Fork Protection and Therapy Resistance in Hereditary Breast Cancer

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The BRCA-Fanconi anemia (FA) pathway preserves the genome and suppresses cancer and is a main determinant of chemotherapeutic efficacy. The hereditary breast cancer genes *BRCA1* and *BRCA2* function in DNA double-strand break repair mediating distinct steps of homologous recombination (HR). More recently, independent of DNA repair, functions in the replication stress response have come to light, providing insight as to how the BRCA-FA pathway also balances genome preservation with proliferation. The BRCA-FA proteins associate with the replisome and contribute to the efficiency and recovery of replication following perturbations that slow or arrest DNA replication. Although the full repertoire of functions in the replication stress response remains to be elucidated, the function of *BRCA1* and *BRCA2* in protecting stalled replication forks contributes along with HR to the sensitivity of BRCA-associated tumors to chemotherapy. Moreover, chemoresistance evolves from restoration of either HR and/or fork protection. Although mechanisms underlying the restoration of HR have been characterized, it remains less clear how restoration of fork protection is achieved. Here, we outline mechanisms of “rewired” fork protection and chemotherapy resistance in BRCA cancer. We propose that mechanisms are linked to permissive replication that limits fork remodeling and therefore opportunities for fork degradation. Combating this chemoresistance mechanism will require drugs that inactivate replication bypass mechanisms.

BRCA-FA PATHWAY FUNCTIONS BEYOND DNA REPAIR

Deficiency in the BRCA-Fanconi anemia (FA) pathway has widespread physiological consequences. Germ-line mutations in the hereditary breast cancer genes such as *BRCA1* and *BRCA2* are highly penetrant and predispose 20%–80% of carriers to breast and ovarian cancer (Apostolou and Fostira 2013). In addition, biallelic inactivation of BRCA genes causes FA, a rare multigenic disease in which loss of any of the 21 distinct complementation groups, FANC-A, FANC-B, FANC-C, ..., FANC-T, confers disease (for reviews, see Bhattacharjee and Nandi 2017; Cheung and Taniguchi 2017). Patients are characterized by progressive bone marrow failure, developmental defects, and cancer predisposition. FA patient cells are also exquisitely sensitive to agents that induce DNA interstrand cross-links (ICLs). Consequently, research has focused on understanding the function of the BRCA-FA pathway in ICL repair. In particular, given the role of BRCA proteins in fixing double-strand DNA breaks by homologous recombination (HR), this function was thought to extend to the repair of ICL-induced breaks. However, essential functions in preserving replication have emerged that are independent of HR. This role was not readily apparent because BRCA-FA-deficient cells are not universally sensitive to fork slowing or stalling drugs. Recent advances in studying the biology of DNA replication and associated machinery have clarified roles in replication and the replication stress response and provide a new perspective for understanding

BRCA-FA disease and the function of BRCA-FA proteins in cell survival.

BRCA-FA PROTEINS PROTECT STALLED REPLICATION FORKS FROM DEGRADATION

A key initial finding linking the BRCA-FA proteins to the replication stress response was the observation that *BRCA2* protects stalled replication forks from break formation following treatment with hydroxyurea (HU) that decreases the generation of deoxyribonucleotides through inhibition of ribonucleotide reductase (Lomonosov et al. 2003). More recently, *BRCA1*, *BRCA2*, *FANCD2*, and *FANCA* were shown to protect nascent DNA strands of replication forks stalled by HU. Mechanistically, *BRCA1* and *BRCA2* protect nascent DNA at stalled forks from MRE11-mediated degradation by loading RAD51 on ssDNA exposed at stalled forks (Schlachter et al. 2011; Ying et al. 2012; Chaudhuri et al. 2016). Correspondingly, in the absence of *BRCA1* or *BRCA2* nascent DNA strands of stalled replication forks undergo extensive nucleolytic degradation leading to long stretches of single-stranded DNA (ssDNA) (Schlachter et al. 2011, 2012; Kolinjivadi et al. 2017b). Consistent with this mode of action, a degradation phenotype also underlies a RAD51 mutation in patients with an FA-like phenotype (Ameziane et al. 2015; Wang et al. 2015; Zadorozhny et al. 2017). Thus, RAD51 is a key mediator of BRCA function in HR at DNA breaks and in protecting nascent DNA at stalled forks. In both settings, RAD51 loading on ssDNA could restrict MRE11

resection to initiate recombination-based mechanisms to promote repair at breaks and restart at stalled forks. The mechanistic overlap of HR proteins operating in DNA repair and DNA replication were recently reviewed (Kolinjivadi et al. 2017a).

