RAP80 Protein Is Important for Genomic Stability and Is Required for Stabilizing BRCA1-A Complex at DNA Damage Sites in Vivo*

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Background: RAP80 can bind Lys-63-linked polyubiquitin chains, thus stabilizing the BRCA1-A complex at DNA damage sites.

Results: RAP80-deficient mice are cancer-prone and hypersensitive to ionizing radiation. RAP80 deficiency impairs BRCA1-A complex focal formation.

Conclusion: RAP80 plays a crucial role in the DNA damage response and in maintaining genomic integrity.

Significance: The in vivo role of RAP80 was investigated.

RAP80 (receptor-associated protein 80) is a ubiquitin-binding protein that can specifically recognize and bind to Lys-63-linked polyubiquitin chains, thus targeting the BRCA1-A complex to DNA damage sites. To study the role of RAP80 in vivo, we generated RAP80-deficient mice. The deficient mice are prone to B-cell lymphomagenesis. B-cell lymphomas in RAP80-deficient mice are nearly diploid but harbor clonal chromosome translocations. Moreover, the deficient mice are hypersensitive to ionizing radiation. Repair of ionizing radiation-induced DNA double-strand breaks is impaired in RAP80-deficient mouse embryonic fibroblasts. Mechanistically, loss of RAP80 suppresses recruitment of the BRCA1-A complex to DNA damage sites and abrogates the DNA damage repair process at DNA damage sites. Taken together, these results reveal that RAP80 plays a crucial role in the DNA damage response and in maintaining genomic integrity.

DNA lesions frequently occur during normal cellular process, such as DNA replication, or following exposure to various environmental hazards, such as UV light and ionizing radiation (IR).4 To maintain genomic integrity and to prevent the accumulation of DNA lesions or transmission of the damaged genetic information from mother cells to daughter cells, sophisticated cell cycle checkpoint pathways and DNA damage repair machineries are developed (1, 2). Among various kinds of DNA lesions, the DNA double-strand break (DSB) is the most deleterious type of DNA damage, and DSBs can drastically alter genomic stability and induce tumorigenesis. In response to DSBs, a large group of DNA damage response factors are quickly recruited to DNA damage sites, which activate cell cycle checkpoints and DNA repair networks (3). Loss of these DNA damage response factors causes genomic instability and tumorigenesis (4, 5). Interestingly, these DNA damage response factors often form large multisubunit protein complexes. One typical example is the BRCA1 complex.

BRCA1 (breast cancer susceptibility gene 1) is a familial breast tumor suppressor and plays an important role in cell cycle checkpoint activation and DNA damage repair in response to DSBs (6, 7). Correlated with the multiple functions of BRCA1 in the DNA damage response, it is the central component of at least three different complexes in the nucleus, known as the BRCA1-A, BRCA1-B, and BRCA1-C complexes (8–10). Whereas the BRCA1-B and BRCA1-C complexes are involved in the different steps of DSB repair, the BRCA1-A complex, including BRCA1, RAP80, CCDC98, BRCC36, BRCC45/BRE, and MERIT40/NBA1/HSPC142, is known to target BRCA1 to DNA damage sites (11–17). Among these subunits, RAP80, a ubiquitin-binding protein, contains two tandem ubiquitin-interacting motifs that facilitate the relocation of BRCA1 to DNA damage sites via the interaction with ubiquitinated histones at DNA damage sites (9, 14, 15, 18). CCDC98 directly interacts with BRCA1 between its C-terminal phospho-Ser motif and the BRCT domain of BRCA1 (11, 12). BRCC36 is a deubiquitinating enzyme (DUB) that specifically digests Lys-63-based polyubiquitin chains (19–21). Both BRCC45 and MERIT40 are adaptors in the BRCA1-A complex (13, 16, 17). Both BRCC36 and BRCC45 may also mediate the interaction between BRCA1 and BRCA2 (22).

Although all of the subunits in the BRCA1-A complex have been identified using unbiased protein affinity purification, the in vivo function of this complex remains elusive. Particularly, the germ-line mutation of RAP80 has been identified in familial breast cancer patients (23), suggesting that RAP80 is important.

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4 The abbreviations used are: IR, ionizing radiation; DSB, double-strand break; DUB, deubiquitinating enzyme; MEF, mouse embryonic fibroblast; Gy, gray(s).
for maintaining genomic stability and tumor suppression. To this end, we generated RAP80-deficient mice and examined the function of RAP80 in vivo.

