for binding to Gst-SUMO2. GFP-tagged Rap80 wild-type or its mutants were transiently expressed in 293T cells. Purified Gst-SUMO2 on beads (10ug) were incubated with lysates from cells expressing GFP-tagged Rap80 wild type or mutants for the pulldown assay. After extensive wash, proteins associated with Gst-SUMO2 beads were loaded to SDS-PAGE gel for separation. The western blot was carried out with antibodies to GFP. Input is 2.5%. (C) Alignment of the SIM domain of Rap80. (D) The SIM domain is required for binding to Gst-SUMO2. Purified Gst-SUMO2 on beads were incubated with lysates from cells expressing GFP-tagged wild-type or mutants of Rap80 for the pulldown assay. SS/AA mutant indicates S44A,S46A; FI/AA mutant indicates F40A,I41A. Western blots were carried out with antibodies to GFP. Input is 2.5%.

Figure 4. Rap80 N-terminus 1-129aa fragment containing tandem SIM-UIM-UIM motif binds to SUMO2 and Ubiquitin chains. (A) SIM domain is required for the SIM-UIM-UIM fragment binding to SUMO2 chain. The SIM-UIM-UIM fragments with mutations of the SIM domain (SIM*: F40A,I41A,S44A,S46A; FI/AA: F40A,I41A; SS/AA:S44A,S46A) or UIM domain (UIM*: A88S,S92A,A113S,S117A) were purified from bacteria cells. The purified Gst-tagged proteins on beads (20 ug) were incubated with 150ng SUMO2, 3-8 chains in NETN buffer. The beads were then washed extensively before loaded onto the SDS-PAGE gel. The input lane shows amount of 100 ng corresponding SUMO2 chains. (B) SIM domain is not required for the SIM-UIM-UIM fragment binding to Ub K63 chains. The experiment was performed similarly as described above. The purified Gst-tagged fragments on beads (20 ug) were incubated with 150ng Ub, K63, 2-7 chains instead. (C) Coomassie blue staining of purified Gst-tagged proteins used in the pulldown assay. (D) Rap80 N-terminus 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 hr after transfection, immunoprecipitations were carried out with anti-FLAG antibodies using total cell lysates. Immunoprecipitates were then analyzed by Western blot with anti-myc antibody for detecting SUMO2 conjugates.
Figure 5. Rap80 1-129aa fragment containing tandem SIM-UIM-UIM motif binds to SUMO2 and Ubiquitin chains independently. (A) Binding to SUMO2 chains is not affected by binding to Ub K63 chains. Purified Gst-Rap80 1-129aa fragment (1 ug) on beads was incubated with 120ng SUMO2 3-8 chain in 400 ul NETN buffer overnight in the absence of Ub chain or in the presence of increased amount of Ub K63 2-7 chain for a pull-down assay. Western blots were carried out with anti-SUMO2 or anti-Ub antibodies. (B) Binding to Ub chain is not affected by binding to SUMO2 chain. Purified Gst-Rap80 1-129aa fragment (0.4 ug) on beads was incubated with 120ng Ub 2-7 chain in 400 ul NETN buffer overnight in the absence of SUMO2 chain or in the presence of increased amount of SUMO 3-8 chain for a pull-down assay.

Figure 6. Rap80 N-terminus tandem SIM-UIM-UIM fragment binds to SUMO2 conjugates and Ub K63 conjugates simultaneously. (A) An illustration of the in vitro pulldown assay used in this experiment. (B) The SIM-UIM-UIM fragment binds to both SUMO2 and Ub K63 conjugates at the same time. The SUMO2 argrose beads were first incubated with purified Gst-SIM-UIM-UIM fragments followed by addition of Ub K63 2-7 chains. After extensive wash, bound proteins of the beads were loaded to SDS-PAGE gel for separation. Western blots were carried out with antibodies to Ub or Gst.

Figure 7. Both SIM domain and UIM domains of Rap80 are required for cellular response to DNA damage. (A) Recruitment of SIM and UIM mutant of Rap80 was decreased in response to laser induced DNA damage within 30 minutes of DNA damage. Rap80 -/- MEF cells stably expressing human GFP-Rap80, GFP-Rap80-SIM mt, GFP-Rap80-UIM mt and GFP-Rap80-SIM&UIM mt were treated with UV laser. 30 minutes after DNA damage, cells were fixed and immunostained with antibodies to GFP and γ-H2AX. (B) Both SIM and UIM domains are required for cellular resistance to IR. Rap80 -/- MEF cells stably expressing GFP-tagged wild type (WT), SIM mutant or UIM mutant of Rap80 were generated for the analyses of cellular resistance to IR using a colony-forming assay. (C) A model for Rap80 recruitment to DNA damage sites through its tandem SIM-UIM-UIM motif binding to SUMO2 and Ub K63 conjugates.
Figure 1

U2OS/GFP-SUMO2

0min 1 min 3 min 6 min 9 min 12 min 15 min

U2OS/GFP-SUMO1

0min 1 min 3 min 6 min 9 min 12 min 15 min
Figure 2

A

Gst-SUMO2 purification

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B

Coomassie blue staining

Pull down

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C

Purification

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D

His-SUMO2 purification

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Con 1 2 3 Si Rap80 Con 1 2 3 Si Rap80

Coomassie blue staining

Pull down

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Figure 4

A. Gst-SIM-UIM-UIM + SUMO2 3-8 chain

B. Gst-SIM-UIM-UIM + Ub K63 2-7 chain

C. Coomassie blue staining

D. IP: Flag

HA-Flag-SIM-UIM-UIM
Myc-SUMO2

anti-SUMO2

anti-Ub

(SUMO2)n

(SUMO2)2

(SUMO2)1

anti-myc

anti-HA

by guest on November 18, 2017 http://www.jbc.org/ Downloaded from
Figure 5

A

SUMO2 chain
(α-SUMO2 blot)

Ub chain
(α-Ub blot)

Gst-Rap80 1-129 (1ug)

B

SUMO2 chain
(α-SUMO2 blot)

Ub chain
(α-Ub blot)

Gst-Rap80 1-129 (0.4ug)
Figure 6

A

Beads + Gst-SIM-UIM-UIM

B

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Agarose-SUMO2 beads
Ub K63 chain

anti-Ub
anti-GST
Gst-SIM-UIM-UIM
Figure 7

A

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</table>

B

![Graph showing survival percentages for different conditions](image)

- MEF Rap80-/- IR (5 Gy)
- Rap80-/-
- Rap80 wt
- Rap80 UIM mt
- Rap80 SIM mt
- Rap80 UIM&SIM mt

C

![Diagram showing Rap80 interactions with other proteins](image)