

Homologous Recombination and Human Health: The Roles of BRCA1, BRCA2, and Associated Proteins

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Homologous recombination (HR) is a major pathway for the repair of DNA double-strand breaks in mammalian cells, the defining step of which is homologous strand exchange directed by the RAD51 protein. The physiological importance of HR is underscored by the observation of genomic instability in HR-deficient cells and, importantly, the association of cancer predisposition and developmental defects with mutations in HR genes. The tumor suppressors BRCA1 and BRCA2, key players at different stages of HR, are frequently mutated in familial breast and ovarian cancers. Other HR proteins, including PALB2 and RAD51 paralogs, have also been identified as tumor suppressors. This review summarizes recent findings on BRCA1, BRCA2, and associated proteins involved in human disease with an emphasis on their molecular roles and interactions.

Soon after homologous recombination (HR) was discovered to be an important DNA repair mechanism in mammalian cells, an association between HR deficiency and human disease was uncovered when the hereditary breast cancer suppressors BRCA1 and BRCA2 were found to be required for HR (Moynahan and Jasin 2010; King 2014). Subsequently, germline mutations in a number of other HR genes have been linked to tumor predisposition. Congenital defects have also been associated with impaired HR. Tumorigenesis can result from ongoing genomic instability from diminished repair, whereas developmental defects can arise from cell

death/senescence. That HR genes act as genomic caretakers has generated widespread interest in both the scientific and medical communities. Because HR defects confer sensitivity to certain DNA-damaging agents, they are being exploited in cancer therapies. Drugs that cause synthetic lethality in the context of HR defects also hold promise for treatment (Bryant et al. 2005; Farmer et al. 2005). This review provides a brief overview of HR in mammalian cells and summarizes the molecular roles of BRCA1, BRCA2, and associated HR proteins involved in human disease. Extensive discussion of HR pathways can be found in Mehta and Haber (2014).

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R. Prakash et al.

THE IMPORTANCE OF HR IN MAMMALIAN CELLS

DNA lesions, such as double-strand breaks (DSBs), threaten the integrity of the genome, but HR provides a mechanism to precisely repair the damage. DSBs repaired by HR are first end resected to generate 3' single-stranded DNA (ssDNA) (Fig. 1) (see Symington 2014). A DNA strand-exchange protein—RAD51 in mammalian cells—binds to the ssDNA to form a nucleoprotein filament, which promotes strand invasion into a homologous duplex to initiate repair synthesis (see Morrical 2015). In the synthesis-dependent strand-annealing (SDSA) pathway of HR, the newly synthesized DNA dissociates to anneal to the other DNA end, and the HR event

is completed by ligation (see Zelensky et al. 2014 and Daley et al. 2014). More complex pathways involve Holliday junction resolution or dissolution (Jasin and Rothstein 2013; see also Bizard and Hickson 2014; Wyatt and West 2014). DSB repair can also occur by a second major mechanism, nonhomologous end joining (NHEJ) (Chapman et al. 2012b). NHEJ differs from HR in that the DNA ends are protected from resection before being rejoined; nevertheless, deletions and insertions can arise during NHEJ. The preferred template for HR is the identical sister chromatid, although the homolog can be used at lower frequency (Johnson and Jasin 2001). The use of the sister chromatid leads to precise repair, restoring the original sequence that was present before damage, but it is limited

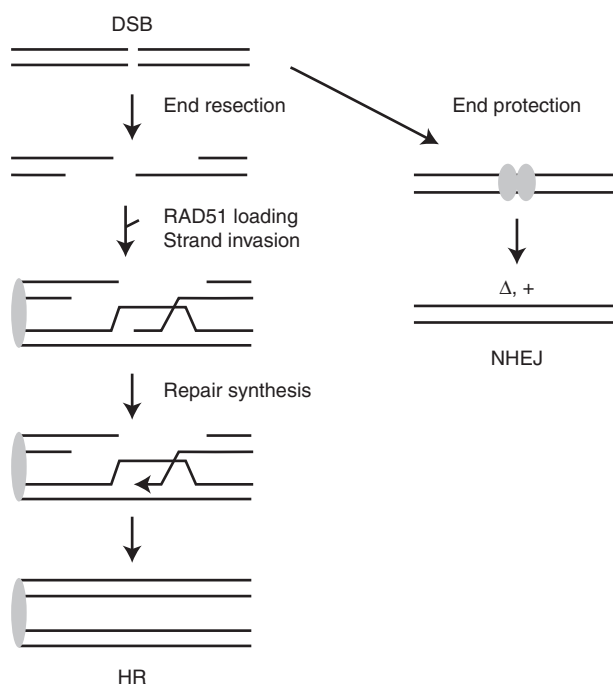


Figure 1. Simplified schemes of double-strand break (DSB) repair by homologous recombination (HR) and nonhomologous end joining (NHEJ). Once a DSB is generated, it can be processed for HR by end resection and strand invasion proteins, leading to ssDNA. The RAD51 strand-exchange protein forms a nucleoprotein filament with ssDNA that invades an unbroken homologous DNA, typically the sister chromatid, as shown. The 3' end primes DNA synthesis from the homologous DNA; using the sister chromatid, the repair can be precise to restore the original sequence before damage. To complete HR, the newly synthesized strand can dissociate to anneal to the other end. Other outcomes are also possible, for example, in which Holliday junctions are formed and either dissolved or resolved. Alternatively, DNA ends are protected from end resection by NHEJ proteins; subsequent steps in NHEJ can result in mutagenic repair with deletions and insertions ($\Delta, +$).

to the S/G₂ phases of the cell cycle, whereas NHEJ is operational throughout the cell cycle (Rothkamm et al. 2003).

Although HR has long been known to be a major DNA repair mechanism in bacteria and yeast (see Mehta and Haber 2014; Reams and Roth 2015), the importance of HR in the maintenance of mammalian genome integrity has only emerged in the last two decades. Direct evidence came from molecular analysis of DSB repair, in which HR and NHEJ are both found to be robust repair mechanisms (Fig. 1) (Rouet et al. 1994; Liang et al. 1998; Johnson and Jasin 2000). This finding forms the basis of current genome-editing approaches in mammalian cells (Cong et al. 2013; Mali et al. 2013).

Strong genetic evidence for the importance of HR comes from the study of mice deficient in the RAD51 strand-exchange protein. *Rad51*

disruption is lethal early in embryogenesis and *Rad51* null cells cannot be propagated (Lim and Hasty 1996; Tsuzuki et al. 1996). The lethality is attributed to the impaired repair of lesions that arise during DNA replication in the rapidly cycling cells of the embryo. Thus, a critical function of HR is likely to be the repair of replication-associated damage (see Syeda et al. 2014), as is the case in bacteria (Cox et al. 2000). HR is also critical for the repair of interstrand cross-links (Long et al. 2011).

HR and NHEJ can “compete” for the repair of the same lesion but also “collaborate” in the repair of distinct lesions (Fig. 2A) (Kass and Jasin 2010). Competition is evidenced in a standard HR reporter assay in which a DSB is induced by the I-SceI endonuclease: HR, measured as GFP⁺ cells, is elevated in NHEJ mutant cell lines relative to wild-type cells (Fig. 2B)