MATERIALS AND METHODS

RAP80-deficient Mice and Mouse Embryonic Fibroblasts—RAP80-deficient mice were generated from the embryonic stem cell line RNN158 (purchased from BayGenomics). Mice were maintained in a specific pathogen-free facility at the University of Michigan Medical School, and prior approval for animal experiments was obtained from the University Committee on the Use and Care of Animals. The health of mice was monitored daily, and moribund mice were killed to examine tumors throughout the body.

Animals were genotyped by PCR of tail DNA. The following primers were used: primer A (5’-CCAAATTCTTTTGGGATCATCTCTCCAG-3’, sense) and primer B (5’-ACTAGGTCTCAGTGCTACACAAAC-3’, antisense) for the wild-type allele and primer A and primer C (5’-AAAAAGGTTTCTTGAAGACAGAGG-3’, antisense) for the Rap80 knock-out allele. The reaction was performed in a 25-μl reaction mixture containing 50 ng of DNA, 200 nM primer (A, B, or C), 0.6 mM MgCl₂, 0.1 mM each dNTP, and 0.25 units of Taq polymerase. The cycling conditions were 5 min at 93 °C (one cycle); 1 min at 93 °C, 1 min at 58 °C, and 1 min at 72 °C (35 cycles); and 5 min at 72 °C (one cycle). The wild-type Rap80 gene is indicated by the presence of 383-bp PCR fragments (amplicon of primers A and B), whereas the mutant allele is indicated by the presence of 402-bp PCR fragments (amplicon of primers A and C).

Antibodies, Plasmids, and Cell Cultures—Antibodies raised against mouse Rap80 and BRCA1 were generated by immunizing rabbits with GST fusion proteins (RAP80 residues 1–354 and BRCA1 residues 1445–1812) (24). Anti-mouse γH2AX, anti-ubiquitin (FK2), and anti-mouse GAPDH antibodies were purchased from Upstate. Anti-FLAG antibody (M2) was from Sigma. Human anti-Lys-63 polyubiquitin antibody (clone Apu3.A8) was from Genentech, and rhodamine-conjugated goat anti-human IgG (GenWay Biotech) was used as a secondary antibody. FLAG-tagged full-length BRCC36, CCDC98, and Apu3.A8 was from Genentech, and rhodamine-conjugated goat anti-human IgG (GenWay Biotech) was used as a secondary antibody. FLAG-tagged full-length BRCC36, CCDC98, and MERIT40 were stably expressed in mouse embryonic fibroblasts (MEFs) using standard protocols. Cells were cultured in Dulbecco’s modified Eagles medium with 10% (v/v) FBS. For IR treatment, cells were irradiated using a J. L. Shepherd137Cs radiation source with the indicated doses and then recovered to room temperature for 5 min. The cells were blocked with PBS containing 8% goat serum at room temperature for 30 min and primary antibodies at room temperature for 1 h. After washing three times with PBS, cells were incubated with secondary antibody, FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, rhodamine-conjugated goat anti-rabbit IgG, or rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at room temperature for 30 min. Cell nuclei were then counterstained with DAPI. After a final wash with PBS, coverslips were mounted with glycerin containing p-phenylenediamine. All images were obtained with an Olympus IX71 fluorescence microscope. For each sample, we examined 1000 cells. Because spontaneous foci exist in untreated cells, cells with more than five foci in each nucleus were considered as focus-positive cells following IR treatment.

Metaphase Spread—Wild-type and Rap80−/− MEFs were first treated with 1 Gy of IR, recovered for 1 h, and then incubated with 50 ng/ml colcemid for another 3 h. Cells were harvested and washed three times with PBS. Cells were then resuspended in 75 mM KCl for 30 min at room temperature. Cells were fixed in freshly prepared Carnoy’s fixative (3:1 methanol/acetic acid), dropped onto a slide, and stained with Giemsa solution. Metaphase spreads were observed using a 60× oil immersion lens under a light microscope. Spreads were photographed, and chromosome breaks were counted.

Spectral Karyotyping and Image Analyses—Freshly prepared metaphase spreads from lymphomas were used for spectral karyotyping analysis. The assay was carried out using spectral karyotyping paints (Applied Spectral Imaging, Carlsbad, CA) according to the manufacturer’s instructions (25). Spectral images were acquired and analyzed with an Applied Spectral Imaging system attached to an Olympus BX61 microscope.