Synchrony in fork processing is established in part by the fact that factors regulating DNA end resection also contribute to fork slowing and subsequent restart mechanisms. Consequently, loss of several BRCA-FA factors causes not only fork degradation phenotypes but also slowing and restart defects following replication stress. In particular, RAD51, FANCD2, FANCM, FANL1, and the FANCD2–FANL1 interaction ensure that replication elongation is slowed in response to HU (Luke-Glaser et al. 2010; Lossaint et al. 2013; Zellweger et al. 2015; Lachaud et al. 2016). BRCA-FA-deficient cells also display defects in slowing replication in response to ICLs that were proposed to underlie the pronounced G₂/M arrest and sensitivity (Sala-Trepat et al. 2000). Even during unperturbed replication, RAD51 loss leads to the accumulation of ssDNA gaps that are visible by electron microscopy (EM) indicating its role in normal replication (Lopes et al. 2006; Hashimoto et al. 2010). Gaps could result from discontinuous replication if DNA synthesis skips over barriers and reinitiates downstream by employing the primase, PrimPol (Guilliam and Doherty 2017). Whatever the source of gaps, they could lead to extensive resection that underlies fork degradation phenotypes that characterize loss of BRCA-FA proteins. Gaps could be initiating sites for remodelers that mediate aberrant processing in BRCA-FA cells (Kolinjivadi et al. 2017a). Future work will ideally reveal how fork slowing, reversal, protection, and recombination are ultimately coordinated and/or linked with other BRCA-FA pathway functions including replication restart (Petermann et al. 2010; Schwab et al. 2013; Raghunandan et al. 2015; Lemacon et al. 2017; Rondinelli et al. 2017), regulation of new origin firing (Thompson et al. 2017), and traversing the replisome past replication blocking lesions (Huang et al. 2013).

DNA REPAIR AND FORK PROTECTION UNDERLIES *BRCA1/2* GENOME STABILITY AND TUMOR SUPPRESSION FUNCTIONS

These recently identified roles for BRCA-FA pathway in replication fork stability raise the possibility that maintenance of replication fork stability may contribute to its genome preservation and tumor suppression functions. The idea that replication dysfunction independent of HR drives cancer formation is in part supported by analysis of mammary epithelial cells from *BRCA1* mutation carriers that have not yet developed cancer. These premalignant cells were found to show functional HR and DNA damage checkpoint signaling but defects in the protection of stalled replication forks (Pathania et al. 2014). Moreover, cells carrying *BRCA2* heterozygous truncating mutations showed extensive replication stress–induced fork degrada-

tion (Tan et al. 2017). Thus, loss of DNA repair may not be the only crucial factor in the etiology of BRCA cancer. More likely, it could be a combined loss of and/or coordination between DNA repair and DNA replication that undermines the maintenance of genome integrity and tumor suppression.

