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BREAST CANCER-ASSOCIATED MISSENSE MUTANTS OF THE PALB2 WD40 DOMAIN, WHICH DIRECTLY BINDS RAD51C, RAD51 AND BRCA2, DISRUPT DNA REPAIR

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Abstract

Heterozygous carriers of germ-line mutations in the *BRCA2/FANCD1*, *PALB2/FANCN*, and *RAD51C/FANCO* DNA repair genes have an increased life-time risk to develop breast, ovarian and other cancers; bi-allelic mutations in these genes clinically manifest as Fanconi anemia (FA). Here, we demonstrate that RAD51C is part of a novel protein complex that contains PALB2 and BRCA2. Further, the PALB2 WD40 domain can directly and independently bind RAD51C and BRCA2. To understand the role of these homologous recombination (HR) proteins in DNA repair, we functionally characterize effects of missense mutations of the PALB2 WD40 domain that have been reported in breast cancer patients. In contrast to large truncations of PALB2, which display a complete loss of interaction, the L939W, T1030I, and L1143P missense mutants/variants of PALB2 WD40 domain are associated with altered direct binding patterns to the RAD51C, RAD51 and BRCA2 HR proteins in biochemical assays. Further, the T1030I missense mutant is unstable, while the L939W and L1143P proteins are stable but partially disrupt the PALB2-RAD51C-BRCA2 complex in cells. Functionally, the L939W and L1143P mutants display a decreased capacity for DNA double-strand break-induced HR and an increased cellular sensitivity to

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CONFLICT OF INTEREST

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ionizing radiation. As further evidence for the functional importance of the HR complex, RAD51C mutants that are associated with cancer susceptibility and FA also display decreased complex formation with PALB2. Together, our results suggest that three different cancer susceptibility and FA proteins function in a DNA repair pathway based upon the PALB2 WD40 domain binding to RAD51C and BRCA2.

Keywords

PALB2; RAD51C; BRCA2; homologous recombination; BRCA genes; DNA repair

INTRODUCTION

Inherited loss-of-function mutations in genes involved in the maintenance of genomic stability are associated with an increased cancer incidence in carriers of heterozygous mutations. *BRCA1* and *BRCA2*, the two most prominent tumor suppressor genes linked to an inherited susceptibility to breast and ovarian cancer (reviewed in¹⁻³), encode proteins which function in the homologous recombination (HR) DNA repair pathway^{4,5}. *BRCA2* regulates oligomerization of the RAD51 recombinase which is necessary for it to form a nucleoprotein filament with single-strand DNA^{6,7}.

PALB2 and RAD51C are other DNA repair proteins linked to breast and/or ovarian cancer⁸⁻¹². PALB2, the partner-and-localizer of BRCA2, interacts with both BRCA1 and BRCA2 through its N-terminal coiled-coil and C-terminal WD40 domains, respectively¹²⁻¹⁶. Notably, the recruitment of BRCA2 to sites of DNA damage, and its function in HR, critically depend on the presence of a normal PALB2 protein^{10,12,15,17}.

WD40 domains are ring-like β -propeller structures with seven blades that mediate protein-protein interactions¹⁸. Truncation or frameshift mutants of PALB2 which perturb all, or a portion, of the WD40 domain abrogate the association of PALB2 with BRCA2, the assembly of RAD51 foci, and cellular resistance to DNA crosslinking agents such as mitomycin C^{12,13,15}. The pathogenicity of these truncation mutants has generally been attributed to disruption of the interaction of PALB2 with BRCA2, but RAD51 also interacts with the WD40 domain of PALB2^{19,20}. Although missense mutants/variants of the PALB2 WD40 domain have also been identified in breast cancer patients²¹⁻³¹, their effect on the function of the PALB2 protein in DNA damage responses, and therefore their pathogenicity, is still largely unknown.

RAD51C is one of five RAD51 paralogs which have been identified in somatic mammalian cells, along with RAD51B, RAD51D, XRCC2, and XRCC3. These proteins are of a similar size as RAD51, but have an overall low homology to RAD51 (20-30%)³². Like RAD51, each of the paralogs is required for DNA repair by HR³³, but their specific roles in HR are not well characterized and seem to be distinct from the function of RAD51.

The RAD51 paralogs form at least two distinct protein complexes in cells: a RAD51B-RAD51C-RAD51D-XRCC2 complex and a RAD51C-XRCC3 complex. RAD51C is the

only paralog present in both³⁴. Aside of an apparent interaction with RAD18³⁵, other RAD51C interactors have not been identified.

Here, we demonstrate that RAD51C has a direct protein-protein interaction with PALB2. In addition to their roles in breast/ovarian cancer, *FANCD1/BRCA2*, *FANCN/PALB2*, and *FANCO/RAD51C* are among the 16 currently identified Fanconi anemia (FA) genes^{36,37}. The functional relationship of RAD51C to other FA proteins, including PALB2, was previously unknown. Our results suggest that the PALB2 WD40 domain may scaffold the RAD51C, RAD51, and BRCA2 HR proteins into a complex. Selected missense mutations of the PALB2 WD40 domain, found in breast cancer patients²¹, perturb this complex, but to a lesser degree than truncation of the WD40 domain. In addition, RAD51C mutants originally identified in individuals with breast/ovarian cancer or FA^{8,38} also diminish the complex of HR proteins. Together, our results suggest that PALB2, RAD51C, and BRCA2 directly cooperate in a network of proteins which mediate homologous recombination, and which may thereby maintain genomic stability. Interestingly, disruption of this network is genetically-linked to three distinct diseases: breast cancer, ovarian cancer, and Fanconi anemia.

RESULTS

To better understand the function of RAD51C in DNA repair and the maintenance of genomic stability, we utilized mass spectrometry to identify proteins that co-purified with His₆-FLAG-RAD51C (HF-RAD51C) from untreated cells (Figure 1A). As expected, peptides for each of the RAD51 paralogs and for RAD51 were detected by mass spectrometry. PALB2 and BRCA2 were the only other proteins detected which were not present in mock protein purifications. Further, these same proteins were also identified following treatment with MMC (data not shown). Interactions with PALB2 and BRCA2 were verified by co-immunoprecipitation with epitope-tagged RAD51C (Figure 1B). As confirmation, we also demonstrated the reciprocal co-immunoprecipitation of RAD51C with PALB2 (Figure 1C).

As evidence that PALB2, BRCA2, RAD51C and RAD51 form a protein complex together, we first immunodepleted RAD51 from extracts of HeLa cells which expressed HF-RAD51C (Figure 1D). While immunodepletion of RAD51 did not have a noticeable effect on the levels of BRCA2, PALB2 or RAD51C that remained in the extract, less PALB2, BRCA2 and RAD51 subsequently co-immunoprecipitated with HF-RAD51C from these extracts. These results suggest that RAD51 and RAD51C form a complex together with PALB2 and BRCA2.

While both RAD51 and RAD51C are minor components of the PALB2 complex(es), BRCA2 is a more abundant component (Supplemental Figure 1). Another RAD51 paralog, XRCC3, is also a minor component of the PALB2 complex (Supplemental Figure 1).

