PALB2: THE HUB OF A NETWORK OF TUMOR SUPPRESSORS INVOLVED IN DNA DAMAGE RESPONSES

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Abstract

PALB2 was first identified as a partner of BRCA2 that mediates its recruitment to sites of DNA damage. *PALB2* was subsequently found as a tumor suppressor gene. Inherited heterozygosity for this gene is associated with an increased risk of cancer of the breast and other sites. Additionally, biallelic mutation of *PALB2* is linked to Fanconi anemia, which also has an increased risk of developing malignant disease. Recent work has identified numerous interactions of PALB2, suggesting that it functions in a network of proteins encoded by tumor suppressors. Notably, many of these tumor suppressors are related to the cellular response to DNA damage. The recruitment of PALB2 to DNA double-strand breaks at the head of this network is via a ubiquitin-dependent signaling pathway that involves the RAP80, Abraxas and BRCA1 tumor suppressors. Next, PALB2 interacts with BRCA2, which is a tumor suppressor, and with the RAD51 recombinase. These interactions promote DNA repair by homologous recombination (HR). More recently, PALB2 has been found to bind the RAD51 paralog, RAD51C, as well as the translesion polymerase pol η, both of which are tumor suppressors with functions in HR. Further, an interaction with MRG15, which is related to chromatin regulation, may facilitate DNA repair in damaged chromatin. Finally, PALB2 interacts with KEAP1, a regulator of the response to oxidative stress. The PALB2 network appears to mediate the maintenance of genome stability, may explain the association of many of the corresponding genes with similar spectra of tumors, and could present novel therapeutic opportunities.

Keywords

PALB2; tumor suppressor; DNA repair; homologous recombination; genome stability; breast cancer

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1. Introduction

1.1. DNA double-strand break repair proteins as tumor suppressors

The identification of tumor suppressor genes has been highly important for understanding how tumors develop (reviewed in [1, 2]). Breast cancer is a good example, where numerous genes have been identified which can cause familial cancer when they are mutated [3–7]. These include the two major genes associated with familial, or inherited, breast cancer, *BRCA1* and *BRCA2* [8, 9]. Inherited germline mutations in *BRCA1* or *BRCA2*, respectively, confer 57% and 49% lifetime risks of developing breast cancer by one estimate [10]. *BRCA1* and *BRCA2* are also major ovarian cancer genes [11]. Mutation of these genes has also been implicated in other cancers, such as those of the pancreas and prostate [12, 13].

The products of many of the identified breast cancer genes, including *ATM*, *CHEK2*, *TP53*, *BRCA1*, *BRCA2*, *PALB2*, *BRIP1*, *ABRA1*, *NBS1*, *RAD50*, *RAD51C* and *XRCC2* [3–7], are involved in DNA damage signaling in response to DNA double-strand breaks (DSBs) and/or DSB repair by homologous recombination (HR). This includes roles for ATM, Chk2 (encoded by the *CHEK2* gene), and RAP80 and Abraxas (encoded by the *ABRA1* gene) in DNA damage signaling. Further, NBS1 and RAD50 are required for end resection during HR, and BRCA2, PALB2, RAD51C and XRCC2 are involved in strand invasion or resolution of Holliday junction intermediates during HR (reviewed in [2, 14–17]). BRCA1 appears to have roles in each of the above activities [18–21].

DSBs are among the most deleterious DNA lesions that occur in cells and are an important source of genome instability. Thus, understanding how DSBs are repaired is important for understanding the origins of many cancers. Further, the repair of DSBs modulates the therapeutic response to radiation and to a variety of chemotherapeutic agents [22, 23], many of which induce DSBs as a result of replication fork collapse or as an intermediate of repair.

1.2. Fanconi anemia, cancer susceptibility, and DNA interstrand crosslink repair

Sixteen Fanconi anemia (FA) genes have been identified (*FANC- A*, *B*, *C*, *D1*, *D2*, *E*, *F*, *G*, *I*, *J*, *L*, *M*, *N*, *O*, *P* and *Q*) [24–46]. FA is a chromosome instability syndrome that is characterized by progressive bone marrow failure, congenital anomalies, and a predisposition to cancer (reviewed in [47, 48]). The risk of acute myeloid leukemia, and of head and neck squamous cell carcinoma, are especially elevated. Cells from FA patients display spontaneous chromosome instability, a defect in DNA interstrand crosslink-induced HR, and hypersensitivity to mitomycin C (MMC) and other DNA interstrand crosslinking agents [47, 49, 50]. The phenotypes of FA cells emphasize the importance of DNA repair pathways as tumor suppressors which prevent genome instability.

A core complex containing eight of the corresponding proteins, FANC- A, B, C, E, F, G, L, and M, is required for monoubiquitination of FANCD2 and FANCI [40, 41, 51]. Four of the FA genes, *BRCA2/FANCD1*, *BRIP1/FANCJ*, *PALB2/FANCN*, and *RAD51C/FANCO* are also breast cancer susceptibility genes [6, 7, 9, 52–54], and the corresponding proteins are not required for monoubiquitination of FANCD2. This subset of FA genes/proteins is also critical for cellular resistance to ionizing radiation (IR) [55–59].

1.3. The PALB2 protein may scaffold, or coordinate, HR proteins and other DNA damage response proteins

PALB2 is among the more recently identified breast cancer genes [52, 53, 60] and is required for HR [24, 53, 58, 60–67]. The PALB2 protein directly binds to BRCA2 [58, 68] and is a functional partner of BRCA2. This issue is considered in more detail in **Section 4.1**.

Here, we will review how recent work on PALB2 has elucidated the cooperation of RAP80 and Abraxas, BRCA1, PALB2 and BRCA2, RAD51, and the RAD51 paralogs RAD51C and XRCC3, in a network of DNA DSB repair by HR [24, 53, 58, 60–64, 66]. Alterations of each of the corresponding genes, except for *RAD51* and *XRCC3*, have been found to cause human cancer [4, 6, 7, 12, 69, 70]. Thus, understanding this DNA repair network, which functions to maintain genome stability, is important for understanding the etiology of cancer.

PALB2 also interacts with MRG15 [65, 71, 72], which is a component of various histone modifying complexes. This interaction represents a potential interface between chromatin regulation and DNA repair, as detailed in **Section 3.2**.

In addition, PALB2 also binds KEAP1 [73, 74], which is a regulator of Nrf2, a transcription factor for antioxidant genes. The interaction of PALB2 and KEAP1 will be examined in depth in **Section 2.3**. Mutations of *Nrf2* and *KEAP1* have been found associated with lung cancer [75]. This is of interest, given that reactive oxygen species (ROS) can lead to oxidative DNA damage that can yield DSBs. Further, ROS can either promote or suppress carcinogenesis, and can modulate the therapeutic response of tumors (reviewed in [76, 77]). Together, these various interactions, including those with HR proteins, a chromatin regulator, and a regulator of the response to oxidative stress, suggest that PALB2 potentially acts as a molecular scaffold that coordinates HR with other activities related to the DNA damage response [65, 66]. Ultimately, this network may prevent the development of cancerous cells by maintaining genome stability.