Until recently, restoration of the HR pathway was the only described mechanism by which *BRCA1/2*-mutant cancers survive genotoxins, such as cisplatin or inhibitors of poly(ADP-ribose) polymerase (PARPi) (Edwards et al. 2008; Sakai et al. 2008, 2009). In *BRCA1*-mutant cells, restoration of HR is achieved by several mechanisms including reversion mutations or loss of the nonhomologous end joining (NHEJ) factor, 53BP1 (Bunting et al. 2010, 2012). Conversely, in *BRCA2*-mutant cells, restoration of HR is solely achieved by reversion mutations (Edwards et al. 2008; Sakai et al. 2008, 2009). The finding that approximately half of *BRCA2*-mutant cancers develop chemoresistance in the absence of restored HR suggested that reversion-independent resistance mechanisms awaited identification (Norquist et al. 2011). To define genetic determinants of cisplatin resistance, we performed a genome-wide short-hairpin RNA (shRNA) screen and found that loss of the chromatin remodeler, CHD4, mediates resistance to cisplatin and PARPi in *BRCA2*-mutated cancer (Guillemette et al. 2015). CHD4 depletion did not restore HR but reduced the levels of chromosomal aberrations in *BRCA2*-mutant cells exposed to cisplatin (Guillemette et al. 2015). The elevated resistance and genomic stability correlated with reduced MRE11 chromatin association and increased fork protection (Chaudhuri et al. 2016). A series of recent publications concur that rewired fork protection confers chemoresistance independent of HR. This is achievable in *BRCA1/2*-deficient cells by several mechanisms, including loss of the chromatin modifier complex MLL3–4/PTIP/MRE11, the fork remodelers, SMARCAL1, HLTf, or ZRANB3, PARP1, the methyltransferase EZH2, and the negative regulator of RAD51, RADX (Chaudhuri et al. 2016; Ding et al. 2016; Dungrawala et al. 2017; Kolinjivadi et al. 2017b; Tagliatela et al. 2017; Vujanovic et al. 2017), as well as through up-regulation of FANCD2 (Kais et al. 2016; Michl et al. 2016). The clinical significance of this mechanism is confirmed by the poor patient response and survival outcomes observed upon restored fork protection (Guillemette et al. 2015; Chaudhuri et al. 2016; Rondinelli et al. 2017). Thus, these factors may prove useful as potential biomarkers of *BRCA1/2*-deficient tumor response to chemotherapy.

DEFINING DEGRADATION FACTORS AND FORK DYNAMICS IN *BRCA1/2*-DEFICIENT CELLS

A common feature of rewired fork protection is that nuclease activity is tempered by various mechanisms. In particular, in *BRCA1/2*-mutant cells, suppressing MRE11-nucleolytic degradation restores fork protection. This can be done with the MRE11 inhibitor mirin or by reestablish-

ing Rad51 filament formation at stalled forks through expression of a RAD51 mutant lacking ATPase activity or by depletion of the anti-RAD51 factor, RADX (Schlachter et al. 2011; Dungrawala et al. 2017). In addition, loss of factors that facilitate the recruitment of MRE11 to stalled forks such as PARP1, PTIP, or CHD4 restores fork protection in *BRCA1/2*-deficient cells (Table 1; Chaudhuri et al. 2016; Ding et al. 2016). Given that MRE11 has limited nucleolytic activity (Cannavo and Cejka 2014), other nucleases likely contribute to fork degradation. In particular, CtIP initiates the MRE11-dependent degradation that is extended by EXO1 (Lemacon et al. 2017). Although it remains debated whether DNA2 contributes to degradation in *BRCA1/2*-deficient cells (Chaudhuri et al. 2016; Spies et al. 2016; Kolinjivadi et al. 2017b; Lemacon et al. 2017), DNA2 degrades forks in cells deficient in Abro1, a paralog of the *BRCA1*-interacting protein, Abraxas (Xu et al. 2017). An MRE11-independent mechanism has also been described in which the nuclease MUS81 contributes to fork degradation in a pathway with EZH2 (Table 1; Rondinelli et al. 2017).

Replication fork structures also play a prominent role in nucleolytic degradation. In particular, a reversed replication fork, a so-called “chicken foot,” is the target of MRE11 digestion in *BRCA1/2*-deficient cells (Mijic et al. 2017). Accordingly, fork reversal has been shown to be a prerequisite for fork degradation in *BRCA1/2*-deficient cells (Kolinjivadi et al. 2017b). In addition to the remodelers HLTF, SMARCA1, and ZRANB3 (Kolinjivadi et al. 2017b; Tagliatela et al. 2017), RAD52, PARP1 and RAD51 drive fork reversal-dependent degradation in *BRCA1/2*-deficient cells (Table 1; Lemacon et al. 2017; Mijic et al. 2017; Ray Chaudhuri et al. 2012). There are numerous proteins known to generate reversed forks such as DNA helicases FBH1, BLM, WRN, RECQL5 and DNA translocases FANCM and RAD54 (Blastyák et al. 2010; Bétous et al. 2012; Ciccica et al. 2012; Fugger et al. 2015; Kile et al. 2015; Neelsen and Lopes 2015). The relevance of these factors to fork degradation in BRCA cancer remains to be investigated. How loss of each remodeler can individually restore fork protection in *BRCA1/2*-deficient cancer also awaits further analysis. Fork remodelers may act in a common pathway; however, HLTF, ZRANB3, and SMARCA1 do not form a complex (Tagliatela et al. 2017). It also remains to be determined how fork remodelers interact with the other pathways of chemoresistance such as EZH2 or MLL3–4/PTIP/MRE11, both of which act independently of each other (Rondinelli et al. 2017). Given that the reversed fork structure has exposed DNA ends that mimic DNA double-stranded break ends, it will also be important to determine if in addition to MRE11, these ends are an entry point for NHEJ factors. If so, the reported elevated NHEJ activity that causes genomic instability in BRCA-FA cells (Adamo et al. 2010; Bunting et al. 2012; Pace et al. 2010) may be suppressed by depletion of fork remodelers. However, as described below, preventing fork reversal and subsequent degradation or NHEJ reactions is not always sufficient for chemoresistance in *BRCA1/2*-deficient tumors.