To better define the interaction of RAD51C with PALB2, we compared immunoprecipitates of full-length PALB2 to a version truncated in the middle of the protein (Y551X) (diagramed in Figure 2A). This mutant was first identified in EUFA1341 cells from a FA

patient¹³. RAD51C, as well as BRCA2 and RAD51, co-immunoprecipitated with full-length Flag-HA-PALB2 from EUFA1341 cells (Figure 2B). Strikingly, however, neither RAD51C nor BRCA2 interacted with Flag-HA-PALB2-Y551X. This truncation mutant of PALB2 was also largely deficient for interaction with RAD51.

RAD51D is a component of the RAD51B-RAD51C-RAD51D-XRCC2 complex of RAD51 paralogs³⁴. RAD51D did not associate with the PALB2-BRCA2-RAD51C-RAD51 complex described above (Figure 2B). This demonstrates that these two protein complexes can be clearly distinguished.

The most prominent structural feature of the C-terminal half of PALB2 is a WD40 domain from amino acids 853–1186. We utilized PALB2 C (diagramed in Figure 2A), which we have described previously¹⁵, to determine whether RAD51C specifically interacts with the WD40 domain of PALB2. PALB2 C is truncated following amino acid P1097 and therefore lacks blades 5–7 of the PALB2 WD40 structure³⁹. PALB2 C was completely deficient for interaction with RAD51C, RAD51, and BRCA2 (Figure 2C), suggesting that RAD51C and these other proteins interact with the WD40 domain of PALB2.

Next, we expressed and isolated fusion proteins containing RAD51C or the PALB2 WD40 domain from bacteria. Using this system, we demonstrate that RAD51C bound directly to the WD40 domain of PALB2 (Figure 2D).

Truncation of the PALB2 WD40 domain completely abrogates interactions with BRCA2, RAD51C and RAD51 (Fig. 2C). Thus, as an alternative that might yield more insight into the interactions of BRCA2, RAD51C, and RAD51 with PALB2, we analyzed the effects of missense mutants/variants of PALB2 identified in a German population of breast cancer patients²¹. While missense mutants have been identified in other studies^{21–31}, our study was not intended to be exhaustive but instead focused on selected mutants/variants of the PALB2 WD40 domain which were predicted to potentially be disease-causing. While the T1030I and L1143P mutants were only found in cancer patients, the L939W variant was found at a lower frequency in controls than in breast cancer families²¹ and has also been linked to breast cancer in other studies^{22,24,28}. First, we examined the positions of the three different missense mutations in the crystal structure of the PALB2 WD40 domain³⁹. L939W, T1030I, and L1143P are present within blade 2, between blades 4 and 5, and between blades 5 and 6, respectively, and therefore potentially identify three independent binding surfaces on the PALB2 WD40 β -propeller structure (Figure 3A). This result is consistent with a potential role for the PALB2 WD40 domain in scaffolding a complex of HR and FA proteins, including RAD51C.

We also modeled each mutation/variant based upon the structure of the PALB2 WD40 domain (Figure 3B–D). The L939 and L1143 residues are present on the surface of the WD40 domain structure and are therefore likely involved in protein-protein interactions. L939W, in particular, represents a change to a more bulky residue that may thereby perturb potential interactions. In contrast, the T1030I mutation likely disrupts hydrogen bonds between the hydroxyl group of threonine 1030 and the carboxylic and amide groups, respectively, of Glu1011 and Met1032. This mutation likely perturbs the pocket formed by

amino acids Pro1009, Glu1011, Ile1013, Asn1034 and Lys1048 and is therefore predicted to be unstable.

Next, we tested the effects of the L939W, T1030I and L1143P PALB2 alterations on *in vitro* interactions with various HR-associated proteins (Figure 4). Examples of a representative experiment, performed with proteins isolated following expression in bacteria, are shown in Figure 4A. Although none of the PALB2 mutations completely abrogated the interaction of its WD40 element with the HR proteins, the L939W variant showed a stronger disruption of direct binding to BRCA2 and the L1143P mutant protein had a weaker effect on the interaction with BRCA2. Both differences were reproducible and statistically significant in three independent experiments (Figure 4B). The T1030I mutant did not show any decrease in the interaction with BRCA2.

In contrast, RAD51C and RAD51 displayed patterns of interaction with each PALB2 mutant that were distinct from BRCA2 and which were distinct from each other (Figure 4). The L939W mutant had no effect on direct binding of the PALB2 WD40 domain by RAD51C, but the T1030I variant, and especially the L1143P PALB2 variant, were associated with a statistically significant decrease in the interaction with RAD51C. In contrast, RAD51 showed significantly less binding to the L939W mutant and especially the T1030I mutant, in addition to a more minor decrease in binding to the L1143P mutant.

The RAD51 paralog XRCC3 also directly bound to the PALB2 WD40 domain and had a pattern of binding to the mutants that resembled that of BRCA2, but which was clearly distinct from that of RAD51C (Figure 4). This suggests that RAD51C and XRCC3 independently interact with PALB2.

Together, results with missense mutants of PALB2, shown in Figures 3–4, appear to identify three distinct surfaces on the PALB2 WD40 β -propeller structure that are involved in binding RAD51C, RAD51, and BRCA2 and XRCC3. Importantly, the distinct patterns of binding of RAD51C, RAD51, and BRCA2 to these PALB2 missense mutants also suggests that each HR protein may directly bind to the PALB2 WD40 domain in an independent manner. The above results support the possibility that the PALB2 WD40 domain scaffolds a complex of HR proteins.

Given that RAD51C and XRCC3 can form a complex together³⁴, we tested whether the presence of PALB2 influences the association of these proteins (Supplemental Figure 2). Co-immunoprecipitation of RAD51C with XRCC3 was clearly increased in cells with a deficiency for PALB2. This suggests that formation of the PALB2 complex with RAD51C and/or XRCC3 may compete with the RAD51C-XRCC3 complex.

To test their function in DNA repair, we then expressed the three breast cancer-associated mutants along with N-terminal Flag-HA epitope tags in PALB2-deficient EUFA1341 cells derived from a FA patient¹³. While the L939W and L1143P mutants expressed at similar levels as the wild-type protein, the T1030I mutant was present at clearly decreased levels in multiple experiments (Figure 5A). We hypothesized that the T1030I mutant was unstable, as predicted in Figure 3D. To further test this possibility, we treated cells containing wild-type PALB2 or the T1030I mutant with cycloheximide to inhibit new protein synthesis (Figure

5B). The turnover of the mutant was more rapid than that of the wild-type protein. Further, treatment with MG132 more modestly increased the levels of wild-type PALB2 but restored the T1030I mutant to near the levels of wild-type protein (Figure 5C). This suggests that the T1030I mutant of PALB2 was degraded in a proteasome-dependent manner. Quantification of these results is shown in Figures 5D and 5E. The levels of mRNA for each form of PALB2 were similar, however (Figure 5F). Together, our results demonstrate that one way in which missense mutations of the PALB2 WD40 domain may act in breast cancer is by decreasing the stability of the encoded protein.