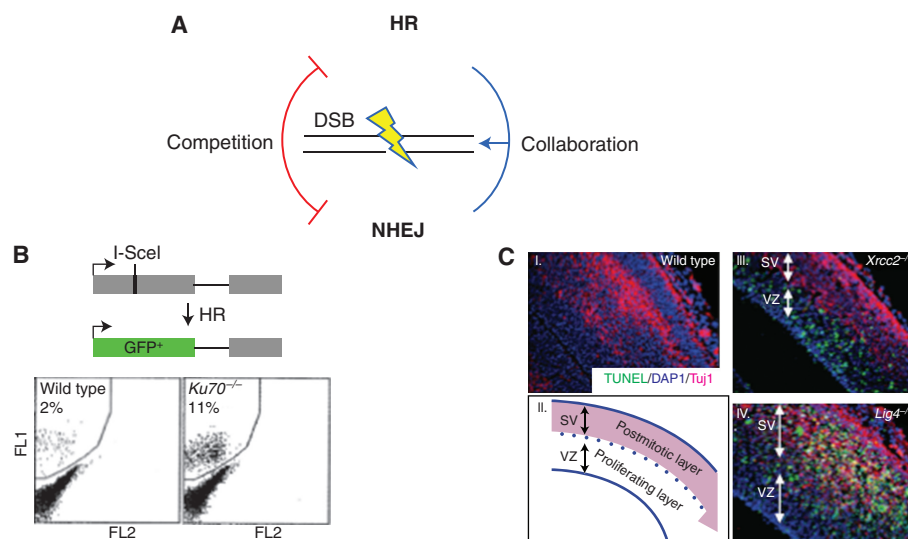


Figure 2. Competition and collaboration between double-strand break (DSB) repair pathways. (A) Schematic of the interactions between the homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways for DSB repair. (B) Competition for repair of a DSB. In the direct repeat green fluorescent protein (DR-GFP) reporter assay, a DSB repaired through HR restores a functional *GFP* gene, as detected by flow cytometry. NHEJ-deficient *Ku70*^{-/-} cells show substantially elevated HR, showing how HR and NHEJ can act on the same DSB, such that NHEJ suppresses HR (modified from Pierce et al. 2001). The DSB is generated by I-SceI endonuclease. (C) Collaboration between HR and NHEJ, as illustrated in the embryonic brain. (i,ii) Apoptosis is rare in the wild-type embryonic brain (E13.5), as indicated by the lack of TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining in either the proliferating ventricular zone (VZ) or the postmitotic subventricular (SV) zone (marked with Tuj1). (iii) HR-deficient *Xrcc2*^{-/-} cells show substantial apoptosis in the VZ. (iv) In contrast, NHEJ-deficient *Lig4*^{-/-} cells predominantly have elevated apoptosis in the SV. Therefore, HR and NHEJ both contribute to the integrity of the embryonic brain (modified from Orii et al. 2006).

R. Prakash et al.

(Pierce et al. 2001). The rescue of certain HR mutants with combined NHEJ deficiency also speaks to the competition between pathways (see below) (Bouwman et al. 2010; Bunting et al. 2010). Collaboration between HR and NHEJ is illustrated in the different cell layers of the embryonic brain. HR is required in the proliferating cell layer and NHEJ is required in the postmitotic cell layer, such that mutation of either an HR or NHEJ pathway component leads to high levels of apoptosis but in a distinct cell layer (Fig. 2C) (Orr et al. 2006). Collaboration between pathways is also observed in some HR/NHEJ double mutant mice, which show more severe phenotypes than either single mutant (Couedel et al. 2004; Mills et al. 2004). HR and NHEJ can even be used to repair the same lesion through a break-induced replication-type event, which is initiated by HR and completed by NHEJ (Richardson and Jasin 2000). Special cases are the programmed DSBs, which are channeled into defined repair pathways, for example, HR for SPO11-generated DSBs during meiosis (see Lam and Keeney 2015) and NHEJ for RAG-induced DSBs in the immune system (Chapman et al. 2012b).

Early studies of mammalian homologs of yeast HR genes did not link HR to tumor suppression. Mouse knockouts showed embryonic lethality (*Rad51*, Lim and Hasty 1996; Tsuzuki et al. 1996) or, at the other extreme, little or no phenotype (*Rad52*, Rijkers et al. 1998; *Rad54*, Essers et al. 1997). However, characterization of *BRCA1* and *BRCA2* led to the discovery of the link between HR and human health, in particular, tumor suppression (Moynahan and Jasin 2010). Subsequent work has implicated a number of other HR proteins, including *PALB2* and the *RAD51* paralogs.

BRCA1

BRCA1 Mutations in Patients

Breast cancer early onset gene 1 (*BRCA1*) was identified in the early 1990s as one of the major hereditary breast cancer susceptibility genes (Futreal et al. 1994; Miki et al. 1994; King 2014). Germline mutations in *BRCA1* confer a high lifetime risk for breast ($\geq 60\%$) and ovar-

ian ($\geq 40\%$) cancer (average cumulative risks by age 70), as well as a lesser increase in risk for pancreatic, prostate, and other cancers (King et al. 2003; Metcalfe et al. 2010). Mutation carriers are heterozygous, whereas tumors often show loss of the wild-type allele (see below) (Futreal et al. 1994). *BRCA1*-mutated breast cancers are typically basal-like rather than luminal, and negative for estrogen and progesterone receptors and human epidermal growth factor receptor 2 (HER2) amplification (i.e., “triple negative”); thus, they do not respond to hormonal therapies or therapies that target HER2, making them particularly difficult to treat (Foulkes et al. 2003).

BRCA1 mutant allele frequencies are sufficiently high that individuals with biallelic mutations could be expected in the population; however, until recently, only one individual with bona fide deleterious mutations in both *BRCA1* alleles has thus far been reported (Domchek et al. 2013), consistent with the embryonic lethality associated with *BRCA1* loss in mice (Moynahan 2002). This individual likely survived to adulthood because one of her *BRCA1* alleles is hypomorphic. However, she had developmental issues, early onset ovarian cancer, and toxicity from carboplatin and paclitaxel therapy (Domchek et al. 2013). The identification of an individual with biallelic *BRCA1* mutations has important implications because the combination of congenital issues, cancer, and sensitivity to interstrand cross-linking agents like platinum-based drugs is associated with a broadly defined syndrome called Fanconi anemia (D’Andrea 2013). More recently, a second individual with biallelic *BRCA1* mutations has been identified, in this case, with breast cancer as well as congenital abnormalities (Sawyer et al. 2015). Thus, biallelic mutations in *BRCA1* are now considered to cause a distinct subtype of Fanconi anemia (FA-S).

BRCA1 Domains and Interactions

Human *BRCA1* encodes an 1863 amino acid protein that can be divided into three regions, the amino terminal Really Interesting New Gene (RING) domain, a central part with a large un-

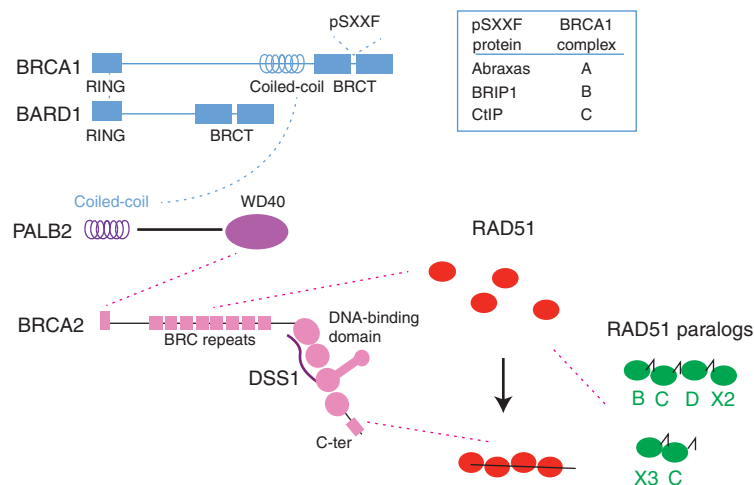


Figure 3. Protein interactions for a functional homologous recombination (HR) pathway. BRCA1 executes its various functions using the RING, coiled-coil, and BRCT domains. BRCA1 and BARD1 interact at their respective RING domains. BRCA1 BRCT motif mediates interaction through a pSXXF motif in Abraxas, BRIP1, and CtIP in the A, B, and C complexes, respectively. The coiled-coil domain of BRCA1 recognizes the coiled-coil domain of PALB2, which, in turn, binds BRCA2 through its WD40 domain. BRCA2 has mediator activity for loading RAD51 onto replication protein A (RPA)-coated ssDNA (BRC repeats) and for the stabilization of the RAD51 presynaptic filament carboxy terminal (C-ter) domain. RAD51 paralog complexes also interact with RAD51 and may promote/stabilize RAD51 filaments. BRCA2 also has a DSS1 and DNA-binding domain that, although not required for HR, likely is required for optimal HR levels. This domain consists of four globular domains and a helical tower domain with a three-helix bundle at its apex.

structured region encoded by exon 11 followed by a coiled-coil domain, and tandem BRCA1 carboxy-terminal repeats (BRCTs) (Fig. 3) (Li and Greenberg 2012). Through these domains, BRCA1 forms complexes with several proteins, implicating BRCA1 in multiple cellular functions, such as transcription regulation, cell-cycle checkpoint activation, and DNA repair (Venkataraman 2014). Importantly, the role of BRCA1 in DNA repair, in particular in HR repair (Moy-nahan et al. 1999), has thus far been one of its most well-recognized functions thought to be critical for its tumor suppressor activity.