PALB2 has 1186 amino acids and a size of 131 kDa. Structural motifs which mediate protein interactions, including coiled-coil and WD40 domains, respectively, are present at the N- and C-termini of PALB2 (Fig. 1A). We will consider interactions with the N-terminus of PALB2, the middle region, and then the C-terminus, respectively, in **Sections 2, 3, and 4**. We will also examine how mutation of PALB2 in breast cancer affects its various protein interactions and may lead to cancer (**Sections 5.1 and 5.2**).

2. The N-terminus of PALB2

The amino-terminal one-third of PALB2 (approximately amino acids 1–394) contains one prominent structural element, a coiled-coil domain. Coiled-coil motifs are known to mediate protein-protein interactions [78]. The coiled-coil motif in PALB2 mediates interactions with BRCA1, and may thereby confer proper localization of PALB2 [63, 64]. Further, this N-terminal region of PALB2 is required for interactions with KEAP1 and with the RAD51 recombinase. The N-terminus of PALB2 is shown in detail in Fig. 1B along with interactions that have been identified.

2.1 Binding to BRCA1 and role in PALB2 localization

BRCA1 and BRCA2 are both recruited to nuclear foci in response to DNA damage [79, 80]. Further, BRCA1- and BRCA2-deficient cells have similar cellular phenotypes, including defects in DSB-initiated HR, chromosome instability, and hypersensitivity to DNA damaging agents [21, 26, 81]. But a direct physical interaction had never been demonstrated between these proteins. As it turns out, they interact indirectly with PALB2 acting as a linker [63, 64, 66].

Different domains on PALB2 mediate direct interactions with BRCA1 and BRCA2 [58, 63, 64, 66, 68]. The interaction of PALB2 with BRCA1 is via coiled-coil motifs present on PALB2 (amino acids 9–44) and BRCA1 (1393–1424) [63, 64, 66]. This interaction promotes DSB-initiated HR [63, 64, 66] and resistance to MMC [63, 66], suggesting that BRCA1 and PALB2 function together in a DNA repair pathway.

The question arose as to whether the interaction of BRCA1 and PALB2 mediates the recruitment of one or the other to nuclear foci. Two initial reports suggested that this interaction mediates the assembly of PALB2 foci [63, 64]. Further, it was reported that this interaction also promotes the assembly of BRCA2 and RAD51 foci in a PALB2-dependent manner [63]. Another report did not support these conclusions, however [66].

Subsequently, it was demonstrated that defective localization of a mutant of the PALB2 coiled-coil domain, and of RAD51 downstream, can be rescued by fusion to the BRCT repeats that normally localize BRCA1 [62]. This lends strong support to the conclusion that BRCA1 localizes PALB2. Importantly, RNAi-mediated depletion of PALB2 [61], or a genetic deficiency for PALB2 in cells from a FA patient [63], does not affect BRCA1 foci. Together, these results suggest that BRCA1 acts upstream of PALB2, which in turn has a key role in a pathway that recruits BRCA2 and RAD51 to sites of DNA damage (Fig. 2).

The recruitment of BRCA1 to DSBs is mediated by ubiquitin-dependent signaling that is centered around the RNF8 ubiquitin ligase (reviewed in [82]). Given that PALB2 is recruited in a BRCA1-dependent manner, the question arose as to whether ubiquitin-based signaling recruits PALB2 [62]. This possibility is supported by the finding that PALB2 colocalizes with ubiquitin, detected using the FK2 antibody, following exposure to IR [62]. Similar to the result for BRCA1 [83–85], RNAi-mediated depletion of RNF8 dramatically reduces IR-induced PALB2 foci [62]. Further, RNF8 binds to the MDC1 checkpoint mediator [83–85], and MDC1 is also required upstream of RNF8 in the recruitment of BRCA1 [86] and PALB2 [62] to sites of DNA damage.

Downstream of MDC1 and RNF8, RAP80 binds to K63-linked polyubiquitin chains and recruits its partner, Abraxas [87–90]. While less prominent than the roles of MDC1 and RNF8, RAP80 and Abraxas also function in recruiting PALB2 to sites of DNA damage that are induced by IR [62]. Since MDC1 binds to the histone variant, H2AX [86], which is phosphorylated after exposure to IR, the MDC1-RNF8-RAP80-Abraxas-BRCA1 network may connect PALB2-dependent HR to early signaling events that occur upon the generation of DSBs [62] (Figure 3).

2.2. Oligomerization of PALB2

In addition to interacting with BRCA1, the N-terminal coiled-coil of PALB2 can also self-associate [67, 91]. In this context, it should be noted that the coiled-coil domain of PALB2 mediates the assembly of PALB2 and RAD51 foci, as well as DSB-initiated HR and resistance to MMC [62–64, 66, 67, 91]. No separation of function mutant of the PALB2 coiled-coil domain has been identified that can directly compare the role of the BRCA1-PALB2 hetero-oligomer and the PALB2 homo-oligomer *in vivo*. Thus, the importance of the PALB2 homo-oligomer in these DNA damage response-related functions of PALB2, as compared to the role of the hetero-oligomer with BRCA1, has been unclear. An initial response to this question came from fusing the BRCT-repeats of BRCA1, which are required for its localization, to a mutant of the PALB2 coiled-coil domain that cannot interact with either BRCA1 or other PALB2 molecules [62]. Expression of this fusion protein in PALB2-deficient cells rescues various PALB2-dependent functions, including the assembly of RAD51 foci, DNA repair by HR, and resistance to MMC. On this basis, it appears that the interaction of PALB2 with BRCA1 may be sufficient to mediate each of these key functions of PALB2 and that the PALB2 homo-oligomer is not essential.

PALB2 promotes the assembly of RAD51 into a nucleoprotein filament with single-strand DNA *in vitro* [92, 93]. A PALB2 mutant lacking the coiled-coil domain (monomeric PALB2) shows increased DNA binding and increased assembly of the RAD51 filament *in vitro*, as compared to PALB2 which contains the coiled-coil [91]. Thus, based upon these results, the PALB2 homo-oligomer could have a regulatory role that is required for optimal maintenance of genome stability. However, whether the PALB2 homo-oligomer has such a regulatory role in intact cells is uncertain, especially since BRCA1 was not present in the *in vitro* experiments described above. It is also unknown how homo- versus hetero-oligomerization of PALB2 is regulated *in vivo*, and whether there are specific signals that induce one or the other at specific stages of DNA repair.