Unlike PALB2-T1030I protein, the L939W and L1143P mutants appeared to be stably expressed in EUFA1341 cells (Figure 5A). To measure the effects of the L939W and L1143P mutants/variants of PALB2 on DNA double-strand break (DSB)-initiated HR, we expressed each form in U2OS-DR cells that contained a GFP reporter for HR⁴⁰ and depleted endogenous PALB2 with a siRNA directed against its 3'-UTR. Each mutant displayed modest but statistically significant decreases in DSB-initiated HR that were intermediate to those of cells which contained the empty vector alone and were depleted of endogenous PALB2 (Figure 6A).

We also tested whether the L939W and L1143P mutants of PALB2 had any effect on RAD51 foci. We depleted endogenous PALB2 in U2OS-DR cells that expressed PALB2-WT or either mutant, as described above. The L1143P mutant displayed a modest but statistically significant decrease in the assembly of RAD51 foci (Figure 6B), consistent with the modest but significant decrease in DSB-HR that is associated with this mutant.

As an additional measure of the importance of altered interactions between the PALB2, BRCA2, and RAD51C proteins, we tested resistance of EUFA1341 cells reconstituted with the stable L939W and L1143P mutants/variants of PALB2 to ionizing radiation (IR) (Figure 6C). A role for PALB2 in promoting resistance to IR has not been reported previously. Interestingly, the L939W and L1143P PALB2 mutants showed a moderate, but significant increase in sensitivity to IR when compared to those which were isogenically-corrected with wild-type PALB2.

Consistent with our findings of compromised DNA repair, the L939W and L1143P mutants of PALB2 showed altered binding to HR proteins in EUFA1341 cells (Figure 6D). Quantification is shown in Figure 6E. The interactions of BRCA2 and RAD51C with PALB2-L1143P were statistically lower than those with PALB2-WT. Although not statistically different from binding to PALB2-WT, PALB2-L939W showed trends of increased binding to BRCA2 and decreased binding to RAD51C.

Finally, we also tested RAD51C mutants for perturbation of the complex with PALB2 and BRCA2. The L138F and D159N mutants were identified in breast/ovarian cancer patients, while the R258H mutant was identified in a FA patient. Each mutant was previously demonstrated to be functionally impaired in DNA repair-related assays^{8,38,41}. As shown for a representative experiment, L138F, D159N and R258H all displayed decreased binding to BRCA2, and to a lesser degree to PALB2 and RAD51, when expressed transiently in 293T cells (Fig. 7). Thus, taken together with Figures 4 and 6, mutations of either *PALB2* or

RAD51C, found in breast/ovarian cancer or FA, perturb the function of a complex of HR proteins that includes PALB2, RAD51C, and BRCA2.

DISCUSSION

Here, we demonstrate that the RAD51C protein interacts with, and can directly bind, PALB2. We term this complex, which also includes RAD51, BRCA2, and XRCC3, the “HR complex”. Our results suggest that these proteins interact and function in a common pathway of DNA repair by HR. This is particularly interesting given that *PALB2*, *BRCA2* and *RAD51C* are all breast/ovarian cancer susceptibility (BRCA) genes^{8–12,42}, as well as FA genes^{13,38,43,44}. Notably, FA is associated with a predisposition to cancer³⁶. Thus, together, our finding that patient-derived mutations of either RAD51C or the PALB2 WD40 domain disrupt the HR complex suggests the importance of this complex in preventing cancer.

The PALB2 WD40 domain coordinates a protein complex that contains both RAD51C and BRCA2

While the role of PALB2 in recruiting BRCA2 to sites of DNA damage has already been established^{13,15,17}, RAD51C was only known to associate with other RAD51 paralogs, in two different complexes³⁴, and with RAD18³⁵. Thus, we demonstrate a novel protein complex that yields insight into the function of PALB2, RAD51C, and BRCA2 as tumor suppressors and FA proteins.

By showing that RAD51C and BRCA2, as well as RAD51, all directly bind to the WD40 domain of PALB2, we propose that a key function of PALB2 is to individually bind multiple proteins which have an essential role in DSB-initiated HR^{4,33,45}. The role of the PALB2 WD40 domain in binding these HR proteins is supported by the finding that three different breast cancer-associated missense mutants/variants of PALB2, L939W, T1030I and L1143P, have distinct patterns of altered binding to BRCA2, RAD51C, and RAD51 *in vitro*. In fact, the WD40 domain could scaffold a complex that contains these HR proteins. Consistent with this possibility, previous work has shown that WD40 domains can coordinate simultaneous interactions with multiple proteins⁴⁶.

We find that the PALB2-L1143P mutant is associated with a moderate but significant decrease in RAD51 foci after exposure to IR. In contrast, this mutant results in a modest but significant increase in the formation of BRCA2 foci (Supplemental Figure 3). It is possible that by binding to PALB2, RAD51C normally leads to a concomitant release of BRCA2 and recruitment of RAD51. This might explain the increased levels of BRCA2 foci and decreased RAD51 foci observed in cells reconstituted with the PALB2-L1143P mutant. This process could involve the DNA binding activity of RAD51C⁴⁷ and/or a change in the composition of the complex with PALB2. The PALB2-L1143P mutant could have indirect effects on RAD51, since it had altered interactions with RAD51C and BRCA2 but not RAD51 both *in vitro* (Figure 4) and in cells (Figure 6). We expect that decreased recruitment of RAD51, despite increased levels of BRCA2 foci, underlies the significant decreases in HR and resistance to IR that are associated with the PALB2-L1143P mutant.

Contrasting reports have suggested that RAD51C and XRCC3 function either before or after RAD51 foci formation^{33,38,48,49} in various cell types, including human cells, following exposure to DNA damage. While the PALB2-L1143P mutant would appear to have some defect before RAD51 foci formation, this does not preclude an additional function afterwards. The interaction of RAD51C with PALB2 could have a role in promoting RAD51 foci formation, since the PALB2-L1143P mutant includes a defect in this interaction.

As added support for the effects of missense mutations of the PALB2 WD40 domain on DNA repair, the L939W variant but not L1143P is associated with an intermediate sensitivity to a PARP inhibitor (Supplemental Figure 4). Since both mutants are associated with increased sensitivity to IR, this could reflect different patterns of interactions of each mutant with HR proteins (Figure 4).

RAD51 is unlikely to be required for the interactions of BRCA2 or RAD51C with the WD40 domain of PALB2. First, both BRCA2 and RAD51C directly, and thus potentially independently, bind the PALB2 WD40 domain (Figure 4). Second, siRNA-mediated depletion of RAD51 decreased the levels of RAD51, but not those of PALB2 or BRCA2, which immunoprecipitated with RAD51C (Supplemental Figure 5).

While the PALB2-BRCA2-RAD51C-RAD51 complex appears to be independent of the previously described RAD51B-C-D-XRCC2 complex³⁴, another RAD51 paralog, XRCC3, appears to interact with this PALB2 complex. This is the third reported complex of RAD51 paralogs, but the first that also includes non-paralog proteins. Given that the L939W and L1143P mutants of PALB2 have altered interactions with RAD51C, XRCC3, BRCA2 and RAD51, and are defective for DNA repair, these RAD51 paralogs would appear to have a function in this PALB2 complex. While RAD51C-XRCC3 has been reported to have a Holliday junction resolvase activity *in vitro*⁵⁰, it is currently unclear what function, if any, this complex by itself has in intact cells.

