MRG15 Is a Novel PALB2-interacting Factor Involved in Homologous Recombination

PALB2 is an integral component of the BRCA complex important for recombinational DNA repair. However, exactly how this activity is regulated in vivo remains unexplored. Here we provide evidence to show that MRG15 is a novel PALB2-associated protein that ensures regulated recombination events. We found that the direct interaction between MRG15 and PALB2 is mediated by an evolutionarily conserved region on PALB2. Intriguingly, although damage-induced RAD51 foci formation and mitomycin C sensitivity appeared normal in MRG15-binding defective PALB2 mutants, these cells exhibited a significant increase in gene conversion rates. Consistently, we found that abrogation of the PALB2-MRG15 interaction resulted in elevated sister chromatid exchange frequencies. Our results suggest that loss of the PALB2-MRG15 interaction relieved the cells with the suppression of sister chromatid exchange and therefore led to a hyper-recombination phenotype in the gene conversion assay. Together, our study indicated that although PALB2 is required for proficient homologous exchange and therefore led to a hyper-recombination phenotype in the gene conversion assay. Together, our study indicated that although PALB2 functions upstream of the BRCA2-MRG15 interaction is important for the suppression of sister chromatid exchange and thereby increases gene conversion rates. Consistently, we found that abrogation of the PALB2-MRG15 interaction resulted in elevated sister chromatid exchange frequencies. Our results suggest that loss of the PALB2-MRG15 interaction relieved the cells with the suppression of sister chromatid exchange and therefore led to a hyper-recombination phenotype in the gene conversion assay. Together, our study indicated that although PALB2 is required for proficient homologous recombination, it could also govern the choice of templates used in homologous recombination repair.

The tumor suppressor protein PALB2 plays a crucial role in homologous recombinational repair. Previous studies indicated that PALB2 functions upstream of the BRCA2-RAD51 axis, where it is essential for the loading of the repair machinery to the damaged chromatin to facilitate DNA repair (1, 2). We and others have recently demonstrated that PALB2 is the link between the BRCA1 and BRCA2 tumor suppressors and orchestrates DNA repair in response to DNA damage (3, 4). Sequence alignment of PALB2 homologues revealed several conserved regions (see Fig. 1A). Although the BRCA2-interacting domain was mapped to the C-terminal leads to impaired DNA repair in vivo, suggesting that these highly conserved regions are important for PALB2 function in homologous recombination.

Apart from the N and C termini of PALB2, bioinformatic analysis revealed another highly conserved region in the middle of the PALB2 coding sequence (see Fig. 1A, region B). Although no function has been ascribed to this region of the PALB2 protein, we found that this conserved region (i.e. region B), missing from the PALB2-deficient Fanconi anemia (FA) patient cells (EUF1341F1) that express a truncated PALB2 mutant (residues 1–500), was restored during spontaneous reversion (5). Notably, this particular revertant supported normal levels of RAD51 foci formation and restored mitomycin C (MMC) resistance in the patient cells. Sequencing analysis indicated that this revertant, containing an internal deletion (residues 71–561), harbors all of the three conserved regions (i.e. regions A–C). From these observations, we speculated that apart from its ability to interact with BRCA1 and BRCA2, the conserved region B might also play an important role in the regulation of PALB2 function in vivo.

MRG15 belongs to a highly conserved protein family that contains the MRG domain responsible for transcriptional regulation via chromatin remodeling by histone acetylation (6). Its yeast homologue Eaf3 has been demonstrated to be a component of both the NuA4 histone acetyltransferase and the Rpd3 histone deacetylase complexes and affects global acetylation (7–9). MRG15 has been demonstrated to bind directly to methylated lysine 36 on histone H3 peptide and was functionally correlated to the acetylation of lysine 16 on histone H4 (10–12). It was demonstrated that Eaf3, via its chromo domain-mediated binding to methylated lysine 36 on histone H3, allows specific recruitment of the Rpd3S histone deacetylase complex (13).