2.3. Interaction with KEAP1, a regulator of NRF2

Other interactions of PALB2, including that with KEAP1, have suggested functions for PALB2 that are not directly related to DNA repair but which may be complementary to its other characterized roles. KEAP1 is an E3 ubiquitin ligase which is a sensor of oxidative stress [94, 95]. KEAP1 can target NRF2, a master transcriptional factor for anti-oxidant genes, for cytoplasmic degradation. KEAP1 also has a role in nuclear export of NRF2 [73, 94]. But in response to oxidative stress, KEAP1-dependent down-regulation of NRF2 function is diminished, thereby promoting the anti-oxidant response. Importantly, PALB2 and NRF2 contain an identical LDEETGE sequence that can directly bind to KEAP1 [73]. As such, PALB2 can compete with NRF2 for binding to KEAP1 and can also disrupt pre-existing KEAP1-NRF2 complexes. Thus, PALB2 is a positive-regulator of NRF2 function and thereby has a role in cellular redox homeostasis [73]. In particular, by binding KEAP1, PALB2 can increase NRF2 occupancy at its target promoters, resulting in decreased cellular levels of ROS. Since ROS can lead to DNA damage (reviewed in [96]), positive regulation of NRF2 is another way in which PALB2 can promote the maintenance of a stable genome.

The LDEETGE motif is present at amino acids 88–94 in PALB2, so it is distinct from the coiled-coil domain at the N-terminus of this protein. Indeed, while mutation of the coiled-coil motif disrupts interactions with BRCA1, this does not disrupt the interaction of PALB2 with KEAP1 [73]. By independently interacting with BRCA1 and KEAP1, PALB2 may serve to coordinate DNA repair with detoxification of ROS. Further, while detoxification of ROS may contribute to the function of PALB2 as a tumor suppressor, it would appear that its role in DNA repair is more important in this regard. No cancer-associated mutants of the LDEETGE motif of PALB2 have been reported. Further, the majority of *PALB2* mutations found in cancer disrupt interactions with proteins that are involved in HR [53, 58, 60, 69].

2.4. PALB2 binds DNA and may have a direct role in HR through its interaction with RAD51

Recent *in vitro* studies have yielded important insight into how PALB2 may function in DNA repair. Notably, PALB2 has a previously unrecognized DNA binding activity [92, 93]. Two DNA binding regions (amino acids 1–200 and 372–561), both within the N-terminal third of PALB2, have been identified. Along with the capacity of PALB2 to homo-oligomerize, these two binding sites could permit PALB2 to bind multiple DNA molecules simultaneously [93]. In this context, it should be noted that BRCA2 also binds DNA *in vitro* [97]. Interestingly, the PALB2-BRCA2 interaction can compensate for the loss of DNA binding by BRCA2 in mediating DSB-initiated HR *in vivo* [98].

Additionally, PALB2 can promote RAD51-dependent formation of a D loop [92, 93], in which invading single-strand DNA pairs with homologous DNA within an intact duplex (Fig. 4). In this way, PALB2 may have a direct role in HR. PALB2 could have two activities in promoting D loop formation. The first of these is a potential role in stabilizing the association of RAD51 with the invading single-strand DNA [93]. This is an activity that PALB2 shares with BRCA2 [99]. Additionally, PALB2 can inhibit the association of RPA with the invading DNA, thereby promoting binding of RAD51 to DNA [93].

PALB2 has two sites which directly bind the RAD51 recombinase, one at the N-terminus, between amino acids 101–184, and another in the WD40 domain [92, 93]. It should be noted, however, that the WD40 domain of PALB2 appears to contain the primary binding site for RAD51 (**Section 4.2**). Whether these two RAD51-binding sites have distinct roles in mediating HR is unknown.

Interestingly, while *BRCA2* and *PALB2* are cancer susceptibility genes, *RAD51* has not been identified as a tumor suppressor. Perhaps RAD51 has an essential function in HR and thus mutations which interfere with its function may not be viable. Support for this possibility comes from findings that RAD51 is overexpressed in BRCA1-deficient cells and is regulated by alternative mechanisms in BRCA2-deficient cells [100, 101]. Still, the *RAD51* 135G>C polymorphism has been shown to increase cancer risk in carriers of *BRCA2* mutations [102], implicating some role for RAD51 in suppressing breast cancer.

While PALB2 alone can promote D-loop formation *in vitro*, it can synergize with RAD51AP1 [92]. In support of this finding, PALB2 and RAD51AP1 bind directly *in vitro*. Only a weak interaction has been found in cells, however, and it is not known what domain of PALB2 is involved. Still, these findings raise the possibility that a ternary complex of

PALB2, RAD51 and RAD51AP1, or perhaps a higher-order complex that also contains BRCA2, may have an important function in HR.

3. The central portion of PALB2

The region at the center of PALB2, which we will define here as being from amino acids 395–790, has fewer defined interactions than the other two regions of PALB2. Still, the central third of PALB2 is of interest since it contains two regions that modulate the interaction of PALB2 with chromatin. This includes the chromatin-association motif (ChAM) at amino acids 395–446 (Fig. 1A) and a region (amino acids 611–764) that binds the MRG15 protein [71, 103] (Fig. 1C). A subsequent report determined that a smaller region of PALB2, from amino acids 562–629, binds to MRG15 [104]. Both the ChAM motif and the region that binds to MRG15 are distinct from the DNA binding domains that have been identified in the N-terminal third of PALB2 (**Section 2.4**).

3.1 The chromatin-association motif (ChAM)

As determined by comparing the proportion of the protein present in the chromatin fraction following a detergent-based extraction, the ChAM domain clearly promotes the association of PALB2 with chromatin [103]. This is the case either with or without exposure to DNA damage induced by IR or MMC. Deletion of the ChAM motif does not completely abrogate PALB2 association with chromatin, however. Further, defective chromatin association of the ChAM mutant of PALB2 can be partially rescued by overexpression of MRG15, suggesting that the ChAM motif of PALB2 and the interaction of PALB2 with MRG15 cooperate in localizing PALB2 to chromatin. Since PALB2 regulates the association of BRCA2 with chromatin [61, 65, 103], it is perhaps not surprising that the ChAM domain of PALB2 also promotes the association of BRCA2 with chromatin [103]. The ChAM domain has a more minor effect on the association of BRCA1 with chromatin, which is consistent with the finding that PALB2 is not required for the assembly of BRCA1 foci ([61, 63, 91] and **Section 2.1**).

Interestingly, while the ChAM motif promotes the association of PALB2 with chromatin, it is not involved in binding to DNA [92, 93, 103]. The association of PALB2 with chromatin may instead be promoted by the apparent capacity of the ChAM motif to interact with nucleosomal histones [103].

The ChAM motif clearly has a role in the function of PALB2 in the cellular response to DNA damage [103]. For example, this motif promotes the recruitment of PALB2, and of RAD51 downstream, to sites of DNA damage. Further, a mutant of PALB2 in which the ChAM motif was deleted conferred no resistance to MMC when expressed in PALB2-deficient cells. Interestingly, cells with a genetic deficiency for *PALB2* are sensitive to inhibition of poly-ADP ribose polymerase (PARP) [57, 58, 93, 103], but deletion of the ChAM motif yielded only an intermediate sensitivity to the PARP inhibitor, olaparib, as compared to cells lacking reconstitution [103]. It is currently unknown why the ChAM motif is fully required for resistance to MMC but not olaparib. One possibility is that these agents induce DNA double-strand breaks (DSBs) after collapse of the replication fork, but by different mechanisms [103].