A protein complex has been reported that includes FANCG, FANCD2, BRCA2 and XRCC3⁵¹. Given that we describe a PALB2 complex that contains BRCA2 and XRCC3, these complexes may be related. Future work will be required to examine this potential relationship.

Comparison of the effects of truncation mutants of the PALB2 WD40 domain to those of missense mutants

Mutants of PALB2 found in breast cancer and FA patients that result in a nonfunctional truncated protein, due to partial removal or absence of the WD40 domain, are completely deficient for DSB-initiated HR^{10,12,13}. These truncations abrogate binding to BRCA2 and we report here that such large-scale truncations also eliminate binding to RAD51C and RAD51 (Fig. 2C), and thereby fully disrupt the HR complex.

More recently, missense mutations of *PALB2* have been identified in breast/ovarian cancer patients and are also present in FA patients^{10,11,13,21–31}. Because these mutants are not clearly pathogenic, it is therefore more difficult to predict any clinical outcome, especially in heterozygous carriers. Here, we have performed the first functional characterization of such

PALB2 missense mutants/variants. In contrast to truncating mutants, selected missense mutants of the WD40 domain are found associated with partial but incomplete disruption of the HR complex. Correspondingly, DSB-initiated HR is only partially compromised.

The breast cancer-associated L939W and L1143P mutants/variants of PALB2 are linked to modest but significantly decreased DSB-initiated HR efficiencies and increased IR sensitivities (Figure 6). We therefore predict that missense mutants of PALB2 may confer lower levels of genomic instability, and thus a lower risk of cancer, than truncation mutants. Notably, our repair-related assays indicate that the L939W variant identified in various studies^{21,22,24,28}, generally more frequently in breast cancer patients than healthy individuals, may be pathogenic. Importantly, our study establishes assays for the functional characterization of other missense mutants/variants of PALB2 in the future.

Results obtained with the L939W, T1030I and L1143P mutants/variants of PALB2 suggest that there are at least two different mechanisms by which missense mutants of the PALB2 WD40 domain may lead to increased genomic instability in breast cancer. First, we find that the T1030I mutant is unstable when expressed in human cells. Decreased levels of PALB2-T1030I due to instability are therefore expected to diminish its function in HR. A second means by which mutants of the PALB2 WD40 domain may lead to increased genomic instability is by altered binding to HR proteins, as illustrated by the L939W and L1143P mutants/variants. The L1143P mutant significantly decreased binding to RAD51C and BRCA2, both *in vitro* and in human cells.

PALB2, BRCA2, and RAD51C are linked to breast/ovarian cancer and to Fanconi anemia

FA is an inherited chromosome instability syndrome clinically characterized by bone marrow failure, congenital anomalies, and a predisposition to leukemia and various solid tumors³⁶. Eight of the sixteen identified FA proteins (FANCA/B/C/E/F/G/L/M) form a core complex required for the monoubiquitination of FANCD2 and FANCI^{36,37}. Other FA proteins, including PALB2, BRCA2, and RAD51C, are not required for the monoubiquitination of FANCD2 or FANCI, and are therefore believed to function downstream of FANCD2^{13,38,43,44}. Interestingly, BRCA2⁵², RAD51C³³, and as we show here, PALB2, are required for resistance to IR, unlike upstream FA proteins that lead to the monoubiquitination of FANCD2 and FANCI. Further, PALB2, BRCA2, and RAD51C are associated with inherited breast cancer, while upstream FA pathway proteins are not⁵³. Thus, a more prominent role in DSB repair and in cellular resistance to IR may underlie the shared association of the *PALB2*, *RAD51C*, and *BRCA2* subset of FA genes with breast cancer. Consistent with this possibility, the products of other breast cancer susceptibility genes, including *BRCA1*, *BRIP1*, *ATM*, *CHEK2*, *NBS1* and *RAD50*², are also involved in mediating the repair of DSBs induced by IR.

In conclusion, we have identified a novel complex of HR and BRCA proteins. Importantly, our results yield insight into the function of PALB2 as a tumor suppressor by demonstrating that its WD40 domain may act to coordinate this complex, which contains two other tumor suppressor proteins: BRCA2 and RAD51C. Finally, the function of this complex in DNA repair may be the basis for the common association of the products of the *PALB2*, *RAD51C* and *BRCA2* genes with three distinct diseases: breast cancer, ovarian cancer, and FA.

MATERIALS AND METHODS

Cell culture

HeLa S3, 293T and U2OS-DR cell lines, and EUFA1341 cells reconstituted with different forms of PALB2 were cultured and irradiated as described previously¹⁵. Stock solutions of cycloheximide (10 mg/ml in H₂O; Sigma), MG132 (10 mM in DMSO; Calbiochem), and Olaparib (5 mg/ml in DMSO; Selleck Chemicals) were kept at -20°C.

Antibodies

Mouse anti-RAD51C (2H11) and rabbit anti-XRCC3 antibodies were purchased from Novus. Rabbit anti-RAD51 (H92) and mouse anti-His (H8) antibodies were purchased from Santa Cruz. Mouse anti-RAD51D (5B3) antibody was obtained from Chemicon. Anti-HA, anti-MBP, anti-GST, anti-β actin, and anti-PALB2 antibodies¹⁵, and anti-BRCA2 antibodies⁵⁴, were described previously.

Immunofluorescence microscopy

Cells were fixed and processed with antibodies as described previously¹⁵.

Cloning and mutagenesis

Human RAD51C cDNA was purchased from Open Biosystems. RAD51C with N-terminal Flag-HA or His-Flag epitope tags were generated and subcloned into pcDNA3.1. Alternatively, RAD51C was cloned into the pMIEG3 retroviral vector with a N-terminal His₆-Flag epitope tag⁵⁵. PALB2 mutants (L939W, T1030I, and L1143P) were generated as described previously¹⁵. To generate fusion proteins for *in vitro* studies, the PALB2 WD40 domain (amino acids 859–1186), RAD51 or RAD51C were cloned into pMAL-c2X (NEB) with N-terminal maltose-binding protein (MBP). Alternatively, RAD51C was cloned into pET28b (Novagen) with C-terminal Histidine (His). An N-terminal fragment of BRCA2 (1–75 a.a) was cloned into pGEX4T-1, which contained N-terminal Glutathione S-transferase (GST).

Expression and purification of recombinant proteins

Fusion proteins were expressed in bacteria (4 h at 30°C with 0.3 mM isopropyl β-thiogalactopyranoside). Recombinant proteins were purified using amylose resins (NEB) for MBP, glutathione sepharose 4B beads (GE healthcare) for GST, and Talon metal affinity resins (Clontech) for His, according to the manufacturers' instructions.

In vitro GST and MBP pull-downs

GST and MBP-tagged proteins (5 μg) were bound to glutathione beads and amylose resins, respectively, and mixed with 5 μg of each purified target protein in binding buffer (10 mM Tris pH 7.4, 75 mM NaCl, 0.5 mM EDTA, 0.025% Tween-20, 1 mM DTT, and 0.5 mg/ml BSA) including 0.5 mM PMSF and 1X protease inhibitor cocktail, and incubated overnight at 4°C. Beads were then washed and bound proteins eluted.