RING Domain Interactions with BARD1

BRCA1 forms a heterodimer with BARD1, another RING and BRCT-containing protein, through helices flanking the core RING motif (Fig. 3) (Wu et al. 1996; Brzovic et al. 2001). BARD1 appears to be an obligate partner of BRCA1 because BRCA1-BARD1 interaction is essential for their mutual stability in vivo, and

Brc1 and *Bard1* knockout mice show identical phenotypes (McCarthy et al. 2003; Shakya et al. 2008). Although not nearly as prevalent as *BRCA1* mutations, germline *BARD1* mutations have also been reported in breast and ovarian cancer families (De Brakeleer et al. 2010; Sabatier et al. 2010; Ratajska et al. 2012).

As a RING domain is a common protein structure for E3 ubiquitin ligases, the E3 ligase activity of the BRCA1-BARD1 heterodimer has been a focus of interest. BRCA1-BARD1 can direct both mono- and polyubiquitylation depending on the E2 conjugating enzyme (Christensen et al. 2007). In addition to more standard linkages, BRCA1-BARD1 catalyzes the formation of noncanonical lysine⁶-linked ubiquitin chains (K6-polyUb), which likely serve as a signal for complex assembly and/or protein stabilization rather than degradation (Wu-Baer et al. 2003; Nishikawa et al. 2004). In vivo, BRCA1-BARD1 appears to play a critical role in the accumulation of K6-polyUb conjugates at DSBs (Morris and Solomon 2004). Substrates

R. Prakash et al.

for BRCA1-BARD1-mediated polyubiquitylation include BRCA1 itself and the end resection factor CtIP (Yu et al. 2006), and, for mono-ubiquitylation, include histone H2A, which has been implicated in the maintenance of silenced heterochromatin (Zhu et al. 2011).

The *in vivo* significance of the BRCA1 E3 ligase activity to tumor suppression has been challenged by recent reports showing that the BARD1-interaction-proficient but E3 ligase-deficient BRCA1 I26A mouse mutant is competent for HR and is not tumor prone (Reid et al. 2008; Shakya et al. 2011). In contrast, the cancer-associated C61G RING domain mutant, which disrupts the interaction with BARD1 rather than specifically impairing interaction with E2 enzymes, causes tumor susceptibility in mice as well as in patients (Wu et al. 1996; Drost et al. 2011). Another deleterious mutant, BRCA1 C64R, also has impaired interaction with BARD1 (Caleca et al. 2014). Thus, whereas the integrity of the BRCA1 RING domain region is critical for tumor suppression, apparently because of its interaction with BARD1, the physiological role of the E3 ligase activity itself is uncertain. One possibility is that auto-ubiquitylation of BRCA1 increases its stability because the BRCA1 I26A mutant protein is present at lower levels in cells (Reid et al. 2008). These cells have elevated DNA-damage-induced genomic instability, suggesting the possibility that wild-type levels of BRCA1 are required for maintenance of genomic integrity.

Coiled-Coil Domain Interaction with PALB2

Through its coiled-coil domain, BRCA1 interacts with the bridging protein PALB2, which connects BRCA1 with the other major hereditary breast cancer suppressor BRCA2 (Fig. 3) (Xia et al. 2006; Sy et al. 2009; Zhang et al. 2009a,b). BRCA1-PALB2-BRCA2 interaction plays an important role in RAD51 cellular dynamics and will be discussed further below.

BRCT Interactions with Several Proteins

The BRCT repeats mediate interactions between BRCA1 and several proteins involved in the

DNA-damage response, including Abraxas/FAM175A, BRIP1/BACH1, and CtIP/RBBP8, which are part of the BRCA1-A, -B, -C complexes, respectively (Moynahan and Jasin 2010; Li and Greenberg 2012). The BRCA1 BRCT repeats recognize a phosphorylated serine in a pSXXF motif (Yu et al. 2003b), which exists in each of Abraxas, BRIP1, and CtIP (Fig. 3). Importantly, BRCT repeats on one BRCA1 molecule can be occupied by only one pSXXF motif, implying the mutually exclusive composition of the BRCA1-A, -B, and -C complexes. The importance of the interaction between BRCA1 and phosphorylated proteins at the BRCT repeats is emphasized by the findings that disruption of this interaction is associated with tumor susceptibility in mice and humans as well as with reduced HR (Shakya et al. 2011). A challenge is to determine which of the multiple protein interactions is/are critical for tumor suppression.

The BRCA1-A complex targets BRCA1 to ubiquitin conjugates at DSBs, which are critical for DNA-damage signaling and repair (Huen et al. 2007; Doil et al. 2009). Deficiency of UBC13, the E2 enzyme required for these ubiquitin conjugates, causes severe defects in DNA-damage signaling and HR (Wang and Elledge 2007; Zhao et al. 2007). In addition to BRCA1, Abraxas binds to another component of the BRCA1-A complex RAP80, which recognizes polyubiquitylated histones like H2AX to recruit the BRCA1-A complex to DNA-damage sites (Kim et al. 2007; Sobhian et al. 2007; Wang et al. 2007). The BRCA1-A complex consists of other proteins with ubiquitin-binding domains and also with deubiquitylation activity, which can provide complex regulation of protein dynamics at damage sites (Huen et al. 2010). Notably, BRCA1-A and -C complexes appear to have opposite roles in an early step of HR, DNA end resection (discussed below).

The BRIP1 component of the BRCA1-B complex is a helicase that unwinds secondary DNA structures, such as four-stranded structures (G4 DNA), which may impede DNA replication (Cantor et al. 2004; London et al. 2008; Wu et al. 2008). BRCA1 recruits phosphorylated BRIP1 to chromatin during S phase,

and the BRCA1-B complex is required for S-phase checkpoint activation when replication forks are stalled or collapsed (Cantor et al. 2001; Litman et al. 2005; Greenberg et al. 2006). Biallelic *BRIP1* mutations, which cause cellular sensitivity to cross-linking agents, have been identified in patients with developmental issues and cancer predisposition, such that *BRIP1* is considered to be a Fanconi anemia gene subtype (FA-J); monoallelic mutations are associated with breast and ovarian cancer predisposition (Cantor et al. 2001; Levitus et al. 2005; Levrán et al. 2005; Litman et al. 2005; Seal et al. 2006; Rafnar et al. 2011).

BRCA1 Function in HR

A link between BRCA1 and RAD51 came from the observation of their subcellular colocalization in nuclear foci (Scully et al. 1997). An essential role for BRCA1 in HR was established by the direct demonstration of substantially reduced HR in BRCA1 mutant cells (Moynahan et al. 1999, 2001a). These studies further showed that BRCA1 mutant cells follow the paradigm of other HR mutant mammalian cells identified around the same time, in terms of having spontaneous chromosome instability and high sensitivity to cross-linking agents (Moynahan et al. 2001a). Multiple breast cancer and engineered mutations in *BRCA1* have been shown to confer defects in HR, connecting the HR and cancer suppressor roles of BRCA1 (Ruffner et al. 2001; Sy et al. 2009; Drost et al. 2011; Towler et al. 2013). BARD1 has also been shown to be important for HR (Westermarck et al. 2003; Laufer et al. 2007). BRCA1-deficient cells are exquisitely sensitive to inhibitors of poly(ADP-ribose) polymerases (PARP) (Bryant et al. 2005; Farmer et al. 2005; McCabe et al. 2006), which function in DNA single-strand break repair. Synthetic lethality between PARP inhibition and HR deficiency is currently being explored as an approach to cancer therapy.

The molecular mechanism by which BRCA1 contributes to HR has been extensively studied in the past decade. Compelling evidence suggests that BRCA1 functions in two distinct steps: (1) 5' to 3' resection of DSBs to

generate 3' ssDNA overhangs, and (2) loading of the RAD51 recombinase onto the ssDNA.

BRCA1 and End Resection

The involvement in resection was first suggested by the observation that BRCA1 mutant cells are defective in a second homology-based DSB repair pathway, single-strand annealing (SSA), which, like HR, relies on a resection intermediate but diverges at later steps (Fig. 4) (Stark et al. 2004). End resection is a key step in DSB repair pathway choice, promoting pathways that use homology while suppressing canonical NHEJ (Kass and Jasin 2010). Consistent with a role in resection, BRCA1 colocalizes with the resection complex MRE11-RAD50-NBS1 (MRN) after DNA damage and directly interacts with the resection factor CtIP (Wong et al. 1998; Yu et al. 1998; Zhong et al. 1999; Sartori et al. 2007).