3.2 PALB2 interacts with MRG15, a component of various histone-modifying complexes

The interaction of PALB2 with MRG15 has been identified both in protein complexes isolated from cells, using mass spectrometry [65, 71], and through yeast-two hybrid screens [72]. This interaction is of particular interest given the potential challenges of recognizing and repairing DNA damage in the context of highly packaged chromatin (reviewed in [105, 106]). Indeed, the interaction of PALB2 and MRG15 provides a potential interface between chromatin regulation and DNA damage repair by HR.

MRG15/MORF4L1 and its yeast homolog, EAF3, are components of the Tip60/NuA4 histone acetyltransferase complex that is conserved from yeast to humans (reviewed in [107, 108]). The Tip60 complex is an important chromatin regulator that has been implicated in transcriptional regulation, as well as in DNA repair [108]. In particular, Tip60-dependent acetylation of histones H3 and H4 can mediate ubiquitination of H2A and recruitment of the RAD51 recombinase [109, 110].

Interestingly, while MRG15 readily associates with PALB2, other componenents of the Tip60 complex are in low abundance, or are absent, in PALB2-containing complexes [65, 71]. Thus, MRG15 may have PALB2-related functions that are independent of the Tip60 complex. In this context, it should be noted that while MRG15 is also a component of histone deacetylase (HDAC) 1 and 2 complexes [111], and the KDM5A histone demethylase complex [112], components of these other MRG15-related complexes were also not specifically detected in the PALB2 complex.

Multiple reports suggest that MRG15 has some role in PALB2-dependent repair of DNA by HR, but differ in the purported role of MRG15 in this process [65, 71, 72]. Sy et al. (2009) [71] suggest that knockdown of MRG15, or deletion of a broad region of PALB2 (amino acids 611–764) that contains the MRG15-interacting domain, leads to hyper-recombination. They therefore propose that MRG15 is a suppressor of HR. Consistent with this possibility, deletion of the region of PALB2 which interacts with MRG15 resulted in increased sister chromatid exchange (SCE). Depletion of MRG15, or reconstitution of PALB2-deficient cells with the MRG15-interaction mutant, did not alter sensitivity to MMC in this study [71].

In contrast, Hayakawa et al. (2010) [65] suggest that RNAi-mediated depletion of MRG15 decreases DNA DSB-initiated HR, but not to the same degree as depletion of PALB2 or BRCA2. They also reported that deletion of MRG15 sensitizes cells to MMC. Opposite from Sy et al., and consistent with a positive role in HR, these authors instead found that depletion of MRG15 decreases SCE [65].

Thus, together, these two reports support a role for the interaction of MRG15 with PALB2 in regulating HR, but disagree about whether MRG15 is a positive or negative regulator. While resolution of this question is important for understanding the mechanistic contribution of MRG15 to HR, the basis for these reported differences is currently unclear.

Consistent with a role for MRG15 in promoting HR, Hayakawa et al. (2010) also found that depletion of MRG15 leads to decreased assembly of PALB2 and RAD51 foci, both in

untreated populations and following exposure to IR. This is of interest, since the presence of a chromodomain in MRG15, and its association with various histone modifying complexes, suggests an intimate relationship of MRG15 with chromatin. It should be noted, however, that while the chromodomain of MRG15 can bind histone H3 methylated at Lysine 36 [113], the role of this chromodomain in the regulation of PALB2 behavior during HR is currently unknown.

An additional report also demonstrated an association between MRG15 and PALB2 [72]. Consistent with Hayakawa et al. (2010), murine embryonic fibroblasts, in which Mrg15 is disrupted, display decreased Rad51 foci and a moderate sensitivity to IR [72]. Given the apparent physical and functional relationship of MRG15 to PALB2, which is the product of a breast cancer gene, *MRG15* was considered to be a strong candidate cancer gene. Martrat et al. (2011) found no alterations in the *MRG15* gene in familial breast cancer [72]. Meanwhile, another study identified variants in patients with inherited breast cancer but these were determined to be unlikely to be disease-causing [114]. Further, while *PALB2* is also a FA gene, *MRG15* was not identified as a FA gene in the screen by Martrat et al.

4. The C-terminus of PALB2

The carboxy-terminal one-third of PALB2 (approximately amino acids 791–1186) is dominated by a seven-bladed WD40-type β-propeller from amino acids 853–1186 (Fig. 1A). WD40 domains are well known to mediate protein interactions (reviewed in [115]). Indeed, it was recently discovered that the WD40 domain of PALB2 mediates direct interactions with many of the key proteins involved in HR, including BRCA2 and RAD51, as well as the RAD51 paralogs RAD51C and XRCC3 [58]. Additionally, binding of the WD40 domain of PALB2 to pol η appears to mediate recombination-associated DNA synthesis [116]. Thus, the C-terminal WD40 domain may coordinate the activities of these key effectors of HR (Fig. 4). Since truncation of even the last four amino acids of PALB2 destabilizes the entire WD40 domain [68], and given the important role of HR in tumor suppression, the WD40 domain appears to be critical to the role of PALB2 in preventing tumor formation.

4.1 BRCA2 functions are mediated by direct binding to the PALB2 WD40 domain

Reflective of the important functional relationship between PALB2 and BRCA2, PALB2 was named "partner and localizer of BRCA2" [61]. Few BRCA2-interacting proteins had been identified, so the discovery of PALB2 yielded important new insight into the function and regulation of BRCA2.

It has been found that PALB2 is required for the recruitment of BRCA2 to nuclear foci that assemble either spontaneously or in response to DNA damage [61, 63]. Further, PALB2 stabilizes the chromatin-associated pool of BRCA2 [61, 65]. BRCA2 has a well-characterized role in the assembly of the RAD51 recombinase into nuclear foci [80]. Thus, the fact that PALB2 also regulates RAD51 foci [61, 63], strengthens the conclusion that it is a functional partner of BRCA2. Additional support for this possibility comes from the finding that PALB2 has a role in DSB-initiated HR and in resistance to MMC which is shared with BRCA2 [61]. Additionally, PALB2 appears to mediate the BRCA2-dependent

intra-S phase checkpoint in response to IR [61], and PALB2 and BRCA2 cooperate in regulating the G2 checkpoint in response to IR [117].

Importantly, mutants of PALB2 found in breast cancer or Fanconi anemia patients, which are defective for interaction with BRCA2, are deficient for DSB-initiated HR and for resistance to MMC [24, 53, 60]. Cells from FA patients with biallelic mutation of BRCA2 have similar defects in the DNA damage response [26, 81]. The close functional relationship of PALB2 and BRCA2 is also reflected by the fact that they are associated with similar spectra of cancers when mutated. Both are breast [8, 52, 53, 60, 118] and pancreatic [119, 120] cancer genes. Additionally, *BRCA2* is a well-known ovarian cancer gene [8], and predicted deleterious mutations or epigenetic silencing of *PALB2* are similarly observed in ovarian cancer [121, 122]. Further, biallelic mutation of *PALB2* and *BRCA2* in Fanconi anemia patients is associated with an especially early onset of acute myeloid leukemia, and with a high risk of medulloblastoma and Wilms tumor that is rarely seen in other FA subtypes [25, 123, 124].