Transfection and viral transduction

Retroviral transduction with pMIEG3-RAD51C, or pMMP-PALB2 wild-type and its mutants with a N-terminal Flag-HA tag, was performed as described previously^{15,55}. Transduced cells were selected with 0.5 µg/ml puromycin for 6 days after the last infection.

Identification of RAD51C-interacting proteins by mass spectrometry

RAD51C complexes were purified from HeLaS3 cells retrovirally transduced with His₆-FLAG tagged RAD51C as described previously⁵⁶. The immunopurified complex was analyzed by mass spectrometry.

Immunoprecipitation

Cell pellets were lysed in 420 NETN buffer as described previously¹⁵. Immunoprecipitations were performed with anti-Flag M2 Affinity Gel (Sigma) or specified antibodies as described previously¹⁵.

RT-PCR analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Synthesis of cDNAs from RNA with the Advantage RT for PCR Kit (Clontech) was by PCR-amplification using the following primers (Forward: 5'-gtgatgctgtactgttctctct-3'; Reverse: 5'-cgactgtcatcgtcgtccttg-3').

Assay of DNA DSB-initiated HR

U2OS-DR cells with an integrated GFP reporter and containing PALB2 or its mutants were assayed as described previously¹⁵.

IR colony and PARP inhibitor sensitivity assays

EUFA1341 cells reconstituted with wild-type PALB2 or its mutants were seeded onto 6 cm dishes (2×10^3 cells/dish) in triplicate, and after allowing attachment cells were irradiated. Cells were incubated for 10–12 days to form colonies and washed with PBS. Colonies were fixed with Wright-Giemsa reagent (EMD), stained with Buffer solution Giordano (Fisher), dried, and counted manually.

To measure Olaparib sensitivity, cells were seeded into 96 well plates at 1×10^6 cells/well and were treated with a range of concentrations of olaparib beginning 4 hr later. Cells were incubated 4 days and relative survival was measured using a colorimetric assay¹⁵.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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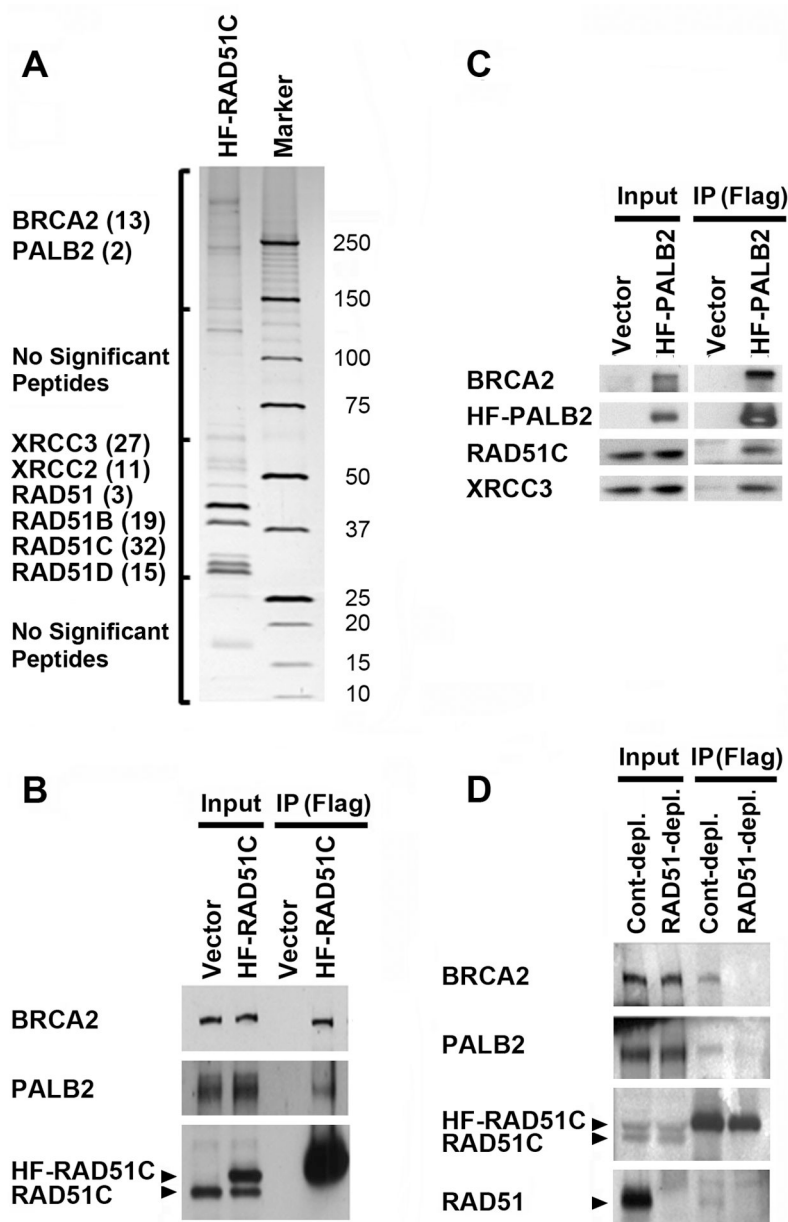


Figure 1. RAD51C interacts with PALB2 and BRCA2

(A) Silver stained gel indicating components of a RAD51C complex identified by mass spectrometry following immunopurification from HeLa S3 cells. RAD51C was expressed with a N-terminal His₆-Flag (HF) epitope tag. The gel was cut into four sections as indicated (left) and mass spectrometry was performed on each piece. The number of unique peptides identified, following subtraction of common peptides isolated from a mock-purified control, is shown in parentheses. (B) Immunoblot demonstrating that PALB2 and BRCA2 co-immunoprecipitate with HF-RAD51C. (C) Immunoblot demonstrating that RAD51C and XRCC3 co-immunoprecipitate with HF-PALB2. (B–C) Immunoprecipitates were prepared using M2 anti-Flag beads and extracts from HeLa (B) and EUFA1341 cells complemented with HF-PALB2 (C). (D) PALB2, BRCA2, RAD51C and RAD51 form a protein complex,

as determined by depletion of a complex that could be immunoprecipitated with HF-RAD51C after prior immunodepletion of RAD51. HeLa cells were first incubated with anti-RAD51 or normal rabbit (Cont.) antisera. Supernatants were then incubated with anti-M2 agarose to immunoprecipitate HF-RAD51C and were immunoblotted with the indicated antibodies. (B–D) Immunoprecipitates represent 200-fold the levels loaded for Input lanes. The positions of HF-RAD51C and endogenous RAD51C, or RAD51, are indicated by arrowheads.