One model is that BRCA1 interacts with phosphorylated CtIP (BRCA1-C complex) through its carboxy-terminal BRCT domain to cooperate with the MRN nuclease to catalyze resection (Wong et al. 1998; Yu et al. 1998; Sartori et al. 2007; Chen et al. 2008). As CDK-dependent phosphorylation of CtIP is required for CtIP activation and BRCA1-CtIP interaction, it has been proposed that BRCA1 promotes resection by recruiting CDK-phosphorylated/activated CtIP to DSB sites (Yu et al. 2003b; Yu and Chen 2004; Yun and Hiom 2009; Buis et al. 2012). Surprisingly, however, a CtIP mouse mutant defective for BRCA1 interaction has recently been reported to support HR to a similar extent as wild-type CtIP (Reczek et al. 2013), questioning the biological significance of the BRCA1-phospho-CtIP interaction. Possibly, other proteins target CtIP to damage sites (Daugaard et al. 2012).

In addition to specifically promoting resection, BRCA1 also appears to act as an antagonist of the resection suppressor 53BP1 (Fig. 4) (Bouwman et al. 2010; Bunting et al. 2010). In the absence of BRCA1, 53BP1 accumulates at DSBs to block resection and HR, ultimately leading to chromosomal aberrations and cell death; deletion of 53BP1, however, rescues the viability of BRCA1 mutant cells and mice, as

R. Prakash et al.

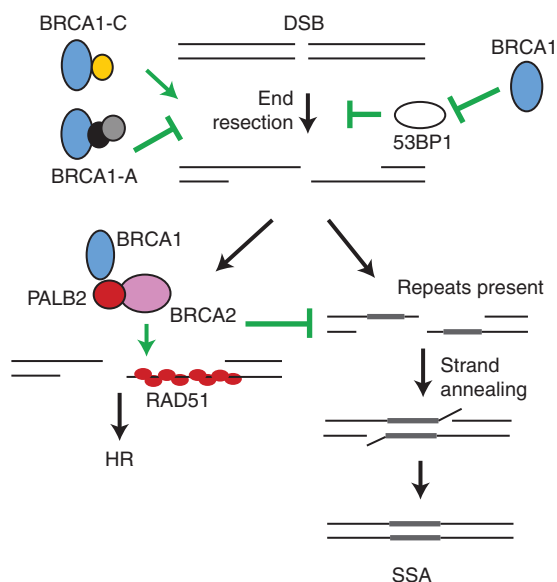


Figure 4. BRCA1 and BRCA2 have distinct roles in HR. BRCA1 acts at an early HR step to promote end resection and at a later step to recruit PALB2 and, hence, promote BRCA2 chromatin localization. BRCA1 acts by antagonizing the resection inhibitor 53BP1. It may further regulate resection by recruiting CtIP (gold ball) in the BRCA1-C complex, while inhibiting end resection in the BRCA1-A complex containing Abraxas and RAP80 (black and gray balls); alternatively, Abraxas-RAP80 may act independently of BRCA1 to suppress resection. BRCA2 promotes loading of RAD51 recombinase onto the resection product to form a RAD51-ssDNA filament, which is essential for HR and prevents the engagement of the 3'-ssDNA into the deleterious single-strand annealing (SSA) pathway. SSA acts when homologous repeats are present and leads to a deletion of sequences between the repeats.

well as the HR defects of BRCA1-deficient cells. Analysis of BRCA1 and 53BP1 in DNA-damage-induced foci by superresolution microscopy suggests that BRCA1 spatially excludes 53BP1 from the proximity of DSBs during S phase (Chapman et al. 2012a).

As CtIP-dependent resection is proficient with the combined absence of BRCA1 and 53BP1 (Bunting et al. 2010), it appears that the role of BRCA1 in relieving 53BP1 is upstream of recruiting CtIP at DSBs. The balance between BRCA1 and 53BP1 at DSBs has been shown to be regulated by acetylation of histone H4K20me₂, which interferes with 53BP1 binding (Tang et al. 2013) and also involves RIF1, which binds ATM-phosphorylated 53BP1 (Chapman et al. 2013; Escibano-Diaz et al. 2013; Feng et al. 2013; Zimmermann et al. 2013). The BRCA1-RIF1 antagonism in human cells has been reported to involve BRCA1 interaction with phosphorylated CtIP (Escibano-

Diaz et al. 2013), although it is uncertain as yet how to reconcile this finding with the lack of an HR phenotype in the phosphor-CtIP mutant mouse cells (Reczek et al. 2013).

Surprisingly, an antiresection activity of BRCA1-A complex members has also been reported (Fig. 4). Depleting members like RAP80 results in a hyperrecombination phenotype associated with increased CtIP-dependent resection (Coleman and Greenberg 2011; Dever et al. 2011; Hu et al. 2011; Kakarougkas et al. 2013). The RAP80 subunit of the BRCA1-A complex has been suggested to inhibit resection by binding to ubiquitin chains at DSBs (Biswas et al. 2011; Coleman and Greenberg 2011); its inhibitory effect can be relieved by the deubiquitylating factor POH1 (Kakarougkas et al. 2013). Based on the mutual exclusiveness of the BRCA1 complexes, a model has been proposed to explain this observation, in which the absence of the BRCA1-A complex allows more

BRCA1 to functionally interact in other complexes that promote resection (Coleman and Greenberg 2011; Hu et al. 2011). Physiologically, the promotion and inhibition of end resection by different complexes can act to fine tune the response. The opposing roles of BRCA1 complexes may help to explain the different degrees of resection defects upon BRCA1 disruption reported in the literature (Chen et al. 2008; Kakarougkas et al. 2013; Zhou et al. 2013).

BRCA1 and RAD51 Loading

In addition to its role in end resection, BRCA1 appears to have a downstream role in HR by promoting the localization of downstream HR factors (Fig. 4). Resected DNA is a substrate for RAD51 binding, but it is initially bound by the ssDNA-binding replication protein A (RPA), requiring mediator proteins like BRCA2 to assist RAD51 loading onto ssDNA concomitant with RPA eviction (see Zelensky et al. 2014). BRCA1 promotes the recruitment of BRCA2 to DSBs through the bridging protein PALB2 (Fig. 3) (Xia et al. 2006; Sy et al. 2009; Zhang et al. 2009a,b). The anchor role of BRCA1 is indicated by the hierarchy of the DNA-damage-induced focus formation: BRCA1 disruption diminishes PALB2, BRCA2, and RAD51 foci; PALB2 disruption reduces BRCA2 and RAD51 foci but not BRCA1 foci; BRCA2 disruption only impairs RAD51 foci. Consistent with the idea that BRCA1 facilitates RAD51-dependent HR through PALB2, clinical BRCA1 mutations, which abrogate BRCA1-PALB2 interactions, cause HR defects. The role of BRCA1 in RAD51 loading appears to be dispensable when 53BP1 is absent, at least in mouse cells, however, as eliminating 53BP1 rescues the HR deficiency of BRCA1 null cells (Bouwman et al. 2010; Bunting et al. 2010,2012). It seems possible that 53BP1 loss creates a favorable condition for RAD51 loading (e.g., creating hyperresected ends), which bypasses a downstream role for BRCA1 in HR.

The BRCA1 coiled-coil domain, which binds PALB2, is distinct from the BRCT domain (Fig. 3). However, mutations in the BRCT domain often result in destabilization of the mu-

tant BRCA1 protein (Williams and Glover 2003; Williams et al. 2003; Lee et al. 2010) and thus impair RAD51 loading despite an intact PALB2-interaction domain (Johnson et al. 2013). Interestingly, therapy-resistant human breast cancer cells have been isolated in which HSP90 stabilization of the mutant BRCA1 protein, combined with reduced 53BP1 levels, restores RAD51 focus formation (Johnson et al. 2013). These results emphasize the complexity of acquired resistance involving BRCA1.