COMPLEXITY OF REWIRED FORK PROTECTION MECHANISMS

The emerging literature indicates that rewired fork protection mechanisms and their relationship to cell viability, genome stability, and chemoresistance are complex. Rewired fork protection in *BRCA1/2*-deficient cells is achieved by loss of either of three remodelers (HLTF, SMARCA1, and ZRANB3), PTIP, or PARP1, but loss of CHD4 or EZH2 is restricted to *BRCA2*-mutant cells (Guillemette et al. 2015; Ding et al. 2016; Ray Chaudhuri et al. 2016; Rondinelli et al. 2017; Tagliatela et al. 2017). These findings suggest that stalled fork structures or compensating pathways in *BRCA1* and *BRCA2* cells may be distinct, but the overall strategy of rewired fork protection remains consistent; forks that escape nucleases are protected from degradation. However, not all routes that escape degradation will confer chemotherapy resistance. RAD51, MRE11, or RAD52 are required for viability of *BRCA1/2*-deficient cells, so their loss will not result in chemoresistance but rather synthetic lethality (Fig. 1; Feng et al. 2011; Ying et al. 2012; Lok et al. 2013). The balance between lethality and survival/chemoresistance also appears dependent on the sequence by which a fork degradation factor is lost, before or after BRCA deficiency. Depletion of PARP1 in *BRCA2*-deficient cells leads to synthetic lethality (Feng and Jasin 2017), whereas deletion of PARP1 before *BRCA2* depletion provides some protective effects (Ding et al. 2016).

An additional level of complexity derives from the fact that some proteins possess multiple functions in the replication stress response. For example, RAD51 participates in both the formation and protection of reversed forks (Hashimoto et al. 2010; Zellweger et al. 2015). Therefore, depending on which RAD51 function is disrupted, fork protection or degradation could be altered. Further findings show the importance of cellular context, specifically mutational status, for therapy resistance. For example, CHD4 depletion conferred cisplatin resistance in *BRCA2*-mutant cell lines harboring truncated *BRCA2*, but did not improve cisplatin resistance in *BRCA2*-depleted cells, indicating resistance requires the maintenance of a residual *BRCA2*-mutant species (SB Cantor, unpubl.). As different cell types show differing levels of reversed forks (Ahuja et al. 2016), this may explain the findings that SMARCA1 depletion resulted in chemoresistance in the MCF10A cancer cell line but not in nonmalignant mammary epithelial cells, despite both cell lines being *BRCA1*-deficient (Tagliatela et al. 2017). Likewise, *Ptip* depletion in *Brca2*-null mouse B cells improves genome stability and resistance to HU, but in *BRCA2*-null primary human cells fails to restore viability (Chaudhuri et al. 2016; Feng and Jasin 2017). Ideally future work will unravel these distinctions so that expectations for therapy are better understood.