Truncation mutants of PALB2 typically remove a portion, or all, of its WD40 domain. Defects of such mutants for DNA repair by HR were initially attributed to perturbation of BRCA2 function [24, 53, 60]. This was reasonable, since mutations of *BRCA2* and *PALB2* result in highly similar phenotypes in FA patients [25]. However, since the PALB2 WD40 domain binds a number of HR proteins, and inactivation of these proteins leads to similar defects in the DNA damage response, it is now unsure how much of the phenotypes associated with truncation of the PALB2 WD40 domain are directly due to perturbation of the interaction with BRCA2.

4.2 PALB2 interacts with RAD51 through a major binding site in its WD40 domain

Available evidence would suggest that the C-terminal WD40 domain of PALB2, rather than the N-terminal region (**Section 2.4**), is the primary binding site for RAD51. For example, truncation or elimination of the WD40 domain leads to a dramatic reduction in the binding of PALB2 to RAD51 [58, 63]. Additionally, deletion of exon 4 leads to reversion of a *PALB2* mutation in EUFA1341 cells from a Fanconi anemia patient and results in deletion of amino acids 71–561 [24]. While this mutant lacks the N-terminal RAD51-binding domain, it is largely resistant to MMC. In contrast, the original Y551X mutant, present in parental EUFA1341 cells, which eliminates the WD40 domain but retains the N-terminal binding site for RAD51, confers no resistance to MMC.

Disruption of the WD40 domain abrogates the assembly of RAD51 foci [24, 63]. The function of PALB2 upstream of RAD51 is also supported by the finding that key regulators of PALB2, including BRCA1 and MRG15, are required for optimal assembly of RAD51 foci [63, 65].

Although both RAD51 and BRCA2 can directly bind to the WD40 domain of PALB2 [58, 68, 93], whether they bind simultaneously in a ternary complex has not been determined. This possibility is consistent, however, with the finding that RAD51 and BRCA2 have different patterns of interactions with missense mutants of the PALB2 WD40 domain [58]. Thus, each protein may have different points of contact with PALB2. Further, it has been

demonstrated that WD40 domains on other proteins can bind to multiple substrates at the same time [125]. Consistent with the formation of such a ternary complex, PALB2 and a BRCA2 chimer promote RAD51-dependent strand invasion *in vitro* [93].

4.3 PALB2 interacts with the RAD51 paralog, RAD51C, through its WD40 domain

Five somatic paralogs of RAD51, including RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3, have been identified in vertebrate cells (reviewed in [17]). These paralogs have a similar size as RAD51, and also possess Walker A and B nucleotide binding motifs, but have an overall homology with RAD51 of 20–30% [17, 126]. Like RAD51, the paralogs have a role in HR and the maintenance of genome stability. For example, deletion of any of the paralogs in chicken DT40 cells compromises HR and sensitizes cells to MMC [55].

The role of the RAD51 paralogs is not well understood. Disruption of any of the genes in DT40 cells severely attenuates IR-induced assembly of RAD51 foci [55], suggesting a role early in HR prior to strand invasion. But some of the paralogs have also been reported to have functions late in HR [127–129]. This includes the finding that RAD51C foci persist longer at DSBs than do RAD51 foci [127], and the finding that RAD51C and XRCC3 can mediate resolution of Holliday junctions *in vitro* [129].

RAD51B-RAD51C-RAD51D-XRCC2 and RAD51C-XRCC3 complexes have been identified in cells [130–132], and direct interactions between various RAD51 paralogs have been identified using yeast 2- and 3-hybrid studies [133]. However, the recent finding that RAD51C, as well as XRCC3, can bind to the PALB2 WD40 domain is a strong indication that these RAD51 paralogs may function in HR outside of paralog-only complexes [58].

In a recent study, PALB2 and BRCA2 were the only proteins besides RAD51 and RAD51 paralogs that were identified in RAD51C protein complexes by mass spectrometry [58]. This was interesting considering that, like *PALB2* and *BRCA2*, *RAD51C* had been identified as a breast/ovarian cancer gene [6, 7].

Further, *RAD51C* was identified as a putative FA gene [30], similar to *PALB2* and *BRCA2*. Several affected children within a single family had a biallelic missense mutation in *RAD51C* (R258H). The only surviving child displayed cellular phenotypes, including DNA interstrand crosslink (ICL)-induced G2-M accumulation and chromosome instability, and developmental abnormalities, which are characteristic of FA. The surviving patient did not have cancer or display hematological abnormalities through age ten, which is not unusual in FA [30]. The finding that RAD51C directly binds to PALB2 further suggests a function for this RAD51 paralog in the FA-BRCA pathway of DNA repair. Consistent with this possibility, the RAD51C missense mutant from a FA patient, R258H, displays defective co-immunoprecipitation of PALB2 and BRCA2, as well as RAD51 and XRCC3 [58, 134]. Missense mutants of RAD51C from breast cancer patients, including L138F and D159N, display similar defects.

RAD51C directly binds to the WD40 domain of PALB2 [58]. Consistent with this observation, truncation of the WD40 domain abrogates binding to BRCA2, RAD51C and XRCC3, and largely diminishes binding to RAD51 [58]. Thus, the PALB2 WD40 domain

could coordinate, or scaffold, a complex of key components of the machinery for HR. These proteins might cooperate to mediate DNA repair by HR and thereby promote genome stability.

Alternatively, it is possible that PALB2 binds to specific HR proteins at different steps in HR. In this way, PALB2 might coordinate various steps involved in this process, including strand invasion, recombination-dependent DNA synthesis, and resolution of the Holliday junction. As an example, the L1143P missense mutant of the PALB2 WD40 domain, which has a clearly diminished interaction with RAD51C, both *in vitro* and *in vivo*, leads to a modest but significant increase in the assembly of BRCA2 foci in response to IR [58]. Thus, it is possible that RAD51C acts subsequent to BRCA2 and displaces it from its interaction with PALB2. Further work will be needed to resolve these questions.

Since truncation of the PALB2 WD40 domain abrogates interactions with numerous HR proteins, to better analyze how each protein binds PALB2, missense mutants of the WD40 domain that were identified in breast cancer patients [135] were tested instead [58]. Interestingly, RAD51C has a different pattern of interaction with the L939W, T1030I and L1143P mutants of the PALB2 WD40 domain *in vitro*, than do BRCA2 or XRCC3, or RAD51. Thus, multiple HR proteins could possibly bind to the WD40 domain of PALB2 simultaneously.

4.4 PALB2 interacts with the RAD51 paralog, XRCC3, through its WD40 domain

XRCC3 also directly binds to the WD40 domain of PALB2 [58]. The pattern of interaction of XRCC3 with PALB2 WD40 missense mutants resembles that of BRCA2, rather than that of RAD51C. This is of potential importance since RAD51C and XRCC3 have been reported to form a complex [130–132]. This suggests that each protein may independently bind to PALB2 rather than one of the proteins in the RAD51C-XRCC3 complex simply having an interaction with PALB2. One possibility is that RAD51C and XRCC3 bind to the PALB2 WD40 domain at different steps during HR-dependent repair of DNA.