BRCA2

BRCA2 Mutations in Patients

BRCA2 is the second major hereditary breast cancer susceptibility gene (Wooster et al. 1995; Tavtigian et al. 1996). *BRCA2* breast cancers are distinct from those with *BRCA1* mutations in that they are generally of the luminal subtype, rather than basal-like, and so are often estrogen-receptor positive (Jonsson et al. 2010; Waddell et al. 2010). The difference in breast cancer type may be related to the observation that *BRCA1* mutations alter mammary progenitor cell fate commitment (see Proia et al. 2011, and references therein). Like *BRCA1*, *BRCA2* mutation carriers are also predisposed to ovarian cancer as well as other tumors at a lower penetrance, such as that of the prostate and pancreas (Ozcelik et al. 1997; Consortium 1999; Antoniou et al. 2003; Edwards et al. 2003; King et al. 2003; van Asperen et al. 2005). Deleterious *BRCA2* mutations, as with *BRCA1*, have been reported throughout the length of the protein, mostly truncating mutations but also point mutations (Breast Cancer Information Core, research.nhgri.nih.gov/bic/). Mutations of uncertain clinical significance have also been identified such that several approaches have been devised to evaluate their functional significance (Hucl et al. 2008; Kuznetsov et al. 2008; Chang et al. 2009; Biswas et al. 2011, 2012; Bouwman et al. 2013). These and other approaches have also been used to evaluate the importance of rationally designed mutations.

Tumors from *BRCA2* mutation carriers were initially reported to have lost the wild-type *BRCA2* allele in most cases (Collins et al. 1995;

R. Prakash et al.

Gudmundsson et al. 1995). However, these findings were recently questioned by a study showing that *BRCA1* or *BRCA2* loss of heterozygosity (LOH) does not always occur in breast tumors, and, when it does occur, LOH can involve the mutant rather than the wild-type *BRCA* allele (King et al. 2007). In the mouse, germline *Brca2* heterozygous mutation promotes tumor formation in a Kras-driven mouse pancreatic cancer model, and loss of wild-type *Brca2* allele is not observed in some tumors (Skoulidis et al. 2010). These studies open the possibility of haploinsufficiency of *BRCA* genes, although the different approaches used in these studies and epigenetic silencing of the remaining wild-type allele could potentially account for some of the observations.

Patients with biallelic *BRCA2* mutations are classified as having a Fanconi anemia subtype (FA-D1), which is associated with brain, kidney, and hematological tumor types very early in life as well as with developmental issues (Howlett et al. 2002; Meyer et al. 2014). In these cases, at least one of the *BRCA2* alleles is expected to be hypomorphic, given the requirement of *Brca2* during embryogenesis in mice (Moynahan 2002).

BRCA2 Domains and Interactions

Human *BRCA2* is a 3418 amino acid protein, which consists of multiple domains (Fig. 3). In addition to binding PALB2 (discussed below), *BRCA2* binds RAD51 at motifs repeated in the middle of the protein (BRC repeats) and at a distinct domain in the carboxyl terminus (Sharan et al. 1997; Wong et al. 1997). In addition, *BRCA2* binds DNA and the DSS1 protein at a conserved region following the BRC repeats (Yang et al. 2002).

RAD51: Binding at the BRC Repeats and Carboxyl Terminus

Mammalian *BRCA2* has eight BRC repeats, which are conserved among vertebrates in both sequence and spacing, but not in the intervening sequences (Bignell et al. 1997). BRC repeats appear to regulate RAD51 filament for-

mation in a complex manner. An individual BRC peptide, when present in excess, disrupts RAD51 filament formation in vitro (Davies et al. 2001) and interferes with damage-induced RAD51 foci formation and HR in vivo (Chen et al. 1999; Xia et al. 2001; Stark et al. 2002; Saeki et al. 2006), presumably by mimicking the interface between RAD51 monomers within the RAD51 filament (Pellegrini et al. 2002). In contrast, the full-length *BRCA2* protein or the peptide containing all eight BRC repeats promotes RAD51-mediated strand exchange by stimulating assembly of RAD51 onto ssDNA, while preventing nucleation of RAD51 on dsDNA (Carreira et al. 2009; Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010). Even a single BRC4 motif has been shown to exhibit these activities under the appropriate experimental conditions (Carreira et al. 2009). The underlying mechanism is thought to be through blocking RAD51-mediated ATP hydrolysis and, thus, stabilizing the active ATP-bound RAD51-ssDNA filament. Evidence from *Rad51* mutant chicken cells indicates that Rad51 is sequestered under normal conditions by interaction with the BRC repeats, but in response to damage, this sequestered fraction undergoes mobilization, suggesting dynamic regulation of Rad51 through interaction with the BRC repeat region (Yu et al. 2003a).

Genetic studies further established the importance of BRC repeats for *BRCA2* function. Mice deleted for the BRC-encoding exon 11 in the germline are inviable, whereas somatic exon 11 deletion causes tumor development (Jonkers et al. 2001). Conversely, a mouse mutant that maintains the amino terminus of *BRCA2* including just three BRC repeats can survive embryogenesis, albeit at low frequency (Friedman et al. 1998). On the cellular level, the essentiality of BRC repeats for *BRCA2* function is supported by proliferation defects observed in exon 11-deleted embryonic fibroblasts (Bouwman et al. 2010) and the failure to rescue defects in *BRCA2*-deficient cells with constructs devoid of BRC repeats (Chen et al. 1998b). Point mutations in BRC repeats that impair RAD51 interaction are found in breast cancer patients (Pellegrini et al. 2002), although functional studies



are required to determine the effects of these mutations in full-length BRCA2.

Both functional redundancy and divergence may exist among BRC repeats in the context of full-length BRCA2 protein. Redundancy among BRC repeats is supported by the sufficiency of an individual BRC repeat for HR function within the BRC-RPA fusion protein (see below; Saeki et al. 2006). A divergence of function is suggested by the poor sequence identity between BRC repeats within a species, in contrast to high interspecies conservation of individual repeats (Bignell et al. 1997). Consistent with this notion, electron microscopy shows that different BRC repeats bind to different regions of RAD51 in filaments (Galkin et al. 2005). Biochemical studies led to the proposal of the existence of two classes of BRC repeats: BRC1, 2, 3, and 4 bind to RAD51 monomers at high affinity and reduce the ATPase activity of RAD51, effectively targeting RAD51 onto ssDNA over dsDNA and stimulating RAD51-mediated strand exchange, whereas BRC5, 6, 7, and 8 bind to the RAD51-ssDNA filament at high affinity (Carreira and Kowalczykowski 2011). These results have led to a model whereby the first class of repeats facilitates nucleation of RAD51 onto ssDNA and the second class stabilizes the nascent RAD51 nucleoprotein filament (Carreira and Kowalczykowski 2011).

The carboxy-terminal RAD51-binding site of BRCA2 shares no homology with the BRC repeats (Mizuta et al. 1997; Sharan et al. 1997). RAD51 binding to this region is regulated: it is abrogated by CDK phosphorylation at S3291 at G₂/M, leading to the hypothesis that it coordinates BRCA2 activity with cell-cycle progression (Esashi et al. 2005). BRCA2 S3291 is a key site for RAD51 binding, as both phosphomimic (S3291E) and phosphodeficient (S3291A) mutations block RAD51 interaction (Esashi et al. 2005). Unlike the BRC repeats, a BRCA2 carboxy-terminal peptide selectively binds RAD51 filaments at the interface region between two RAD51 protomers, such that carboxy-terminal-binding functions to stabilize RAD51-ssDNA filaments (Davies and Pellegrini 2007; Esashi et al. 2007). Surprisingly, however, S3291 mutation confers little or no DNA-dam-

age sensitivity and does not compromise HR in the context of full-length protein (Hucl et al. 2008; Ayoub et al. 2009; Schlacher et al. 2011), although the mutation does compromise HR in crippled BRCA2 peptides that have defects in other functional domains (Siaud et al. 2011), implying that carboxy-terminal RAD51 binding is not essential for HR but can promote HR under some circumstances. However, RAD51 binding by the BRCA2 carboxyl terminus has been implicated in the protection of nascent DNA strands at stalled replication forks (Schlacher et al. 2011).

DMC1, the meiosis-specific RAD51 homolog, has been shown to have a distinct binding site on BRCA2 from the BRC repeats and carboxyl terminus (a PheProPro motif) (Thorslund et al. 2007). However, disrupting the motif in the mouse does not have a discernible effect on DMC1 function and meiosis (Biswas et al. 2012), suggesting that DMC1 binds elsewhere, perhaps at sites also bound by RAD51.