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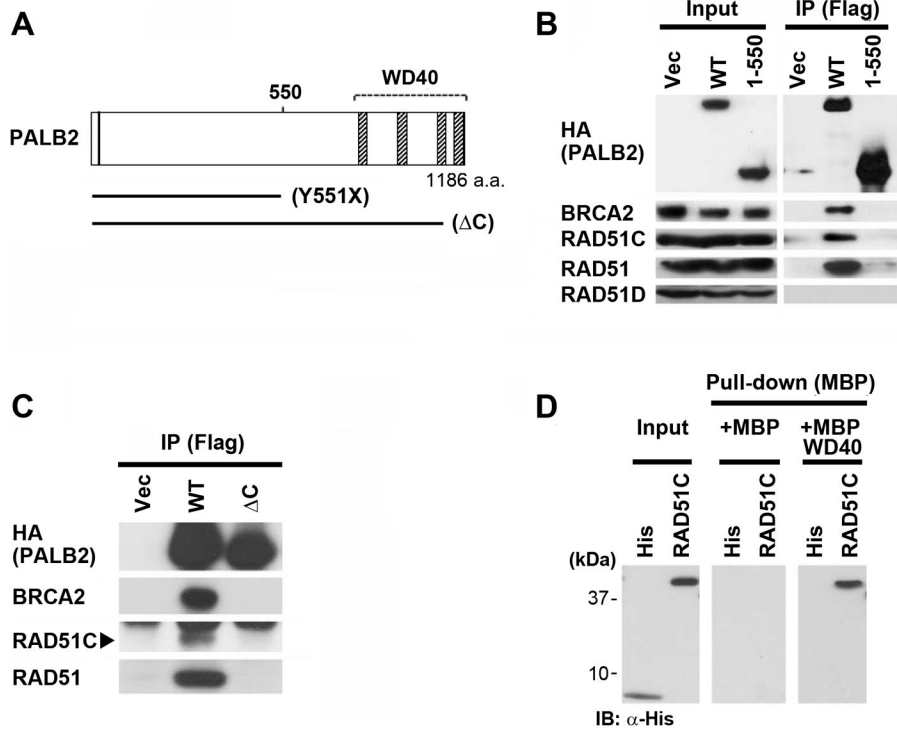


Figure 2. RAD51C directly binds to the WD40 domain of PALB2
 (A) Schematic diagram of PALB2 and selected mutants. Hatched boxes indicate WD40 repeats of PALB2. (B) Wild-type PALB2 and the Y551X truncation mutant containing N-terminal Flag and HA epitope tags were transiently transfected into 293T cells. Immunoprecipitates represent 500-fold the levels loaded for Input lanes. (C) Cell lysates were prepared from EUFA1341 cells reconstituted with wild-type PALB2 or the Δ C mutant truncated after P1097. The position of RAD51C is indicated by an arrowhead. (B–C) Cell lysates were immunoprecipitated with α -Flag beads, followed by immunoblotting with the indicated antibodies. (D) Analysis of direct binding using bacterially-expressed maltose-binding protein (MBP) alone or MBP fused to the WD40 domain of PALB2 (amino acids 859–1186). Bacterially-expressed His₆ alone, or fused to RAD51C, was incubated with MBP or MBP-PALB2 immobilized on maltose beads. Bound RAD51C was detected by immunoblotting with anti-His antibodies following elution.

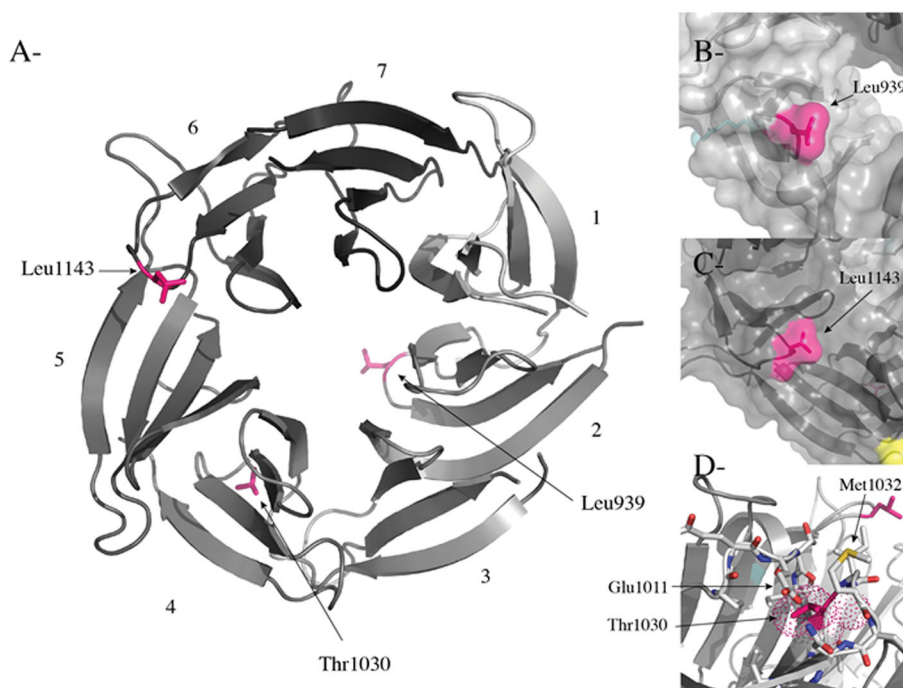


Figure 3. Analysis of the positions and potential effects of breast cancer mutations in the PALB2 WD40 domain

(A) A ribbon diagram of the WD40 domain of PALB2 shown in grey (PDB ID 3EU7³⁹). The blades are numbered 1 to 7 and colored light grey to dark grey. The missense mutations/variants (L939, T1030 and L1143) found in breast cancer patients are shown in stick representation and in pink. (B–C) Surface representation of the region of PALB2 centered on Leu939 and Leu1143, respectively, colored in pink showing how both hydrophobic residues are solvent exposed. (D) Details of residues surrounding T1030 of blade 4. Residues E1011 and M1032, which make hydrogen bonds with the hydroxyl group of T1030, are highlighted. The figure was prepared with Pymol (<http://www.pymol.org/>).