In the current study, we have identified MRG15 as a novel interacting partner of PALB2 that binds to a previously uncharacterized conserved region on PALB2 (see Fig. 1A, region B). In keeping with the importance of the conservation of functional protein motifs, we demonstrated that the PALB2-MRG15 interaction is important for the suppression of sister chromatid-mediated homologous recombination.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies against the FLAG epitope (M2) were purchased from Sigma. Rabbit polyclonal anti-RAD51 (D51), anti-PALB2, anti-BRCA2 (C25), and anti-pH2AX antibodies were described previously (4, 14). Mouse

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anti β-actin antibody was purchased from Sigma. To facilitate the study of endogenous MRG15 protein, we generated a rabbit polyclonal antibody raised against recombination GST-MRG15 purified from Escherichia coli.

Cell Cultures—Cells were maintained in RPMI supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin and kept in a 37 °C incubator with 5% CO2.

Constructs—pOZC-PALB2 (a gift from Dr. David Livingston (Dana-Farber Cancer Institute)) was subcloned into the entry vector pDONR201 (Invitrogen). Mutations or deletions of PALB2 were generated using site-directed mutagenesis (QuikChange, Stratagene).

Tandem Affinity Purification (TAP)—293T cells expressing SFB-PALB2 F3 or SFB-MRG15 were used for the purification of protein complex(es). TAP procedures were described in Ref. 4. Tandem Affinity Purification (TAP)—293T cells expressing SFB-PALB2 F3 or SFB-MRG15 were used for the purification of protein complex(es). TAP procedures were described in Ref. 4.

RNA Interference—A non-targeting siRNA and siRNA specific targeting human MRG15, MRGX, PALB2, and BRCA2 were purchased from Dharmaco. Cells were seeded at 30% confluence for 24 h before double siRNA transfection using Oligofectamine (Invitrogen). Cells were harvested 48 h after the second siRNA transfection.

Immunoprecipitation and Pulldown Experiments—Cells were lysed in NETN (20 mm Tris-HCl, pH 8, 100 mm NaCl, 1 mm EDTA, 0.5% Nonidet P-40, 1 mm MgCl2) buffer containing Benzonase (Novagen). For immunoprecipitation or pulldown experiments, cell extracts were incubated with either 5-agarose (EMD Biosciences) or GST fusion proteins immobilized on glutathione-beads for 2 h at 4 °C. Beads were washed with NETN buffer, and proteins were eluted by boiling in 2× Laemmli buffer. Samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Immunoblottings were subsequently performed with antibodies as indicated.

Immunofluorescence Staining—Cells grown on coverslips were pre-extracted with buffer containing 0.5% Triton X-100 and fixed using 3% paraformaldehyde solution. Immunostaining experiments were performed using MRG15, RAD51, and pH2AX antibody. Cells were mounted onto glass slides in DAPI-containing antifade. Immunofluorescent analyses and image capturing were performed on a Nikon Eclipse 800 microscope.

MMC Sensitivity Assay—Cells were seeded at a density of 1 × 103 cells in triplicate onto 96-well plates. A different concentration of MMC was added 24 h after cell seeding. Cells were incubated for another 5 days. MMC sensitivity was monitored by the Alamar blue assay (Biosource). Absorbances at 570/600 nm were measured using the TECAN SAFIRE plate reader. Results were the averages of data from three independent experiments.

Gene Conversion Assay—1 × 106 cells were electroporated with 10 μg pCBASce plasmid at 270 V, 975 microfarads using a Bio-Rad gene pulser II (15). Cells were plated and incubated in culture media for 48 h prior to fluorescence-activated cell sorter analyses. Cells were analyzed in a BD Biosciences FACScan on a green (FL1) versus orange (FL2) fluorescence plot. Results were the average of data obtained from two independent experiments.

Sister Chromatin Exchange Assay—Cells were incubated with 10 μM BrdUrd for 48 h and then chased for 24 h. Cells were treated with Colcemid for 8 h and harvested for the preparation of metaphase spread. For MMC treatment, 10 μM MMC was added to the media 6 h before harvest. Metaphases were spread onto slides and aged for 5 days. Prior to the incubation with mouse anti-BrdUrd antibody, slides were denatured with 70% formamide/4× SSC and dried with an ethanol series. Rhodamine-conjugated anti-mouse antibody was used to visualize chromatids labeled with BrdUrd. Chromosomes were counterstained with DAPI.