Interestingly, PALB2-deficient cells show diminished levels of the RAD51C-XRCC3 complex, as compared to complemented cells [58]. Thus, binding of PALB2 to one or both components of the RAD51C-XRCC3 complex may compete with a RAD51C-XRCC3 complex that is devoid of PALB2. While the RAD51C-XRCC3 complex has Holliday junction resolvase activity *in vitro* [136], whether the PALB2-free complex formed by these proteins has any function in cells is presently unknown.

The observation that *RAD51C* is a FA gene and breast/ovarian cancer susceptibility gene, like *PALB2*, yields some support for the possibility that the RAD51C protein has critical functions in complex with PALB2. But *XRCC3* has not been identified as a FA gene or as a cancer gene, and the role of the complex it forms with PALB2 is currently unknown.

4.5 PALB2 binds pol η and promotes recombination-dependent DNA synthesis

An additional interaction of the PALB2 WD40 domain, with pol η, has recently been reported [116]. Pol η directly binds to PALB2 and also to the BRC repeats of BRCA2 [116]. It was already known that PALB2 promotes strand invasion during HR [92, 93], thereby

forming a D-loop intermediate. The 3′ tail end ssDNA is then utilized as a primer for recombination-associated DNA synthesis. Thus, the ability of PALB2 to interact with both RAD51 and with pol η may coordinate strand invasion and subsequent synthesis (Fig. 4). Indeed, PALB2 and BRCA2 promote DNA synthesis by pol η on D loop substrates in vitro [116]. Further, PALB2, as well as BRCA2, are required for the assembly of pol η into foci at blocked replication forks and may recruit it to the 3′ end ssDNA at the D loop. Consistent with a key role in recombination-associated DNA synthesis, deletion of pol η in DT40 cells impairs HR [137].

Pol η is a translesion polymerase which can bypass thymidine dimers that are induced by ultraviolet radiation. A deficiency for this activity, resulting from biallelic mutation of the *RAD30* gene, greatly increases the risk of sunlight-induced skin cancer in Xeroderma pigmentosum variant (XP-V) patients [138, 139]. While this demonstrates the role of pol η as a tumor suppressor, biallelic mutation of *PALB2* or *BRCA2* in Fanconi anemia does not confer a particular predisposition to skin cancer. Thus, the contribution of defects in pol η-dependent recombination-associated DNA synthesis to the development of cancer is less clear than the role of defects in pol η-dependent repair of thymidine dimers. The latter activity is related to nucleotide excision repair rather than HR.

5. Implications of the PALB2 network for cancer biology and cancer management

The finding that PALB2 is involved in a network of tumor suppressors that regulate or mediate HR has already yielded important insight into how this repair pathway maintains genome stability and thereby prevents cancer. The fact that *BRCA1*, *PALB2*, *BRCA2* and *RAD51C* are all breast/ovarian cancer genes underlines the importance of the interaction of the corresponding proteins in a common pathway of DNA repair. The observation that mutation of many of these HR genes leads to a similar spectrum of tumors suggests the importance of particular DNA lesions, including DSBs and stalled/collapsed replication forks, to the genesis of breast/ovarian cancer. Importantly, further investigation of protein interactions within the PALB2 network should continue to improve the understanding of mechanistic steps that are involved in HR. This may ultimately lead to the identification of novel biomarkers for diagnosing and treating cancer, and could identify novel therapeutic targets.

5.1 How missense mutations in PALB2 affect DNA repair

With the exception of the G1145R missense mutant of PALB2, which was found in a breast cancer patient and does not diminish HR or cellular resistance to MMC [53], until recently no functional studies had been conducted on PALB2 missense mutants. A recent study, however, found that the L939W and L1143P missense variants/mutants of the PALB2 WD40 domain are stably expressed in PALB2-deficient fibroblasts and have modest but significant decreases in HR and in cellular resistance to IR [58]. This is consistent with the fact that altered, but not abrogated interactions with BRCA2, RAD51 and RAD51C, are observed [58]. In contrast, truncation mutants of PALB2, found in FA or breast cancer, which remove the WD40 domain, have no HR activity and confer no resistance to MMC

[24, 53, 60]. Thus, missense mutants of the PALB2 WD40 domain may, in general, be associated with less genome instability than the truncation mutants. Although less than that conferred by truncation mutants, these missense mutants may still confer an increased cancer risk. Importantly, the recent study by Park et al. has established an assay system for further functional evaluation of PALB2 missense variants [58].

Interestingly, a missense mutant, T1030I, which was examined by Park et al. could not be stably expressed in PALB2-deficient fibroblasts and appears to be degraded in a proteasome-dependent manner [58]. Thus, there are at least two mechanisms by which missense mutants of the PALB2 WD40 domain may act. The first mechanism involves instability of PALB2, and thus lack of a functional complex with BRCA2, RAD51 and RAD51C, as seen for PALB2-T1030I. The second mechanism involves altered but not abrogated interactions with these HR proteins, as seen for PALB2-L939W and PALB2-L1143P. Unstable missense mutants are expected to have a larger defect in DNA repair and, thus a greater risk of developing cancer.

Similarly, truncation mutations of *PALB2* also appear to act by at least two mechanisms. Truncations which remove the WD40 domain lead to stable mutants [24, 53, 60], whereas the Y1183X mutant of PALB2 interferes with closure of the WD40 structure and is unstable [25]. Either type of truncation mutant is expected to fully abrogate PALB2 function, however.

5.2 Role of the PALB2 network in preventing genome instability and replication defects

Genome instability enables the development of cancer by leading to a series of genetic changes, including amplifications, deletions, rearrangements and substitutions, which result in genetic diversity (reviewed in [140]). This diversity can lead to clonal selection and the development of more aggressive cancers, and even to resistance to therapy [141]. The PALB2 network is thought to suppress tumor formation by promoting DNA repair, thereby preventing genome instability and the initiation of cancer. Consistent with this thinking, BRCA1, PALB2, BRCA2, and RAD51C all prevent chromosome instability characterized by chromosome breaks or rearrangements that occur spontaneously, or in response to DNA interstrand crosslinking agents or PARP inhibitors [21, 26, 30, 81, 142].

There is also evidence that PALB2 and related proteins may maintain genome stability by promoting fidelity in DNA replication. Heterozygous mutation of PALB2 is associated with a six-fold increase in the risk of developing malignant disease [143]. Interestingly, cells that are heterozygous for *PALB2* mutations display increased chromosome instability. Further, this increased chromosome instability is associated with perturbation of DNA replication, including increased origin firing and decreased distances between active origins [143]. Loss of heterozygosity would be expected to yield even greater chromosome instability and might similarly be related to defects at replication forks.