RESULTS

Generation of RAP80-deficient Mice—In the embryonic stem cell line RNN158, the Rap80 gene is disrupted by an insertion of a β-geo selection cassette (a fusion of β-galactosidase and neo-mycin phosphotransferase II) between exons 2 and 3 (Fig. 1A). The insertion site was mapped by genomic PCR and DNA sequencing. The embryonic stem cells were injected into C57BL/6 blastocysts to generate chimeric mice, which were then back-crossed with C57BL/6 mice to obtain Rap80−/− mice. The heterozygotes were intercrossed to generate Rap80−/− mice and confirmed by genotyping using genomic PCR (Fig. 1B). Because the ATG translation start site is in exon 2, the β-geo cassette insertion created a fusion transcript containing a region encoding only the N-terminal 49 residues of RAP80 and β-geo, which abolished the transcription and translation of all of the important domains of RAP80, such as the N-terminal ubiquitin-interacting motifs that target it to DNA damage sites. Thus, this chimera could not be detected by Western blotting using anti-mouse RAP80 antibody in the Rap80−/− MEFs (Fig. 1C).

RAP80-deficient Mice Are Prone to Tumorigenesis—Although we did not observe the obvious developmental defect of RAP80-deficient mice, compared with wild-type or heterozygous mice, Rap80−/− mice had a substantially reduced life span.
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Mice and MEFs Are Hypersensitive to IR

B-cell lymphomas were likely induced by clonal translocations. Because we found only one karyotype per tumor sample, or close to diploid and displayed chromosome translocations. The tumor penetrance in Rap80+/− mice was 66% (27 of 41), compared with the 33% tumor penetrance in Rap80−/− mice (8 of 24) (Fig. 2B). Interestingly, most tumors identified in Rap80−/− mice were B-cell lymphomas with a significantly increased mitotic index (Fig. 2, C and D). The tumor cells were B220 and IgM double-positive cells (Fig. 2D). To elucidate the mechanism of B-cell lymphomagenesis, we examined the karyotype of five tumors from Rap80-deficient mice by spectral karyotyping analysis. As shown in Fig. 2 (E–H), all five B-cell lymphomas were diploid or close to diploid and displayed chromosome translocations. Because we found only one karyotype per tumor sample, these lymphomas were likely induced by clonal translocation of chromosomes. Thus, it is likely that clonal chromosome translocations induced by chromosome breaks and inefficient DNA repair eventually cause B-cell lymphomagenesis in Rap80−/− mice.

Rap80−/− Mice and MEFs Are Hypersensitive to IR—To further examine the role of RAP80 in the DNA damage response, Rap80-deficient mice were treated with 7.5 Gy of IR. Within 20 days, 80% of the Rap80+/− mice (n = 10) died, whereas most of the Rap80+/− and Rap80−/− mice were viable after the same dose of IR (Fig. 3A). Moreover, consistent with the whole body irradiation, Rap80−/− MEFs were also hypersensitive to IR compared with wild-type MEFs, whereas reconstitution of RAP80 in the Rap80−/− MEFs rescued the IR-induced cell lethality (Fig. 3B). To examine the role of RAP80 in the repair of DSBs, we directly counted the chromosome breaks in MEFs following low dose IR treatment. As shown in Fig. 3C, with 1 Gy of IR, 52% of the Rap80−/− MEFs harbored at least one unrepaired chromosome break during mitosis. After the same dose of IR treatment, only 12% of the Rap80+/− MEFs contained IR-induced chromosome breaks. Thus, these results suggest that loss of RAP80 suppresses DSB repair.

RAP80 Is Required for Stabilizing BRCA1-A Complex at DNA Damage Sites—Following DSBs, a protein ubiquitination cascade governed by RNF8 and Ubc13 is activated and facilitates damage repair (26–32). The Ubc13-dependent Lys-63-linked polyubiquitin chains are synthesized at DNA damage sites for recruiting other DNA damage response factors. The tandem ubiquitin-interacting motifs of RAP80 specifically recognize the Lys-63-linked polyubiquitin chains and facilitate the recruitment of BRCA1 to DNA damage sites (9, 14, 15, 18, 33). To examine whether the recruitment of the entire BRCA1-A complex to DNA damage sites is dependent on RAP80, we treated Rap80+/− and Rap80−/− MEFs with 2 Gy of IR. Consistent with previous observations using siRNA to down-regulate RAP80 (9, 14, 15, 17–19), the complete loss of RAP80 in MEFs significantly suppressed the relocation of BRCA1 to DNA damage sites (Fig. 4, A and B). Other subunits in the BRCA1-A complex, including CCDC98, BRCC36, and MERIT40, failed to relocate to DNA damage sites in the Rap80−/− MEFs, whereas reconstitution of RAP80 in the Rap80−/− MEFs rescued the their focal formation at the DNA damage sites (Fig. 4, A and B), suggesting that RAP80 is critical to target the entire BRCA1-A complex to DNA damage sites.