RESULTS AND DISCUSSION

To identify proteins that interact with PALB2 through its conserved region B, we used 293T cells stably expressing an SFB-tagged PALB2 fragment (F3) (Fig. 1A) for tandem affinity purification. Mass spectrometry analysis identified MRG15 as a major binding partner of PALB2 F3, along with its close homologue MRGX (Fig. 1B and supplemental Fig. 1A). In addition, MRG15 was also co-purified with full-length PALB2 when we performed TAP using 293T cells expressing SFB-tagged full-length PALB2. To confirm the interaction between PALB2 and MRG15, we performed reverse TAP using 293T cells expressing SFB-tagged MRG15. Consistently, PALB2 was co-purified with MRG15 along with other known MRG15-binding partners including p400 and BRD8 (Fig. 1B). Reciprocal co-immunoprecipitation experiments also verified a specific interaction between PALB2 and MRG15 (Fig. 1, C and D). MRG15 chromo
domain fragment (F1) and MRG domain fragment (F2) were generated, and a co-immunoprecipitation experiment indicated that MRG15 interacts with PALB2 via its MRG domain (supplemental Fig. 1, B and C).

MRG15 belongs to the MRG domain-containing protein family, which is highly conserved among organisms including yeast, Caenorhabditis elegans, and Drosophila (6). Although the MRG domain is highly conserved, MRG15 is the only member in the family that harbors a chromo domain on its N-terminal, which has been shown to be capable to bind methylated H3K36 lysine 36 (10, 12). Intriguingly, MRG15 knock-out mice manifested DNA repair defects in which the recruitment of repair proteins including 53BP1 to sites of DNA damage was delayed (11). It is not clear what is the MRG15 function in DNA repair. The interaction between MRG15 and PALB2 prompted us to investigate whether MRG15 serves as a cofactor in the PALB2-dependent DNA repair.

Because PALB2 localizes at sites of DNA breaks upon DNA damage, we next asked whether MRG15 might similarly be recruited to DSBs. Immunofluorescent staining revealed that following ionizing radiation, endogenous MRG15 localized to sites of DNA breaks that are marked by γH2AX (Fig. 1E). These results indicated that PALB2 and MRG15 may function together in response to DNA damage.

To further understand exactly how PALB2 interacts with MRG15, we used a series of SFB-tagged PALB2 mutants to map the MRG15-binding region (Fig. 2A). Pulldown experiments using GST-MRG15 fusion protein suggested that the PALB2 and MRG15 interaction requires the conserved region B of PALB2 (residues 611–764; Fig. 2B). Further experiments using a series of PALB2 deletion mutants reassured us that this region B of PALB2 is the only region that mediates its interaction with MRG15 (Fig. 2C). In addition, co-immunoprecipitation experiments showed that although wild-type PALB2 associated readily with an MRG15, PALB2 deletion mutant that lacks residues 611–764 did not (Fig. 2C). Furthermore, disruption of either the oligomerization and BRCA1-interacting motif (ΔN42) or the BRCA2-binding domain (ΔC32) of PALB2 has no obvious impact on the PALB2-MRG15 interaction (Fig. 2, B and D). The fact that the MRG15-binding

**FIGURE 2.** PALB2 interacts with MRG15 via its conserved domain C, which contains residues 611–764. A, schematic presentation of PALB2 deletion mutants used in the study. The MRG15 binding property of these PALB2 mutants is summarized. WT, wild type. B and C, lysates prepared from 293T cells expressing various SFB-PALB2 mutants were subjected to a pulldown assay using beads coated with GST-MRG15. CMB, Coomassie Blue. D, co-immunoprecipitation (IP) experiments were performed using lysates prepared from 293T cells expressing Myc-tagged wild-type or mutant PALB2 together with SFB-MRG15. WB, immunoblot.
motif on PALB2 is well separated from its other interaction domains suggests that MRG15 possibly serves as an additional factor to modulate the PALB2-dependent DNA repair process.