DNA and DSS1 Binding

That BRCA2 is a DNA-binding protein was revealed in structural studies (Yang et al. 2002). The DNA-binding domain (DBD) consists of five components: a helical domain, three oligonucleotide-binding (OB) folds that bind ssDNA, and a tower domain with a three-helix bundle (3HB) at its end (Fig. 3). The 3HB is similar to the DNA-binding domain of Hin recombinase, suggesting dsDNA-binding activity. The helical domain, OB1 and OB2, interacts with the small, highly conserved DSS1 protein (Marston et al. 1999; Yang et al. 2002), which has been shown to promote HR in human cells (Gudmundsdottir et al. 2004; Li et al. 2006; Kristensen et al. 2010).

BRCA2 peptide mutants abrogated for DSS1 binding have impaired HR (Siaud et al. 2011), indicating that at least part of the HR function of DSS1 is through interaction with BRCA2. Biochemical studies have shown that DSS1 promotes the RAD51-loading activity of BRCA2 (Liu et al. 2010). DSS1 also appears to maintain the stability of BRCA2 protein in cells (Li et al. 2006), although an effect is not always

R. Prakash et al.

observed (Gudmundsdottir et al. 2004). In the fungus *Ustilago maydis*, the ortholog of DSS1 is also required for HR by regulating Brh2, the *U. maydis* ortholog of BRCA2 (Kojic et al. 2003, 2005). In budding yeast, which does not have a BRCA2 homolog, the DSS1 homolog localizes to DSB break sites and promotes HR- and NHEJ-mediated DSB repair, suggesting a BRCA2-independent function of DSS1 in DSB repair (Krogan et al. 2004). DSS1 is also found to interact with the 19S proteasome, the relevance of which to BRCA2 function remains to be clarified.

Patient missense mutations are found throughout BRCA2 DBD domain (Yang et al. 2002). Mutations predicted to compromise either the structural integrity of the DBD domain and/or DSS1/DNA-binding affect function (Kuznetsov et al. 2008; Biswas et al. 2011, 2012; Siaud et al. 2011). Moreover, mutation of ssDNA contact residues or deletion of the 3HB has detrimental effects on HR in reporter-based assays measuring BRCA2 peptide activity (Siaud et al. 2011). Surprisingly, a BRCA2 peptide deleted for the entire DBD is still functional in HR (Siaud et al. 2011); in fact, deletion of the DBD is one type of reversion mutation identified for BRCA2 (Edwards et al. 2008). These findings emphasize the plasticity of BRCA2 function in HR. An intact DBD is required if it is present, but loss of the entire DBD can be tolerated for substantial HR function, as long as PALB2 interaction is intact (Siaud et al. 2011).

BRCA2 Function and HR

The earliest clues about the importance of BRCA2 in maintaining genome integrity came from observations that *Brca2* mutant mice show early embryonic lethality and DNA repair defects (Connor et al. 1997; Ludwig et al. 1997; Sharan et al. 1997; Suzuki et al. 1997) similar to *Rad51* mutant mice (Lim and Hasty 1996; Suzuki et al. 1996). At the same time, BRCA2 interaction with RAD51 was uncovered (Sharan et al. 1997; Wong et al. 1997) and BRCA2 was found to colocalize with RAD51 in damage-induced nuclear foci (Chen et al. 1998a). The requirement of BRCA2 in HR was directly dem-

onstrated using HR reporters (Fig. 2B) (Moy-nahan et al. 2001b).

BRCA2 clearly acts at a distinct step in HR from that of BRCA1 (Stark et al. 2004). Although BRCA1-BARD1 promotes repair by both HR and SSA, BRCA2 promotes HR while suppressing SSA (Fig. 4) (Tutt et al. 2001; Stark et al. 2004). End resection presumably initiates normally in BRCA2 mutant cells, but ssDNA overhangs cannot be channeled into HR; instead, where present, complementary ssDNA overhangs anneal to each other. RAD51 disruption results in the same phenotype as BRCA2 loss, indicating that genetically BRCA2 acts at the same step in HR as RAD51 (Stark et al. 2004). Not surprisingly then, loss of 53BP1 does not rescue the viability of BRCA2-deficient cells, as it does with BRCA1-deficient cells (Bouwman et al. 2010).

Genetic and biochemical studies have also pointed to a RAD51 mediator activity of BRCA2. Remarkably, HR activity and genomic integrity are restored to BRCA2-deficient hamster cells by fusing a single BRC motif to the ssDNA-binding protein RPA (Saeki et al. 2006), suggesting that the main role of BRCA2 is to load RAD51 onto ssDNA. Purified Brh2 protein, the BRCA2 ortholog in the fungus *U. maydis*, stimulates Rad51-mediated strand exchange at an ssDNA-dsDNA junction by loading Rad51 onto DNA and displacing RPA (Yang et al. 2005). Recently, this RAD51-mediator function was further confirmed with purified human BRCA2, which specifically promotes RAD51 filament assembly on ssDNA over dsDNA (Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010), leading to displacement of RPA to stimulate strand exchange (Jensen et al. 2010; Liu et al. 2010). Stabilization of RAD51-ssDNA complexes by BRCA2 occurs by inhibition of the DNA-dependent ATPase activity of RAD51. Although challenges exist studying this very large protein involved in multiple protein interactions, these studies are promising for understanding the biochemistry of BRCA2.

Consistent with its major role in HR, BRCA2-deficient cells are sensitive to DNA-damaging agents that lead to lesions normally repaired by HR, including cross-linking agents



(Kraakman-van der Zwet et al. 2002) and to PARP inhibitors (Bryant et al. 2005; Farmer et al. 2005). BRCA2 has also been implicated in processes other than HR repair per se. Earlier studies suggested that stalled replication forks are stabilized by BRCA2 (Lomonosov et al. 2003). Recent work has provided evidence that this function is achieved through a RAD51-dependent, but HR-repair-independent mechanism, such that BRCA2 protects nascent DNA strand from degradation by stabilizing the RAD51 filament to maintain genomic integrity under replication stress (Schlachter et al. 2011). Roles for BRCA1 and canonical Fanconi anemia proteins have also been shown in nascent strand protection (Schlachter et al. 2012). The link between BRCA1, BRCA2, and Fanconi anemia proteins in replication fork protection is notable because loss of canonical Fanconi anemia proteins results in only mild HR defects in mammalian cells (Nakanishi et al. 2005), except for HR repair of cross links coupled to replication (Nakanishi et al. 2011).

PALB2

PALB2 Mutations in Patients

As with *BRCA2*, monoallelic *PALB2* mutations are associated with breast cancer susceptibility (Erkko et al. 2007; Rahman et al. 2007; Tischkowitz et al. 2007). The breast cancer risk associated with *PALB2* mutation has recently been estimated to overlap with that of *BRCA2* mutation (Antoniou et al. 2014). Although mutations are generally infrequent compared with *BRCA1* and *BRCA2*, in the Finnish population ~1% of unselected breast cancers are associated with a founder *PALB2* mutation (Erkko et al. 2007). The clinical phenotype of breast cancers with *PALB2* mutation is more similar to that of *BRCA2* in that a more substantial fraction is estrogen receptor positive rather than triple negative (Antoniou et al. 2014). Monoallelic *PALB2* mutations have also been associated with pancreatic and ovarian cancer susceptibility (Jones et al. 2009; Walsh et al. 2011).

Biallelic *PALB2* mutation leads to a Fanconi anemia subtype (FA-N), which shares a similar

tumor spectrum with the FA-D1 subtype arising from *BRCA2* mutation. Patients are predisposed to developing early childhood cancers, such as Wilms' tumor and medulloblastoma (Reid et al. 2007; Xia et al. 2007). Further, homozygous germline deletion of *Palb2* in mice leads to early embryonic lethality (Bouwman et al. 2011; Bowman-Colin et al. 2013), whereas conditional deletion of *Palb2* causes mammary tumors with long latency accelerated by p53 loss (Bowman-Colin et al. 2013; Huo et al. 2013). As with *BRCA2* but unlike *BRCA1*, 53BP1 loss fails to rescue genome instability caused by *PALB2* deficiency (Bowman-Colin et al. 2013). These phenotypes are consistent with the notion that *PALB2* and *BRCA2* function in the same step of the HR pathway to maintain genome integrity and suppress tumor development.