Loss of RAP80 Suppresses DNA Damage Repair Process at DNA Damage Sites—The major biological function of the BRCA1-A complex remains elusive. Although BRCA1 is a RING domain E3 ubiquitin ligase, other subunits in this complex, including RAP80, CCDC98, BRCC36, and MERIT40, are organized similarly to the complex of the 19 S proteasome (16). In particular, BRCC36 is a DUB that specifically digests Lys-63-linked polyubiquitin chains (20). Thus, it is intriguing that the BRCA1-A complex contains both E3 ligase and DUB. Because RAP80 facilitates the relocation of both BRCA1 and BRCC36 to DNA damage sites, the protein ubiquitination at DNA damage sites could be precisely regulated. To examine the role of RAP80 in the dynamic protein ubiquitination at DNA damage sites, we monitored the conjugation of ubiquitin at DNA damage sites in Rap80+/+ and Rap80−/− MEFs. Using monoclonal antibody FK2, we first examined the kinetics of the overall ubiquitin conjugation at DNA damage sites. As shown in Fig. 5 (A and B), after treatment with 0.5 Gy of IR, protein ubiquitination occurred at DNA damage sites within 1 h in both Rap80+/+ and Rap80−/− MEFs, suggesting that RAP80 does not regulate early DNA damage-induced protein ubiquitination cascades. In Rap80+/+ MEFs, the ubiquitin signals started to fade at 8 h following DNA damage and almost disappeared at 24 h after DNA damage. However, ubiquitin was still conjugated at DNA damage sites (marked by γH2AX foci) at 24 h after IR treatment in Rap80−/− MEFs. Reconstitution of RAP80 in Rap80−/− MEFs facilitated the fading of ubiquitination and γH2AX foci. These results indicate that RAP80 is important for the DNA damage repair process and the deubiquitination at DNA damage sites. Because RAP80 facilitates the relocation of BRCC36 to DNA damage sites and BRCC36 is the DUB that digests Lys-63-linked polyubiquitin chains, we also examined the Lys-63-linked polyubiquitin signals at DNA damage sites. Again, Lys-
FIGURE 3. Rap80-/- mice and MEFs are hypersensitive to IR. A, Kaplan-Meier survival curve of Rap80+/+, Rap80+/−, and Rap80−/− mice following whole body IR (7.5 Gy). B, increased radiation sensitivity was observed in Rap80−/− MEFs, and reconstitution of RAP80 in Rap80−/− MEFs rescued the severity of IR-induced cell lethality. The y axis represents the percentage of surviving cells relative to unirradiated control cells of the same genotype. C, DNA damage repair is abrogated in RAP80-deficient cells. Rap80+/+ and Rap80−/− MEFs were treated with 1 Gy of IR, and chromosome breaks were counted in at least 100 metaphases in each genotype of MEFs. Representative chromosomal spreads of wild-type and Rap80−/− MEFs are shown in the left panel, and chromosome break frequencies are summarized in the right panel. Arrows indicate DNA breaks.

FIGURE 2. RAP80-deficient mice are prone to tumorigenesis. A, Kaplan-Meier plot of the overall survival of Rap80−/−, Rap80+/−, and Rap80+/+ mice. B and C, spontaneous tumor incidence and tumor spectrum of Rap80−/−, Rap80+/−, and Rap80+/+ mice. D, representative images of B-cell lymphoma (left panel; arrows indicate lymph nodes), H&E staining of the B-cell lymphoma (middle panel; arrows indicate mitotic cells), and flow cytometry analysis of tumor cells (right panel). APC, allophycocyanin. E–H, B-cell lymphomas in Rap80−/− mice display clonal translocations. The results from spectral karyotyping analysis of a representative metaphase from a B-cell lymphoma are shown as inverted DAPI staining (E), spectra-based classification color (F), and alignment of DAPI and classification color (G). A summary of spectral karyotyping analysis results from five Rap80−/− mouse B-cell lymphomas is provided in H.