To assess whether the interaction with MRG15 is critical for the PALB2-dependent DNA repair, we reconstituted the EUF1341F PALB2-deficient cell with constructs encoding wild-type PALB2, PALB2 Δ611–764, or empty vector. Both wild-type PALB2 and the Δ611–764 deletion of PALB2 displayed discrete ionizing radiation-induced DNA damage foci that co-localized with pH2AX (data not shown), which agrees with our earlier study that suggests that only the N terminus of PALB2 is required for its focus localization following DNA damage (2). Because PALB2 is known to be required for RAD51 foci formation, homologous recombination, and MMC resistance in vivo (1–5), we further examined whether the reconstitution of PALB2-deficient cells with PALB2 Δ611–764 mutant could restore these PALB2-dependent functions. Surprisingly, we found that EUFA1341F cells reconstituted with either wild-type PALB2 or PALB2 Δ611–764 mutant fully restored RAD51 foci formation, whereas cells reconstituted with empty vector alone did not (Fig. 3A).

In mammalian cells, the major repair pathways exploited to repair DSBs are the homologous recombination pathway and non-homologous end joining (3). As shown in Fig. 3B, PALB2-MRG15 interaction results in a hyper-rec phenotype. Likewise, hyper-rec phenotype was also observed in U2OS cells with depletion of endogenous PALB2 and the reintroduction of PALB2 Δ611–764 mutant when compared with the wild-type PALB2 reconstituted cells (supplemental Fig. 2A). Consistently, siRNA-mediated MRG15 knockdown significantly enhanced gene conversion efficiency (Fig. 3D), suggesting that MRG15 and its interaction with PALB2 may be responsible for the hyper-recombination phenotype we observed here. As a control, we examined the expression levels of PALB2, BRCA2, and MRG15 in these siRNA-transfected cells (Fig. 3E). Results indicated that although PALB2 or BRCA2 knockdown led to the destabilization of each other (1), knockdown of MRG15 did not affect the expression levels of either PALB2 or BRCA2, and thus, this rules out the possibility that the hyper-recombination phenotype observed in MRG15-depleted cells was due to an indirect effect on PALB2 or BRCA2 expression.

In mammalian cells, the major repair pathways exploited to repair DSBs are the homologous recombination pathway and...
the non-homologues end-joining pathway. Homologous recombination allows accurate repair of DSBs with the use of homologous templates (16). During the cell cycle, cells employ homologous chromosomes as templates for recombinational repair in S and G2 phases. The use of sister chromatids during homologous recombination may also occur under some circumstance in mitotic cells for repairing DSBs, especially when the recombination between homologues might lead to loss of heterozygosity in mitotic cells, which could result in inactivation of both alleles of tumor suppressors.

The hyper-recombination phenotype observed in cells reconstituted with PALB2 Δ611–764 mutant raised the possibility that PALB2 may be normally responsible for the suppression of recombination or the sister chromatid exchange (SCE). We thus performed the SCE assay using EUFA1341F cells reconstituted with either wild-type PALB2 or Δ611–764 mutant of PALB2. Analyses of metaphase chromosomes clearly indicated that cells expressing only PALB2 Δ611–764 mutant have a significantly higher rate of SCE than that observed in cells expressing wild-type PALB2 (Fig. 3, F and G), although both of these cells are fully capable of restoring RAD51 foci and therefore affects the frequency of recombination. Interestingly, MRG15 and its homologue MRGX exist in similar protein complexes involving chromatin remodeling. Because MRG15 and its homologue MRGX are also identified as components of the PALB2-dependent repair pathway. A histone acetyltransferase was shown to bind to chromatin surrounding sites of DSBs in vivo. Tip60-Trrap deficiency impaired damage-induced histone H4 hyperacetylation, impeded chromatin accessibility to repair proteins, and resulted in defective homologous recombination (19). As such, there is a possibility that MRG15, MRGX may also regulate sister chromatid exchange frequency in the cell. The exact mechanism as to how MRG15 and also MRGX participate in DNA repair and recombination requires further investigations.

In conclusion, our findings here highlight that PALB2 not only regulates the efficiency of homologous recombination but also dictates the specific homologous recombination pathway in which it participates. These attributes are likely critical for the maintenance of genomic stability in the cell. Further studies into the underlying mechanism as to how PALB2 dictates the repair mechanisms should warrant a more comprehensive understanding on the role of PALB2 in tumor suppression.

Acknowledgments—We thank Prof. David Livingston for the pOZC-PALB2, Prof. Maria Jasin for the DR-GFP and pCRASce plasmids, Prof. Johan P. de Winter for EUFA1341 cell, and Prof. Ben Turk for the TECAN SAFIRE plate reader.

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