PALB2 Domains and Interactions

PALB2 is an 1186 amino acid protein with a coiled-coil domain at its amino terminus, which interacts with *BRCA1*, and a WD40 β -propeller domain at its carboxyl terminus, which interacts with *BRCA2* (Fig. 3) (Xia et al. 2006; Oliver et al. 2009; Sy et al. 2009; Zhang et al. 2009a,b). Emphasizing the importance of the *PALB2* interaction for *BRCA2* function, disruption of this interaction results in severe HR defects (Xia et al. 2006; Siaud et al. 2011). Furthermore, human *BRCA2* mutations that abrogate *PALB2* interaction fail to support viability of *Brca2*-null mouse embryonic stem cells (Biswas et al. 2012), although paradoxically *Palb2*-null embryonic stem cells have been reported to be viable (Bowman-Colin et al. 2013).

PALB2 and HR

Biochemical studies have shown that purified *PALB2* binds DNA and *RAD51*, and is able to stimulate *RAD51*-dependent strand invasion, including synergistically with a *BRCA2* peptide (Buisson et al. 2010; Dray et al. 2010). A recent report also links both *BRCA2* and *PALB2* to a downstream step in HR, DNA synthesis, by stimulating polymerase η activity on strand invasion intermediates (Buisson et al. 2014).

R. Prakash et al.

RAD51 PARALOGS

RAD51 Paralog Mutations in Patients

RAD51 has five paralogs (*RAD51B*, *RAD51C*, *RAD51D*, *XRCC2*, and *XRCC3*) that were discovered both as *RAD51*-related genes and through their complementation of radiation-sensitive Chinese hamster cell mutants (Tebbs et al. 1995; Albala et al. 1997; Cartwright et al. 1998; Dosanjh et al. 1998; Pittman et al. 1998). Thus far, the strongest evidence that *RAD51* paralogs are tumor suppressors comes from studies of *RAD51C* and *RAD51D*. Monoallelic, germline mutations in these genes predispose to ovarian cancer (Meindl et al. 2010; Loveday et al. 2011, 2012), although the predisposition to breast cancer is less clear. However, in both cases, mutations have been observed in ~1% of families with *BRCA1/2*-negative breast and ovarian cancer. Truncating mutations have been reported for both *RAD51C* and *RAD51D* and, in addition, missense mutations that impair the ATP-binding site have been reported for *RAD51C* (Meindl et al. 2010; Loveday et al. 2011, 2012; Somyajit et al. 2012).

RAD51B, *XRCC2*, and *XRCC3* monoallelic mutations have also been observed in breast cancer families, although the significance is not certain (Hilbers et al. 2012; Park et al. 2012; Golmard et al. 2013). One unusual finding is the presence of chromosomal translocations involving *RAD51B* in some benign tumors (Ingraham et al. 1999; Schoenmakers et al. 1999), including a germline translocation in a family with multiple cases of thymoma (Nico-deme et al. 2005). As reduced gene dosage of *RAD51B* has been reported to have phenotypic consequences (Date et al. 2006), translocations involving one allele may reduce repair activity.

Biallelic missense mutations of *RAD51C* (R258H) were identified in one consanguineous family with three children with severe congenital abnormalities whose cells were sensitive to cross-linking agents (Vaz et al. 2010). Thus, biallelic *RAD51C* mutation is now considered to confer a Fanconi anemia-like disorder, subtype FA-O. Two of the children with *RAD51C* mutation died as infants from the congenital defects; the surviving child was reported at 10 years of age to be

cancer free. Some of the congenital abnormalities are similar to those described in FA-D1 and FA-N children with biallelic *BRCA2* and *PALB2* mutation, respectively; however, the absence of a malignancy in the older child is distinct (Moldovan and D'Andrea 2009). *RAD51C* R258 is close to the ATP-binding site and is highly conserved; the mutation has been shown to reduce but not abolish HR function (Somyajit et al. 2012). *XRCC2* is the only other *RAD51* paralog besides *RAD51C* for which a Fanconi anemia-like phenotype has been described to date. A child from a consanguineous family with an *XRCC2* truncating mutation has been reported with developmental issues and cellular sensitivity to cross-linking agents (Shamseldin et al. 2012).

RAD51 Paralog Complexes

RAD51 paralogs show 20%–30% amino acid sequence similarity to *RAD51* and with each other, and this sequence conservation is predominantly found at a globular domain with Walker A and B motifs, which are critical for the ATP-binding/hydrolysis activity. Protein homology modeling studies have predicted that the *RAD51* paralogs, with the exception *XRCC2*, have an amino-terminal domain containing a four-helix bundle linked to the carboxy-terminal globular domain (Miller et al. 2004), similar to *RAD51* (Shin et al. 2003). Yeast two-/three-hybrid, coimmunoprecipitation studies, and biochemical studies have shown that *RAD51* paralogs exist in two major complexes, *RAD51B*-*RAD51C*-*RAD51D*-*XRCC2* (BCDX2) and *RAD51C*-*XRCC3* (CX3) (Fig. 3), although subcomplexes have also been identified (BC, CD, DX2, BCD, and CDX2) (Braybrooke et al. 2000; Schild et al. 2000; Masson et al. 2001; Sigurdsson et al. 2001; Liu et al. 2002; Wiese et al. 2002). The amino-terminal domain of one paralog apparently makes contact with the carboxy-terminal domain of another, providing specificity to the interactions (e.g., *RAD51B*-N-ter with *RAD51C*-C-ter, *RAD51C*-N-ter with *RAD51D*-C-ter, *RAD51D*-N-ter with *XRCC2*-C-ter) (Kurumizaka et al. 2003; Miller et al. 2004). Furthermore, *RAD51* interactions with the BCDX2 and CX3 complexes and indi-

vidual paralogs have also been reported (Dossanjh et al. 1998; Schild et al. 2000; Masson et al. 2001; Liu et al. 2002).

Biochemical activities of these various complexes and subcomplexes have been described, although their integration into HR pathways involving BRCA2 and other factors is not well understood. The RAD51 paralogs bind a variety of DNA structures, including ssDNA and branched structures like Holliday junctions (Braybrooke et al. 2000; Kurumizaka et al. 2001; Masson et al. 2001; Sigurdsson et al. 2001; Yokoyama et al. 2003, 2004; Liu et al. 2004). As expected, the RAD51 paralogs have been shown to hydrolyze ATP (Braybrooke et al. 2000; Sigurdsson et al. 2001; Lio et al. 2003; Yokoyama et al. 2003; Shim et al. 2004); an intact ATP hydrolysis domain is required for the function of several paralogs (French et al. 2003; Yamada et al. 2004; Gruver et al. 2005; Wiese et al. 2006), although not for XRCC2 (O'Regan et al. 2001). The BC subcomplex has been reported to stimulate RAD51 strand-exchange activity in the presence of RPA (Sigurdsson et al. 2001), apparently by stabilizing the RAD51 nucleoprotein filament rather than facilitating RAD51 nucleation (Amunugama et al. 2013).

RAD51 Paralog Cellular Phenotypes

A key role for the RAD51 paralogs in HR was initially shown for XRCC2 and XRCC3 in Chinese hamster cell mutants (Johnson et al. 1999; Pierce et al. 1999) and later for each of the paralogs in chicken B lymphocytes (Takata et al. 2001). As with biochemical studies in human cells, two distinct paralog complexes are predicted in chicken cells, given that mutations in two paralogs in the same complex are epistatic (*Rad51B* and *Rad51D*), whereas mutations in paralogs in two different complexes are not (*Xrcc3* and *Rad51D*) (Yonetani et al. 2005). Overexpression of RAD51 in each of the chicken cell mutants partially suppresses DNA-damage sensitivity, indicating a role for each of the paralogs in RAD51 loading or filament stabilization (Takata et al. 2001). Overall, mammalian RAD51 paralog mutants display reduced DNA-damage-induced RAD51 focus formation and

have increased spontaneous chromosomal abnormalities and are sensitive to DNA-damaging agents, such as cross-linking agents like cisplatin, and to PARP inhibitors (Bishop et al. 1998; Liu et al. 1998; Cui et al. 1999; Takata et al. 2001; French et al. 2002; Yoshihara et al. 2004; Smiraldo et al. 2005; Loveday et al. 2011; Urbin et al. 2012; Min et al. 2013).