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63-linked polyubiquitin signals failed to be removed in Rap80<sup>−/−</sup> MEFs after 24 h of recovery following low dose IR treatment, and reconstitution of RAP80 in Rap80<sup>−/−</sup> MEFs or Rap80<sup>−/−/+</sup> MEFs expressing RAP80 were subjected to immunofluorescence staining. Endogenous BRCA1 and stably expressed FLAG-tagged BRCC36, CCDC98, and MERIT40 were detected with the corresponding antibodies. γH2AX was used as a focus-positive control. B, focus-positive cells are summarized. Data are from three independent experiments, and error bars represent S.D.

**DISCUSSION**

The BRCA1-A complex plays an important role in the DNA damage response. In this study, we analyzed the function of the BRCA1-A complex by generating RAP80-deficient mice because RAP80 is the key subunit in this complex and facilitates the relocation of the complex to DNA damage sites. In contrast to BRCA1-deficient mice (34, 35), Rap80<sup>−/−</sup> mice were viable and fertile, suggesting that the BRCA1-A complex is not essential for cell viability. In addition to the BRCA1-A complex, BRCA1 also associates with other DNA damage factors to form the BRCA1-B and BRCA1-C complexes, which may participate in the different steps during DSB repair and are important for cell viability (36–39). However, consistent with the role of RAP80 and the entire BRCA1-A complex in the DNA damage response, Rap80<sup>−/−</sup> mice developed B-cell lymphomas. Among somatic cells, B-lymphocytes are particularly prone to DSB formation (40–42). During B-cell development and immune responses, B-cells undergo programmed DNA damage processes such as V(D)J recombination, somatic hypermu-
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FIGURE 5. Prolonged IR-induced ubiquitin foci exist in RAP80-deficient cells. Wild-type and Rap80−/− MEFS and Rap80−/− MEFS expressing Rap80 were treated with 0.5 Gy of IR, and cells were fixed at the indicated times. The ubiquitin (Ub) and γH2AX foci were detected by immunofluorescence staining with antibody FK2 (A) or anti-Lys-63 ubiquitin antibody (C). Ubiquitin focus-positive and γH2AX focus-positive (B) and Lys-63-linked ubiquitin focus-positive (D) cells are summarized. Data are from three independent experiments.

FIGURE 6. Model for BRCA1-A complex in response to DNA damage.

The BRCA1-A complex contains both E3 ligase and DUB. How could an E3 ligase and DUB function together in response to DNA damage? It has been shown that BRCC36 digests only Lys-63-linked polyubiquitin chains, but not monoubiquitin or other types of polyubiquitin chains (15, 19–21, 47). However, BRCA1 has been shown to monoubiquitinate histones or to catalyze Lys-6-based polyubiquitin chains (48–52). Thus, it is likely that BRCA1 and BRCC36 play independent roles in response to DNA damage. For example, BRCC36 deubiquitinates Lys-63-linked polyubiquitin chains on one substrate, whereas BRCA1 catalyzes monoubiquitin or Lys-6-based polyubiquitin chains on the same or different substrates (Fig 6) (49, 53, 54). The specificity of protein ubiquitination may be important for different steps during DNA damage repair. Loss of RAP80 suppresses the relocation of both E3 ligase and DUB to DNA damage sites. However, we did not observe the significant suppression of protein ubiquitination at DNA damage sites. It is possible that BRCA1-dependent protein ubiquitination accounts for a very small portion of protein ubiquitination at DNA damage sites. A specific antibody that recognizes only BRCA1-dependent ubiquitination events might reveal the defects in Rap80−/− MEFS in the future. Alternatively, although a large portion of BRCA1 fails to be retained at DNA damage sites upon loss of RAP80, it is possible that relocation of a small amount of BRCA1 to the DNA damage sites via the association with other protein complexes may be sufficient for retaining most BRCA1-dependent function during DNA damage repair. In Rap80−/− MEFS, we found that deubiquitination, especially deubiquitination of Lys-63-linked polyubiquitin...
chains, was suppressed, which was associated with the retention of γH2AX at DNA damage sites. It is possible that RAP80/BRCC36 antagonizes the function of RNF8/Ubc13 for the dynamic assembly and disassembly balance of Lys-63-linked polyubiquitin chains, which could be important for the different steps of DNA damage repair. Alternatively, the retention of ubiquitination indicates that the BRCA1-A complex is important for DNA damage repair that is associated with the ubiquitination at DNA damage sites.

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