As a further indication of the potential role of the PALB2 network in mediating chromosome stability through a role in DNA replication, BRCA1 and BRCA2 prevent the degradation of newly replicated DNA at stalled forks [144, 145]. Evidence would suggest that their action in maintaining the stability of DNA at the replication fork is separable from

their role in DSB repair [144]. The function of BRCA1 and BRCA2 in the response to replication stress is relevant both to understanding the genesis of cancer and the response to treatment. For example, BRCA1 is required for the stability of common fragile sites, which are loci at which replication stalling occurs more frequently [146]. Also, chemotherapeutic agents that result in ICLs induce replication stress and/or fork stalling [49, 145, 147]; BRCA1- [21] and BRCA2-deficient cells [26], as well as PALB2- [24, 53, 60, 61, 63] and RAD51C-deficient cells [6, 30, 55], are all hypersensitive to ICLs. While the role of PALB2 was not specifically tested, one might anticipate that it is also involved in this process.

5.3 Implications of the PALB2 network for the treatment of cancer

The identification of a network of tumor suppressors that includes PALB2 may have important consequences for treating cancer. For example, defects in DSB repair are typically associated with increased sensitivity to radiation. Additionally, because the DSB-initiated HR pathway can rescue defects in base excision repair, mutation of genes in this network are typically also associated with hypersensitivity to PARP inhibitors [57, 58, 93, 103, 148–150]. Further, DNA interstrand crosslinking agents, including platinum compounds and nitrogen mustards are a major class of chemotherapeutic agents utilized to treat cancer [49]. BRCA1, PALB2, BRCA2 and RAD51C, which are key components of the HR machinery, are required for cellular resistance to ICLs [6, 21, 26, 61]. Thus, there are numerous therapeutic options which may be utilized to treat tumors with deficiencies in the PALB2-based network of HR.

At present, there is abundant interest in inhibitors of histone methyltransferases and histone demethylases for treating cancer [151, 152]. Thus, the fact that MRG15 possesses a chromodomain and influences HR could potentially be utilized to rationally design combination treatments for cancer. For example, inhibitors which modulate histone methylation might be utilized to suppress recruitment of MRG15, and MRG15-dependent recruitment of PALB2 and RAD51, and thereby inhibit HR. This would be expected to sensitize cells to DNA damage induced by radiation or DNA interstrand crosslinking agents.

5.4 Assays for defective assembly of RAD51 foci to predict deficiencies in PALB2 network proteins

The majority of the tumor suppressors in the PALB2 network, including BRCA1, PALB2, BRCA2, and RAD51C, are involved in the assembly of RAD51 foci in response to DNA damage [30, 55, 61, 63, 80, 153]. Thus, it may be possible to utilize assembly of RAD51 foci as a functional assay to identify a defect in the central PALB2 network, without having information on the presence or clinical significance of mutations in any of the associated genes. Since this network of HR proteins mediates resistance to radiation, as well as to ICLs, a defect in RAD51 foci may be predictive of a better clinical response to such treatments [154]. For example, there have been reports that mutations in *BRCA1* or *BRCA2* lead to increased response of ovarian cancers to platinum compounds and/or increased survival [155–158]. An approach for *ex vivo* irradiation of living tumor tissue to assess levels of RAD51 foci in breast cancer tissue has recently been described [159].

6. Conclusions

Eight proteins, BRCA1, KEAP1, MRG15, BRCA2, RAD51, RAD51C, XRCC3, and pol η, have been identified which directly bind to PALB2 [58, 63–66, 68, 71, 73, 92, 93, 116]. The majority of these proteins, except for MRG15, RAD51 and XRCC3, have been demonstrated to have tumor suppressor activities [6, 8, 9, 75, 138]. Additionally, RAP80, Abraxas, and RAD51AP1 have functional interactions with PALB2 [62, 92]. Among these, RAP80 and Abraxas are also tumor suppressors [4, 160]. Thus, PALB2 appears to function in a network of tumor suppressors. PALB2 may act as the hub of this network based upon its numerous interactions and its apparent function near the center of the network. PALB2 recruitment to sites of DNA damage is downstream of ubiquitin-dependent signaling and of BRCA1. PALB2 recruitment also involves MRG15, which is a component of histone remodeling complexes [62, 65, 71]. Once recruited, though, PALB2 then has a role in regulating the response to oxidative stress through its interaction with KEAP1 [73], and appears to coordinate DNA repair by HR through its interactions with BRCA2, RAD51, RAD51C, XRCC3, and pol η[58, 92, 93, 116].

Loss of function of tumor suppressors in the PALB2 network is associated with a shared pre-disposition to breast and/or ovarian cancer via mutation of *RAP80*, *Abraxas*, *BRCA1*, *PALB2*, *BRCA2* or *RAD51C* [4, 6, 8, 9, 52, 53, 160]. Understanding why loss of function of proteins related to DSB-initiated HR specifically predisposes individuals to breast and/or ovarian cancer may provide insight into risk factors for these cancers and could ultimately lead to improved cancer prevention. Also, further analysis of protein complexes which contain proteins in the PALB2 network may likely identify additional tumor suppressor genes.

Finally, as discussed in **sections 5.3 and 5.4**, the identification of the PALB2 network of DNA damage response proteins may have important implications for treating cancer, including the potential for identification of biomarkers to guide the selection of therapeutic options. Definition of this network has also provided a framework for a better understanding of DNA repair by HR and this insight could, ultimately, lead to novel therapeutic targets.

Acknowledgments

Abbreviations

BRCA1	Breast cancer susceptibility 1
BRCA2	Breast cancer susceptibility 2
DNA	Deoxyribonucleic acid
DSB	DNA double-strand break
FA	Fanconi anemia
HR	Homologous recombination

ICL	DNA interstrand crosslink
IR	Ionizing radiation
KEAP1	Kelch-like ECH-associated protein 1
MMC	Mitomycin C
MMR	Mismatch repair
PALB2	Partner and Localizer of BRCA2
PARP	poly-ADP ribose polymerase
RAD51AP1	RAD51-associated protein 1
RAP80	Receptor associated protein 80