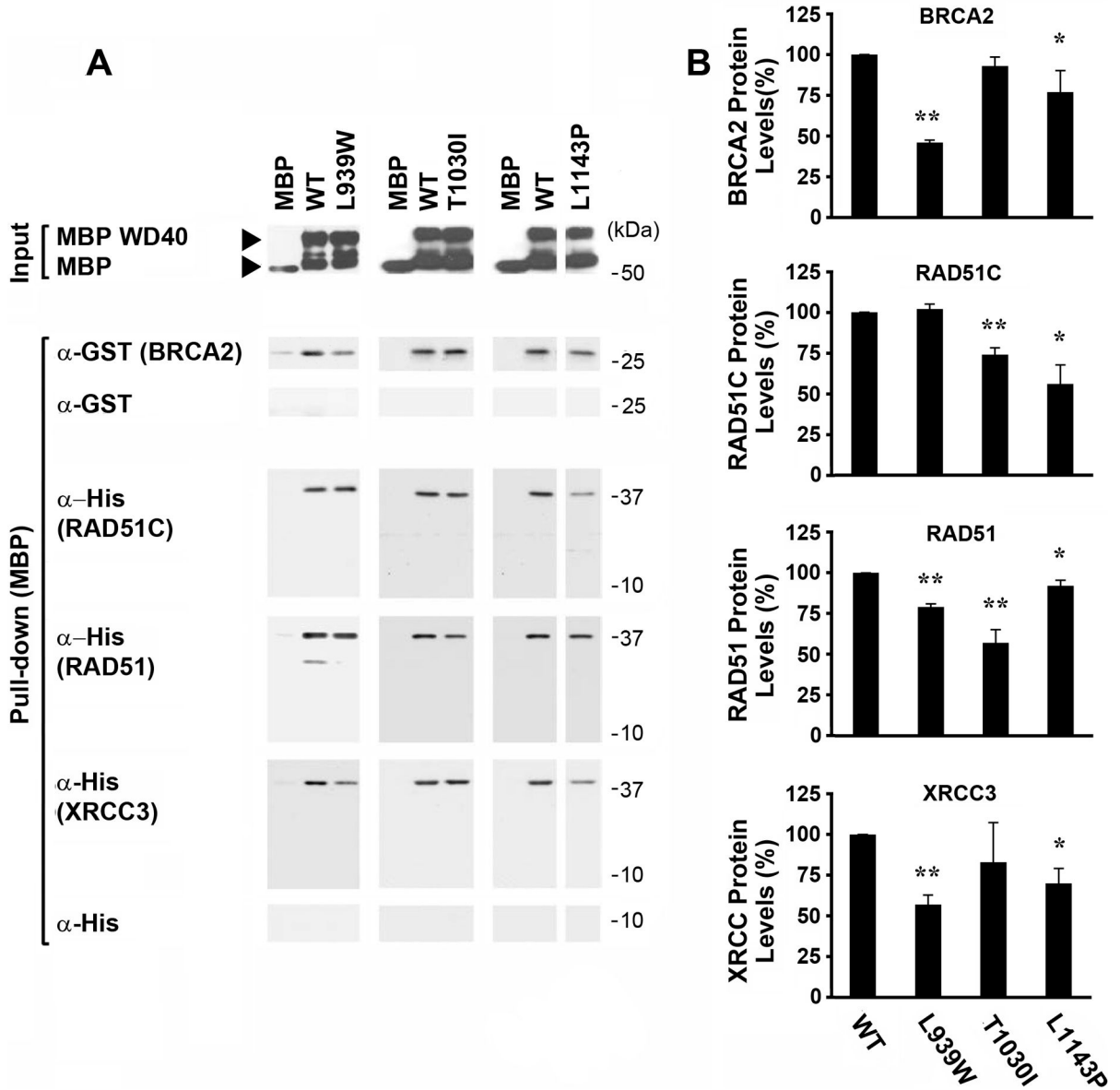


Figure 4. Different patterns of *in vitro* binding of RAD51C, BRCA2, RAD51, and XRCC3 to disease-associated missense mutants of PALB2 suggest that the PALB2 WD40 domain may scaffold these proteins into a complex

(A) A representative *in vitro* binding experiment. MBP alone, or MBP-fused to the wild-type PALB2 WD40 domain or to different breast cancer-associated mutants/variants of this domain, were expressed in bacteria, purified, and immobilized on maltose beads. Isolated MBP-WD40 proteins or MBP alone are shown as “Inputs”. A GST-tagged BRCA2 fragment (amino acids 1–75), or His-tagged RAD51C, RAD51, or XRCC3, were purified from bacteria and incubated with the purified MBP fusion proteins. Proteins present in the MBP fusion protein pull-down were detected with anti-GST or Anti-His antibodies, as appropriate. GST alone served as a negative control for GST-BRCA2, while His alone served as a negative control for His-fusion to RAD51C, RAD51, or XRCC3. (B) A graph showing quantification of binding from three independent experiments. Values for each HR

protein were determined by densitometry and adjusted relative to the levels of each form of PALB2 in the Input lanes and are shown normalized to the amounts that immunoprecipitated with the wild-type PALB2 WD40 domain. The mean and standard deviation are shown for each value (* = $P < 0.05$; ** = $P < 0.01$).