Finally, it is important to realize that mechanisms of fork protection may vary for different genotoxic agents; mechanisms revealed following treatment of cells with HU may not be informative for mechanism following treatment of cells with cisplatin or PARPi, as these agents

Table 1. Simplified overview of factors whose loss restores fork protection and/or chemoresistance in BRCA1/2-deficient cells

Factor	Function	Fork Protection	Chromosome stability	Chemoresistance	Clinical Relevance	Publication(s)
MRE11	Nuclease that degrades nascent DNA in stalled replication forks	Inhibition restores fork protection in BRCA2-depleted (V-C8), BRCA1-depleted U2OS and BRCA1-mutant (UWB1) cells.				Schlacher et al. 2011 and 2012; Ying et al. 2012; Lemacon et al. 2017; Mijic et al. 2017
CHD4	Recruits MRE11 to stalled fork	Depletion restores fork protection in BRCA2-mutant (PEO1) cells.	Depletion protects against chromosomal aberrations in BRCA2-mutant (PEO1) cells (cisplatin).	Depletion increased resistance in BRCA2-mutant (PEO1, Capan1, FA-D1) cells (cisplatin/PARPi).	Low CHD4 correlated with reduced progression-free survival (PFS) in BRCA2-mutant ovarian cancers	Guillemette et al. 2015; Chaudhuri et al. 2016
PARP1	Recruits MRE11 to stalled fork, fork reversal activity	Deletion restores fork protection in <i>Brca1</i> ^{-/-} or <i>Brca2</i> ^{-/-} B cells and <i>Brca2</i> ^{-/-} mESC.	Deletion protects against chromosomal aberrations in <i>Brca2</i> ^{-/-} mESC and <i>Brca1</i> ^{-/-} B cells (cisplatin, camptothecin).		PARP1 deletion reduces tumor-free survival in <i>Brca2</i> ^{-/-} mouse model.	Chaudhuri et al. 2016; Ding et al. 2016
PTIP	Recruits MRE11 to stalled fork	Depletion restores fork protection in <i>Brca1</i> ^{-/-} or <i>Brca2</i> ^{-/-} B cells.	Deletion protects against chromosomal aberrations in <i>Brca1</i> ^{-/-} or <i>Brca2</i> ^{-/-} B cells (HU, cisplatin, camptothecin).	Deletion increased resistance in <i>BRCA1</i> ^{-/-} B cells (HU).	Low PTIP correlated with reduced PFS in BRCA2-mutant ovarian cancer	Chaudhuri et al. 2016
HLTF	Fork reversal activity, E3 ubiquitin ligase for PCNA	Depletion restores fork protection in BRCA1- or BRCA2-depleted MCF10A cells.				Tagliatela et al. 2017
SMARCA1	Fork reversal activity	Depletion restores fork protection in BRCA1- or BRCA2-depleted MCF10A cells or in BRCA2-depleted <i>Xenopus</i> extracts.	Depletion protects against chromosomal aberrations in BRCA1- and BRCA2-depleted MCF10A cells (camptothecin).	Depletion increased resistance in BRCA1-mutant (MDA-MB-436) cells (cisplatin/PARPi).	Low SMARCA1 correlated with reduced overall survival (OS) in BRCA1-mutant breast cancer	Kolinjivadi et al. 2017b; Tagliatela et al. 2017
ZRANB3	Fork reversal activity	Depletion restores fork protection in BRCA1- or BRCA2-depleted MCF10A cells.	Depletion protects against chromosomal aberrations in BRCA1- and BRCA2-depleted MCF10A cells (camptothecin), Deletion causes increased chromatid breaks/gaps in BRCA2-depleted U2OS cells (HU).			Vujanovic et al. 2017; Tagliatela et al. 2017; Mijic et al. 2017