An early role for RAD51 paralogs in HR pathways is supported by the rapid recruitment of XRCC3 to DSBs, which is independent of RAD51 (Forget et al. 2004). A later role in HR for RAD51 paralogs has also been investigated. RAD51C accumulates in nuclear foci in response to ionizing radiation where it colocalizes with RAD51; however, RAD51C foci persist for longer than RAD51 foci, suggesting that RAD51C, a component of both main complexes (BCDX2 and CX3), has a role in both early and late stages of HR (Badie et al. 2009). Like hamster and chicken cells, XRCC3 knockout HCT116 human cells were initially reported to have impaired RAD51 focus formation (Yoshihara et al. 2004); however, a recent study in these cells and in XRCC3 siRNA knockdown MCF7 cells reported no RAD51 focus formation defect, leading investigators to conclude that BCDX2 acts to load RAD51 onto DNA ends while CX3 acts after RAD51 loading (Chun et al. 2013). Consistent with a distinct role for XRCC3 among the paralogs, XRCC3 knockdown in HeLa cells results in a persistent spindle assembly checkpoint, whereas RAD51B or RAD51C knockdown induces a G₂/M cell-cycle arrest (Rodrigue et al. 2013). Overall, these studies point to the need for a coherent mammalian model to dissect the roles of the individual paralogs in HR.

RAD51 Paralog Mouse Knockout Models

Mouse knockouts of four RAD51 paralogs (*Rad51b*, *Rad51c*, *Rad51d*, and *Xrcc2*) have been reported and each is embryonic lethal, implying a critical function at developmental stages when cells are rapidly dividing (Shu et al. 1999; Deans et al. 2000; Pittman and Schimenti 2000; Kuznetsov et al. 2009; Smeenk et al. 2010). No mouse model for *Xrcc3* is currently

R. Prakash et al.

available. The reported developmental stage of death is not equivalent in the various knockouts, suggesting possible distinct functions of the paralogs, although differences in genetic background of the various mutants may account for some of the phenotypic differences. Embryonic lethality does not appear to be defined by a specific developmental defect but, rather, the embryos show growth delays over the course of a few days, consistent with what might be expected from stochastic DNA damage that is not repaired.

Supporting repair defects leading to apoptosis as a cause of lethality, death is delayed a few days by *Trp53* mutation, although postnatal viability is not restored (Shu et al. 1999; Smiraldo et al. 2005; Adam et al. 2007; Kuznetsov et al. 2009). The partial rescue by *Trp53* mutation is similar to that observed with *Rad51*, *Brca1*, and *Brca2* mutants (Moynahan 2002) but is unlike NHEJ mutants, in which the late embryonic lethality is rescued to give rise to viable (although feeble) mice (Frank et al. 2000; Gao et al. 2000). The one exception is *Xrcc2*, in which the viability of one of the two reported knockouts is rescued by *Trp53* mutation (see below) (Orii et al. 2006).

A summary of the RAD51 paralog knockouts is as follows in order of embryonic death (*Rad51b*, *Rad51c*, *Rad51d*, *Xrcc2*):

Rad51b mutants have severe growth retardation by embryonic day E5.5 and by E8.5 are completely resorbed (Shu et al. 1999). Embryonic stem cell lines could not be derived, suggesting that loss of RAD51B is cell lethal, similar to RAD51, BRCA1, or BRCA2 (Moynahan 2002).

Rad51c mutants show abnormalities by E5.5, are severely retarded by E8.5, and are resorbed shortly thereafter (Kuznetsov et al. 2009). *Rad51c* and *Trp53* are on the same chromosome, and adult mice that are heterozygous for both genes in *cis* develop a tumor spectrum (including mammary and preputial gland tumors) that differs from mice either with both mutations in *trans* or with *Trp53* mutation alone. The difference in the *cis* and *trans* mice is because LOH allows tu-

mors to develop, which are null for both *Rad51c* and *Trp53*. A hypomorphic *Rad51c* mutant has also been described in which RAD51C levels are reduced significantly ($\leq 30\%$) (Kuznetsov et al. 2007). This level of expression is sufficient to give rise to viable mice; however, a significant fraction is infertile owing to meiotic defects, including reduced RAD51 foci and the presence of unrepaired DSBs in spermatocytes.

Rad51d embryos display a range of abnormalities between E7.5 and 10.5, and none are viable by E11.5 (Pittman and Schimenti 2000). *Trp53* mutation leads to a significant rescue, such that embryos are detected as late as E16.5, although they are growth retarded (Smiraldo et al. 2005). *Rad51d* is located between *Rad51c* and *Trp53*; mice with *Rad51d* and *Trp53* heterozygous mutations in *cis* are not tumor prone up to 12 months of age.

Xrcc2 mutants develop normally to E8.5; thereafter, they display developmental delay/defects and are found at sub-Mendelian ratios (Deans et al. 2000). The few mice that are born succumb soon after birth. However, when *Xrcc2* mice are backcrossed onto a C57BL/6 genetic background, embryos do not reach late embryogenesis, although combined *Trp53* mutation allows some embryos to survive until just before birth (Adam et al. 2007). In another knockout model, presumably on a mixed genetic background, *Trp53* mutation actually rescues the viability of *Xrcc2* mice (Orii et al. 2006).

Apoptosis is elevated throughout *Xrcc2* mutant embryos (Deans et al. 2000), especially in the brain in proliferating cells, which is distinct from *Lig4* mutant embryos where it predominates in postmitotic cells (Fig. 2C) (Orii et al. 2006). *Trp53* mutation suppresses apoptosis in the developing brain and rescues the viability of *Xrcc2* mutant mice in this model, as it does to *Lig4* mutant mice (Frank et al. 2000; Orii et al. 2006), permitting a comparison of postnatal phenotypes, such as tumorigenesis. Although *Lig4/Trp53* null mice succumb to *Myc*-amplified B cell lymphoma and medulloblastoma by



3 months of age (Frank et al. 2000; Lee and McKinnon 2002), *Xrcc2/Trp53* null mice have a broad tumor spectrum with an earlier onset without obvious *Myc* amplification (Orii et al. 2006). Thus, both HR and NHEJ are important for the viability of mice and tumor suppression, although their roles differ.

Rad51c/Trp53, *Rad51d/Trp53*, and *Xrcc2/Trp53* mouse embryonic fibroblasts have been established (Smiraldo et al. 2005; Adam et al. 2007; Kuznetsov et al. 2009), as have *Xrcc2* embryonic stem cell lines (Deans et al. 2003). These RAD51 paralog mutant cells are sensitive to DNA-damaging agents, such as ionizing radiation and interstrand cross-links, have defects in RAD51 foci formation, increased genomic instability, and, where checked, decreased HR in reporter assays. *Rad51d/Trp53* fibroblasts have shortened telomeres, suggesting that RAD51D plays a role in telomere maintenance (Tarsounas et al. 2004).

SUMMARY AND FUTURE DIRECTIONS

Genetic studies have revealed a link between germline mutations in several HR genes and predisposition to breast/ovarian and other tumors. Somatic mutations in HR genes have also been uncovered (Cancer Genome Atlas Research Network 2011; Pennington et al. 2014). Insight into the molecular functions of the various HR proteins has been forthcoming. A role for BRCA1 in the end resection step of HR is well supported. At least three mechanisms are implicated: antagonizing the resection inhibitor 53BP1, promoting resection in the BRCA1-C complex with CtIP, and inhibiting resection in the BRCA1-A complex through Abraxas-RAP80. The BRCA1-PALB2-BRCA2 complex and RAD51 paralogs cooperate to load RAD51 onto ssDNA coated with RPA to form the essential recombination intermediate, the RAD51-ssDNA filament. The crucial role of these genes in key steps of HR provides important clues for understanding the cause of cancer in patients with mutations in these genes and for investigating more effective cancer treatments.

However, many key questions remain to be addressed. For instance, how are the various

roles of BRCA1 orchestrated? How do so many players, including the BRCA1-PALB2-BRCA2 complex and RAD51 paralog complexes, collaborate to load and stabilize RAD51 onto ssDNA? Importantly, how exactly does HR deficiency specifically contribute to ovarian/breast carcinogenesis and other cancer-prone diseases, such as Fanconi anemia? And how are other potential functions of HR proteins integrated into their tumor suppressor roles? A comprehensive understanding of disease-linked components and mechanisms of HR in mammalian system will be vital, including for therapeutic approaches that target the HR pathway.

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R. Prakash et al.

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