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 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 recombination 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 conserved C terminus, which contains the WD40 repeats (see Fig. 1A, region C) (2), the BRCA1-binding motif was mapped to the N-terminal conserved coiled-coil domain (see Fig. 1A, region A). Interestingly, the same region was found to be responsible for PALB2 oligomerization and foci formation (2). Accordingly, disruption of either the N-terminal or 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 (EUFA1341F) 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)4 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 Ea3 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 Ea3, 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 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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Figure 1. MRG15 is a PALB2-binding protein. A, schematic presentation of PALB2. Three conserved regions A, B, and C are identified in the alignment of PALB2 homologues, as identified in this study, B, the identification of PALB2- or MRG15-interacting protein(s) by TAP. Proteins identified by mass spectrometry analysis and the numbers of peptide hits are listed. C, MRG15 interacts with PALB2. 293T cells were transfected with plasmids encoding Myc-PALB2 together with plasmids encoding SFB-RAD51 or SFB-MRG15. Lysates were subjected to immunoprecipitation (IP) using anti-Myc antibodies and immunoblotted (WB) with antibodies as indicated. D, co-immunoprecipitation of endogenous proteins was conducted using HeLa lysates and anti-MRG15, anti-PALB2 antibodies, or prebleed serum. Immunoblotting was performed using indicated antibodies. E, HeLa or U2OS cells were irradiated with 10 grays of ionizing radiation and recovered for 5 h. Immunofluorescence staining was performed using the antibodies as indicated.

The study of endogenous MRG15 protein, we generated antibody to MRG15, along with other known MRG15-binding partners (Fig. 1B), reciprocal co-immunoprecipitation experiments also verified a specific interaction between PALB2 and MRG15 (Fig. 1, C and D). MRG15 chromosome experiments were performed using MRG15, RAD51, and pH2AX antibodies. Cells were mounted onto glass slides in DAPI-containing antifade. Immunofluorescent analyses of image capturing were performed on a Nikon Eclipse 800 microscope.

MMC Sensitivity Assay—Cells were seeded at a density of 1 × 10^4 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 average of data obtained from three independent experiments.

Gene Conversion Assay—1 × 10^6 cells were electroporated with 10 μg of pCBASce plasmid at 270 V, 975 microfarads using a BioRad 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.

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. 1C 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 pH2AX (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 (∆N42) or the BRCA2-binding domain
(∆C32) of PALB2 has no obvious impact on the PALB2-MRG15
interaction (Fig. 2, B and D). The fact that the MRG15-binding

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**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 EUFA1341F 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 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 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). The same is observed in 293T cells reconstituted with wild type or PALB2 Δ611–764 mutant after the depletion of endogenous PALB2 (supplemental Fig. 2A). In addition, the MMC sensitivity assay indicated that similar to wild-type PALB2, Δ611–764 mutant of PALB2 was also able to restore cell survival following MMC treatment (Fig. 3B).

We further performed a gene conversion assay to directly measure the efficiency of homologous recombination in cells reconstituted with either wild type or Δ611–764 mutant of PALB2. Intriguingly, cells reconstituted with PALB2 Δ611–764 mutant consistently displayed higher homologous recombination efficiency than cells reconstituted with wild-type PALB2 (Fig. 3C), suggesting that loss of the PALB2-MRG15 interaction results in a hyper-rec phenotype. Likewise, a 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. 2B). 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 formation and MMC resistance (Fig. 3, A and B). Therefore, we proposed that PALB2 suppresses SCE events during homologous recombination to assure error-free DNA repair. Deletion of the MRG15-binding motif on PALB2 would lead to the loss of such suppression, and thus, result in hyper-recombination and genomic instability.

The exact role of MRG15 in chromatin remodeling involved in DNA damage or repair remains unknown. However, MRG15-deficient mouse embryonic fibroblasts demonstrated DNA repair deficits, and 53BP1 and pH2AX foci formation was delayed in these cells upon DNA damage (11). There are a number of possibilities. MRG15 and its homologue Eaf3 have been identified as components of the Tip60-NuA4 histone acetylase complex in human and yeast, respectively (9, 17). It has been demonstrated that the Tip60 complex acetylates nucleosomal phospho-H2Av at Lys-5 in a DSB-dependent manner and catalyzes H2AX variant exchange upon DNA damage (18). It is possible that MRG15 is required for such a function given that dMRG15 mutant embryos demonstrated defects in the acetylation of nucleosomal phospho-H2Av similar to the dTip60 mutant embryos (18). On the other hand, Tip60 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 functions in regulating chromatin structure and accessibility and therefore affects the frequency of recombination. Interestingly, MRG15 and its homologue MRGX exist in similar protein complexes involving chromatin remodeling. Because MRGX was also identified as a component of the PALB2 complex, we further checked whether MRGX shares a similar function of MRG15 in the PALB2-dependent repair pathway. A gene conversion assay performed in U2OS cells transfected with control siRNA- or MRGX-specific siRNA showed that depletion of MRGX led to a hyper-rec phenotype when compared with control cells (supplemental Fig. 1D). This observation suggests that like 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.

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