Continued

Table 1. *Continued*

Factor	Function	Fork Protection	Chromosome stability	Chemoresistance	Clinical Relevance	Publication(s)
RAD52	Fork reversal activity, loads Rad51 onto ssDNA, recruits MRE11 to stalled fork	Depletion restores fork protection in BRCA2-depleted U2OS cells	Depletion protects against chromosomal breakage in BRCA2-depleted U2OS cells (HU).			Mijic et al. 2017
RAD51	Fork reversal activity, binds and protects ssDNA	Depletion restores fork protection in BRCA2-depleted RPE-1 cells				Mijic et al. 2017
EXO1	Nuclease that extends MRE11 fork degradation	Depletion restores fork protection in BRCA2-mutant (PEO1) or BRCA1-mutant (UWB1) cells, and BRCA1- or BRCA2-depleted U2OS cells.	Depletion protects against chromosomal aberrations in BRCA2-depleted U2OS (HU).			Lemacon et al. 2017
MLL4	Induces histone H3 methylation at Lysine 4 (H3K4) to recruit MRE11 to stalled fork	Deletion restores fork protection in <i>Brca1</i> ^{-/-} or <i>Brca2</i> ^{-/-} B cells.	Deletion protects against chromosomal aberrations in <i>Brca2</i> ^{-/-} B cells (PARPi, cisplatin).			Chaudhuri et al. 2016
RADX	Inhibits the accumulation of Rad51 at forks	Depletion restores fork protection in BRCA2-depleted U2OS cells, BRCA2 mutant Capan1 cells		Depletion increased resistance in BRCA2-depleted U2OS cells (PARPi).		Dungrawala et al. 2017
EZH2	Induces trimethylation of histone H3 at Lysine 27 (H3K27me3) to recruit Mus81 to stalled fork	Depletion restores fork protection in BRCA2-mutant (VU423) cells.	Inhibition protects against chromosomal aberrations in BRCA2-depleted HeLa or BRCA2-mutant (VU423) cells (mitomycin C).	Depletion increases resistance in BRCA2-depleted HeLa cells (PARPi, cisplatin).	Low EZH2 correlated with reduced PFS in BRCA2-mutant ovarian cancer. EZH2 inhibition promotes relapse in <i>Brca2</i> ^{-/-} breast tumor mouse model (PARPi).	Rondinelli et al. 2017

have distinct modes of disrupting replication. For example, EZH2 and MUS81 are required for the restart of replication in *BRCA2*-deficient cells following their release from HU (Lemacon et al. 2017; Rondinelli et al. 2017). HU treatment promotes replication fork reversal (Zellweger et al. 2015), and reversed forks are extensively resected by nucleases in *BRCA2*-deficient cells. MUS81 cleaves the over-resected reversed DNA forks in *BRCA2*-deficient cells and promotes POLD3-dependent fork rescue, explaining their dependence on MUS81 for replication fork progression and resistance to HU (Lai et al. 2017; Lemacon et al. 2017). Conversely, following treatment with PARPi, it is less clear how MUS81 depletion

achieves PARPi resistance in *BRCA2*-deficient cells (Rondinelli et al. 2017). In particular, MUS81 is probably not required for fork restart upon treatment with PARPi because PARPi suppresses fork slowing and reversal depriving cells of a MUS81 substrate (Sugimura et al. 2008; Ray Chaudhuri et al. 2012). Likewise, it remains to be determined if similar to depletion of SMARCA1, ZRANB3 depletion confers cisplatin/PARPi resistance. ZRANB3 depletion restores fork protection in distinct cell systems; however, genomic instability is increased or decreased in *BRCA1/2*-deficient cells depending on the source of replication stress (Mijic et al. 2017; Tagliatela et al. 2017).

Figure 1. Model of the mechanisms and potential consequences of fork protection in *BRCA1/2*-deficient cells. (A) In wild-type (WT) cells, when replication stress (represented by X) is encountered in the course of cancer therapy, there is a reversal of replication forks, and the protection of nascent DNA in a *BRCA1*-, *BRCA2*-, and *Rad51*-dependent manner, that limits resection by nucleases such as *MRE11*, *CtIP*, and *EXO1*. Following TS or recombination-based restart, the replication fork is restarted, thereby conferring chemoresistance. In absence of *BRCA1/2*, (B) *Rad51* is no longer stabilized on reversed forks, allowing access of nucleases to nascent DNA, resulting in extensive degradation and chemosensitivity. (C) Extensive nascent cell degradation can be avoided by either loss of fork degradation factors or gain in stabilization factors that will allow forks to restart via template switch (TS) and confer chemotherapy resistance if viability is not also compromised. (D) Loss of fork reversal will limit fork degradation if translesion synthesis (TLS) is active at the fork and gaps generated by repriming reactions are avoided.