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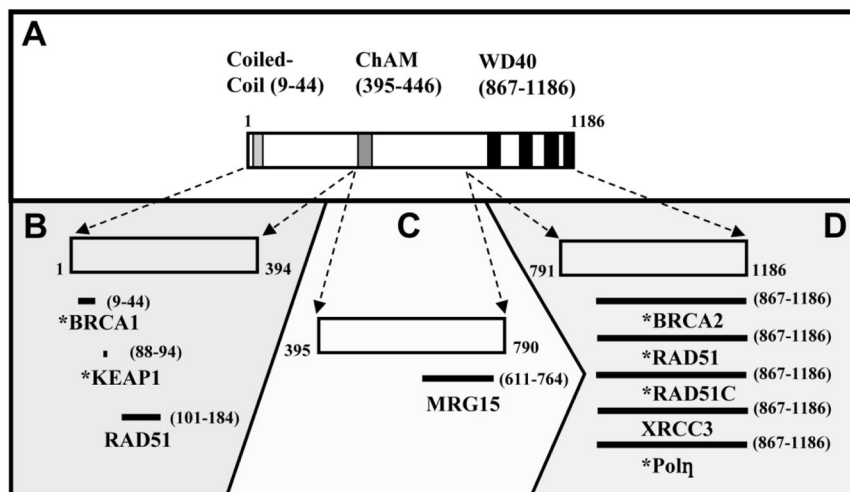**Figure 1.**
PALB2 has numerous interactions in its role in mediating DNA damage responses. (A) Three structural domains have been identified, one each in the N-terminal, central, and C-terminal thirds of the protein. (B) Protein interactions with the N-terminal portion of PALB2 (amino acids 1–394) which have been identified are displayed. (C) MRG15, the only known interaction of the central region of PALB2 (amino acids 395–790) with a specific protein, is shown. (D) Numerous proteins with functions in homologous recombination (HR) associate with the WD40 domain present in the C-terminus of PALB2. (B–D) Interacting proteins which have been identified as tumor suppressors are indicated (*).
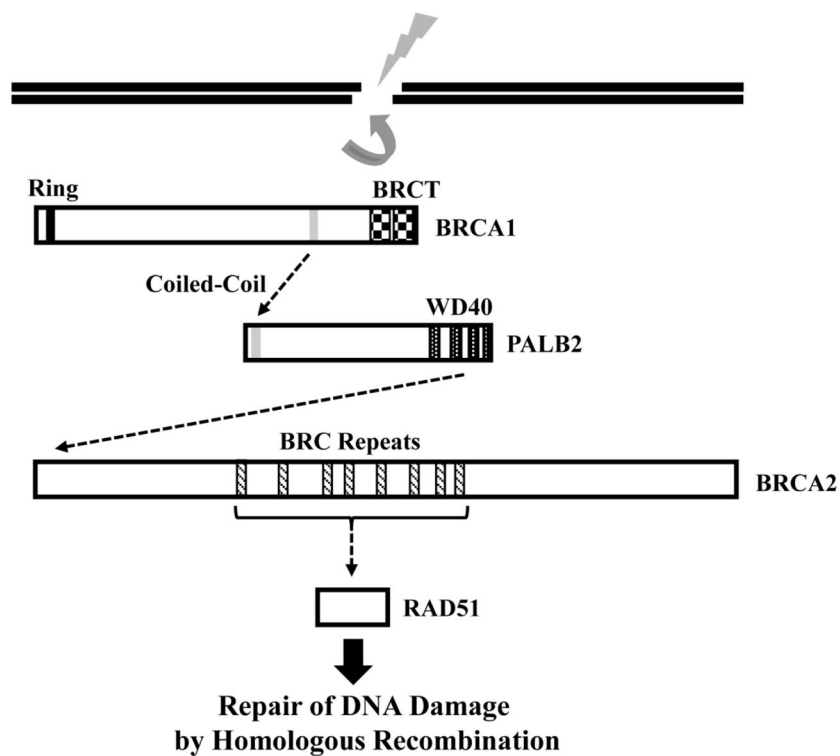
Figure 2.
PALB2 links BRCA1 and BRCA2 into a pathway of products of breast cancer susceptibility genes. This pathway mediates HR, and as a result, the maintenance of genome stability. BRCA1 binds to PALB2 through a coiled-coil motif present on each protein and recruits it to sites of DNA damage. PALB2 then directly binds to the N-terminus of BRCA2 through its WD40 domain and recruits BRCA2. BRCA2 directly binds and recruits the RAD51 recombinase, which mediates strand invasion. PALB2 can also directly bind RAD51, but whether PALB2 can recruit RAD51 independent of BRCA2 is unknown. *BRCA1*, *PALB2*, and *BRCA2* are all breast cancer susceptibility genes.
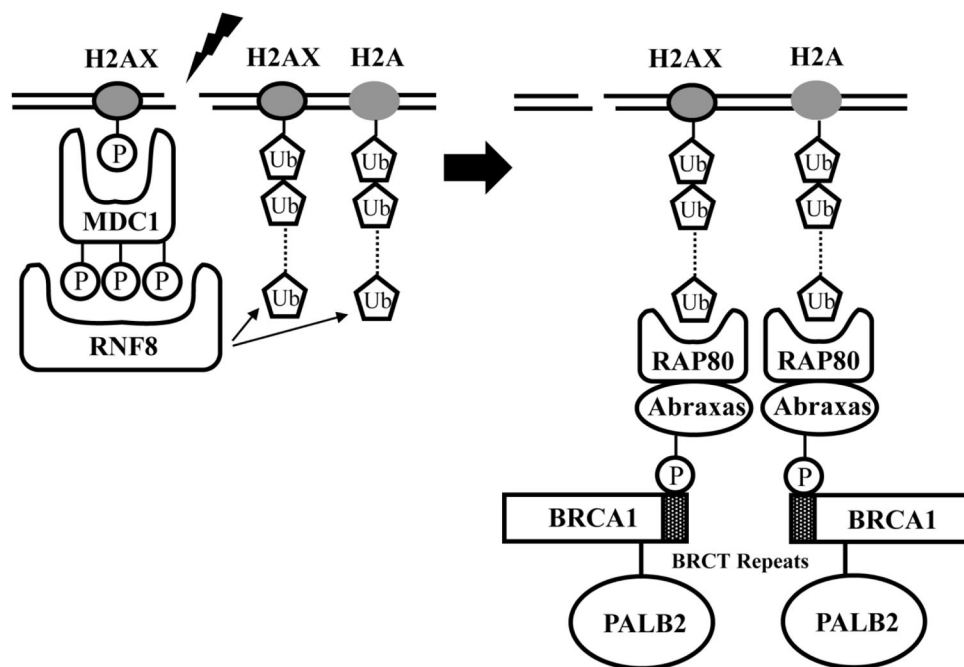
Figure 3.
Ubiquitin-dependent signaling culminates in the recruitment of PALB2 to sites of DNA damage. (Left) MDC1 binds to the phosphorylated histone variant, H2AX, and is then recognized by the RNF8 E3 ubiquitin ligase. RNF8 mediates polyubiquitination of H2A and of H2AX. (Right) Polyubiquitinated H2A and H2AX are then recognized by RAP80, which has an ubiquitin-interaction motif. The partner of RAP80, Abraxas, is phosphorylated in a cell cycle-dependent manner. BRCA1 binds to phospho-Abraxas, which thereby results in the recruitment of PALB2.
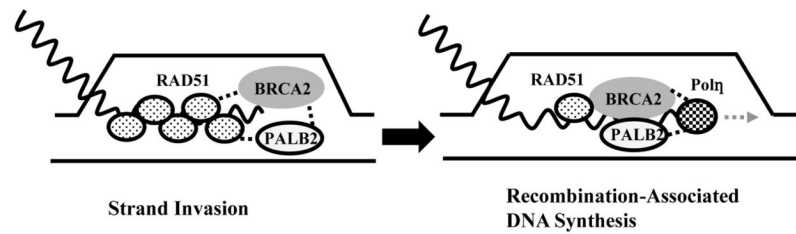
Figure 4.
PALB2 and BRCA2 can directly bind RAD51 and pol η. The interaction of PALB2 and
BRCA2 with RAD51 promotes formation of a D-loop during strand invasion (left), while
interaction with pol η promotes recombination-associated DNA synthesis (right). PALB2
and BRCA2 may thereby coordinate these two steps during HR.