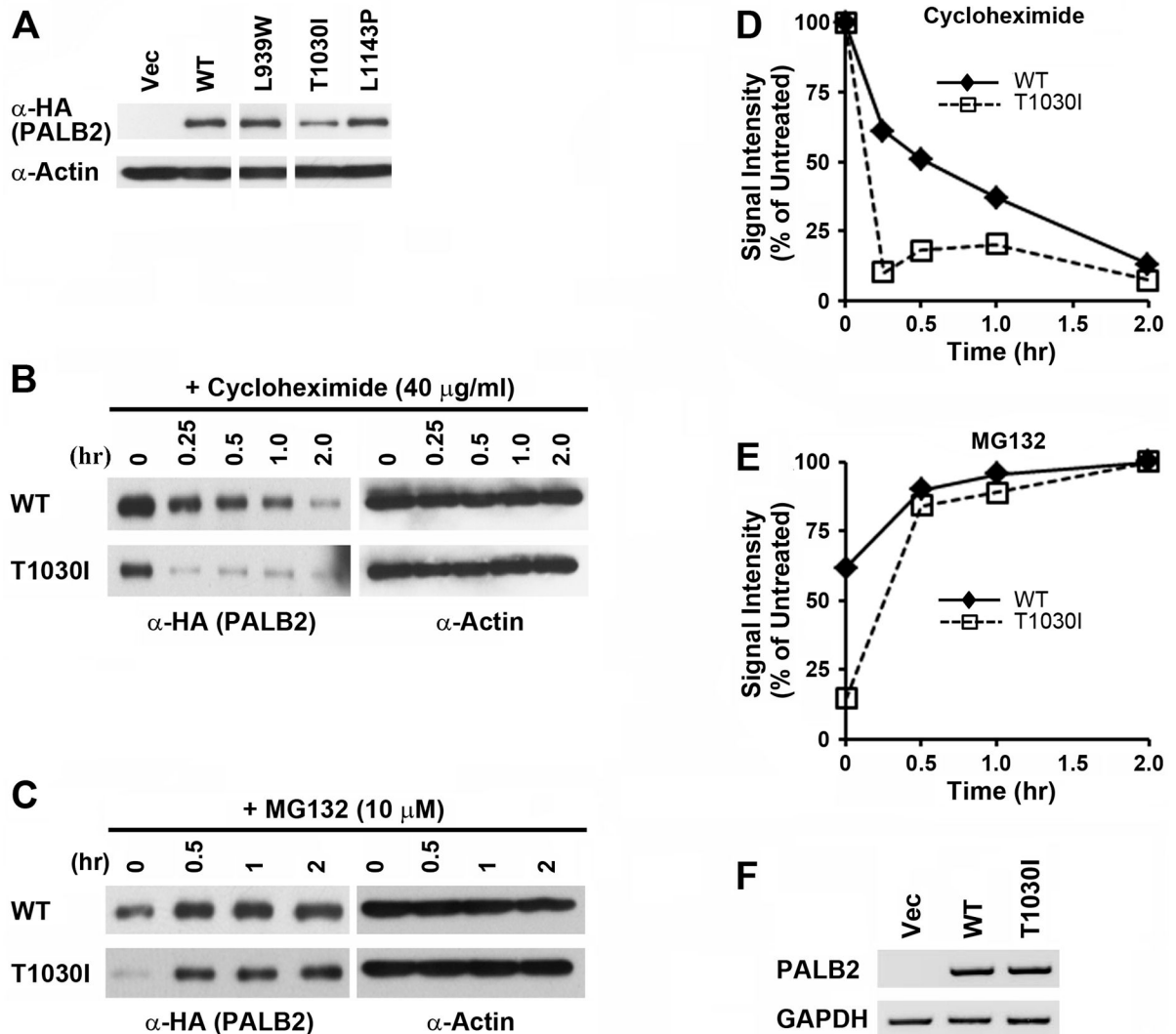


Figure 5. The breast cancer-associated T1030I mutant of PALB2 is unstable
 (A) Wild-type PALB2 or the L939W, T1030I, and L1143P mutants/variants were retrovirally expressed in EUFA1341 cells along with a N-terminal Flag-HA epitope tag. The level of each protein was determined by immunoblotting with α -HA antibodies. Actin was used as a loading control. (B) To examine relative rates of turnover, new protein synthesis was inhibited in EUFA1341 cells reconstituted with Flag-HA tagged wild-type PALB2 or with the T1030I mutant by treating with 40 μ g/ml cycloheximide. Cell lysates were prepared at the indicated time points. (C) EUFA1341 cells reconstituted with wild-type PALB2 or the T1030I mutant were treated with 10 μ M MG132 and harvested at the indicated time points. (D–E) Quantification of the levels of wild-type PALB2 or the T1030I mutant following treatment with cycloheximide (D) or MG132 (E) at various time points was determined by densitometry of immunoblots. Results for each protein were normalized to the values at 0 hr (D) or 2 hr (E) of treatment. (F) Levels of mRNA of wild-type or mutant PALB2 in reconstituted EUFA1341 cells were analyzed by RT-PCR. Primers that detected Flag-HA-

tagged PALB2 but not endogenous PALB2 were utilized. GAPDH was used as a control for the levels of an unrelated endogenous protein.

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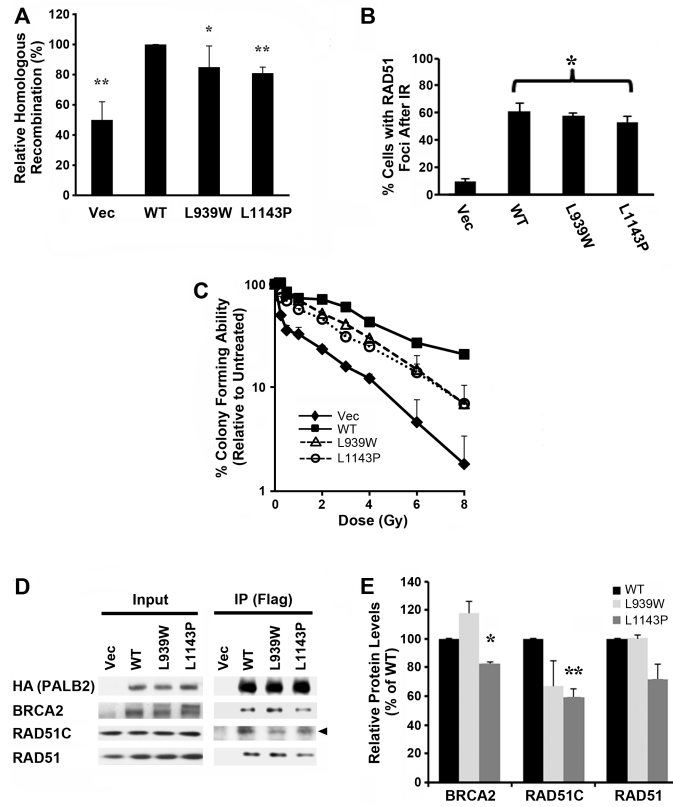


Figure 6. The L939W and L1143P mutants of the PALB2 WD40 domain are associated with defective DSB-initiated HR and with increased sensitivity to ionizing radiation

(A) Gene conversion assays were performed in U2OS-DR cells stably expressing wild-type PALB2, or the L939W or L1143P mutants, and depleted of endogenous PALB2 using a siRNA directed against its 3'-UTR. GFP-positive cells were quantified by flow cytometry. Each value represents the average of three independent experiments performed in duplicate. The data shown are the mean and standard deviation of three independent replicates (* = $P < 0.05$; ** = $P < 0.01$). (B) The percentage of cells with RAD51 foci at 16 hr after exposure to 10 Gy IR. U2OS-DR cells were stably reconstituted with the different forms of PALB2, as indicated, and endogenous PALB2 was depleted as described above. Each value represents the mean and standard deviation of 3 counts of 150 or more cells each with 3 or more RAD51 foci (* = $P < 0.05$). (C) IR colony assay performed in EUFA1341 cells reconstituted with wild-type PALB2, the L939W or L1143P mutants, or with vector alone. Cells were treated with the indicated doses of IR. Differences for EUFA1341 cells reconstituted with the vector or with either mutant, as compared to cells corrected with WT PALB2, were statistically significant ($P < 0.05$) at doses of IR ranging from 1–8 Gy. (D–E) Wild-type PALB2, the L939W or L1143P mutants, or the vector alone, were stably expressed in EUFA1341 cells along with a N-terminal Flag-HA epitope tag. Levels of associated HR proteins in extracts (Input), or in immunoprecipitates (IP) prepared using α -Flag M2 agarose beads, are shown in (D). These results are representative of two independent experiments. The position of RAD51C is indicated by an arrowhead. Immunoprecipitates represent 200-fold the levels loaded for Input lanes. Quantification of the levels of BRCA2, RAD51C or RAD51 which immunoprecipitated with the WT, L939W or L1143P forms of PALB2 in

two independent experiments (E). Levels of immunoprecipitating protein were normalized to the input for each form of PALB2 and each interacting protein. The average \pm S.D. are shown relative to the values for PALB2-WT (which was set to 100%). Statistical significance: * = $P < 0.05$; ** = $P < 0.01$.

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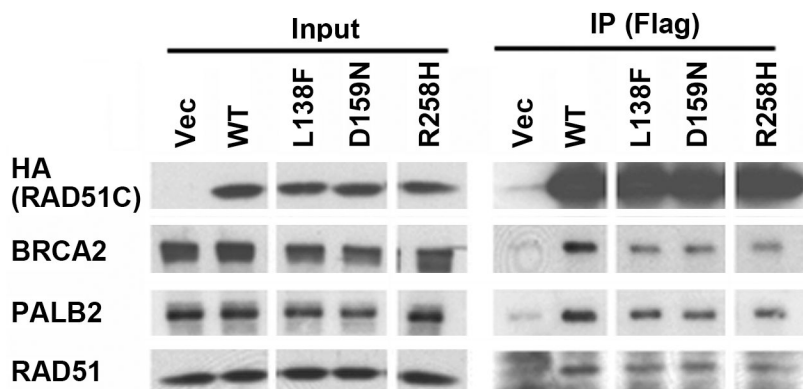


Figure 7. Disease-associated mutants of RAD51C have decreased interactions with BRCA2 and PALB2

(A) Wild-type RAD51C, the L138F and D159N mutants found in breast cancer patients, or the R258H mutant found in a Fanconi anemia patient, were transiently expressed in 293T cells along with a N-terminal Flag-HA epitope tag. The levels of protein in extracts (Input), or in immunoprecipitates prepared using α -Flag M2 agarose beads (IP), were determined by immunoblotting with the indicated antibodies. Immunoprecipitates represent 500-fold the levels loaded for Input lanes.