REWIRED FORK PROTECTION AND REPLICATION PROGRESSION WITHOUT FORK REVERSAL

When rewired fork protection confers therapy resistance in *BRCA1/2*-mutant cells, the mechanism of replication should be quite different than as found in *BRCA1/2*-proficient cells. In this regard, it is important to note that upon replication stress, replication forks typically slow and reverse (Zellweger et al. 2015). As described above, rewired fork protection achieved through suppression of *HLTF*, *SMARCA1*, *ZRANB3*, *PARP1*, and *RAD51* is expected to suppress fork reversal. Even though reversed forks are dangerous species that present DNA ends for resection, ssDNA generation, and fork degradation, they also serve to liberate DNA lesions from replisome entanglements, allowing more efficient DNA repair processing. DNA ends in reversed forks also provide DNA substrates for recombination. In addition, the reversed fork is thought to facilitate other strategies for repair or bypass of DNA lesions, such as template switch (TS). TS is an error-free DNA damage tolerance pathway that uses the newly synthesized daughter strand DNA for homology-directed repair to bypass lesions (for review, see Lovett 2017). Access to recombination, TS, and other repair mechanisms through fork remodeling activities is an important genome stabilizing response to stress that limits fork breakage (Ray Chaudhuri et al. 2012; Neelsen and Lopes

2015). Thus, rewired fork protection that limits replication fork slowing and reversal should compromise genomic stability. In *BRCA*-deficient cancer cells, however, fork reversal could have dire consequences given that its loss provides genomic stability and chemoresistance.

Clues to replication progression mechanisms that confer therapy resistance come from how other activities are altered in *BRCA1/2*-deficient cells that have rewired fork protection. In particular, depletion of *HLTF* not only results in loss of fork reversal, but TS is also disrupted. This stems from the role of *HLTF* as an E3 ubiquitin ligase responsible for the addition of ubiquitination chains on *PCNA*, an essential step in TS (Branzei 2011; Lin et al. 2011). Rewired fork protection established through *ZRANB3* depletion will also alter the replication stress response. Polyubiquitinated *PCNA* recruits *ZRANB3* to sites of replication stress, both of which are critical for fork reversal (Ciccia et al. 2012; Vujanovic et al. 2017) and proposed to mediate TS. In addition to catalyzing the regression of stalled replication forks, *ZRANB3* also prevents inappropriate recombination (Ciccia et al. 2012). Therefore, loss of *ZRANB3* could elevate aberrant recombination as well as interfere with a polyUb-*PCNA*-dependent axis required for engagement of TS. Furthermore, loss of *ZRANB3*-*PCNA* interaction may allow access of de-ubiquitination enzymes thereby decreasing polyUb-*PCNA* and in turn increase monoUb-*PCNA*. If so, loss of *ZRANB3*, similar to loss of *HLTF*, could enhance the

overall monoUb-PCNA. This result could have great significance as monoUb-PCNA serves as a molecular switch for the error-prone mechanism of translesion synthesis (TLS), a DNA tolerance mechanism that allows the timely bypass of DNA lesions using error-prone TLS polymerases (Choe and Moldovan 2017).

REWired FORK PROTECTION VIA TLS AT THE FORK

Could TLS operate at the fork to limit gap formation, maintain replication, and promote chemoresistance in *BRCA1/2*-deficient cells (Fig. 1)? TLS is best described for filling in gaps postreplication and operates effectively when restricted from S phase (Karras and Jentsch 2010). However, TLS may operate at the fork, as the replisome is passing through the site of DNA damage or other barrier to replication (Sale et al. 2012). Indeed, TLS polymerases enable replication to continue despite DNA damage or other barriers induced by chemotherapy because they have low-fidelity, which allows the insertion of nucleotides opposite to bulky DNA lesions that block high-fidelity replicative DNA polymerases. Moreover, when nucleotide pools are reduced as in HU-treated cells, TLS could also tolerate insertion of mismatched nucleotide (Edmunds et al. 2008; Quinet et al. 2014). Consistent with the role of TLS reactions occurring at the elongating fork, replication fork stalling is observed in cells depleted of TLS polymerases (Quinet et al. 2014, 2016). TLS activity at the fork may also be restricted to a subset of polymerases. Whereas Rev3L operates in postreplicative gap filling, Rev1 and Pol η are required for TLS at stalled forks (Quinet et al. 2016).

In considering the role of TLS in rewired fork protection, it is important to note that TLS may be compromised in BRCA-FA cells. Indeed, in some respects, loss of the BRCA-FA pathway phenocopies loss of TLS and vice versa. Sensitivity to ICL-inducing agents is a phenotype attributed to loss of either pathway. Moreover, FA-like phenotypes in the hematopoietic stem cells result from loss of Ub-PCNA (Pilzecker et al. 2017). Pathway interconnections between BRCA-FA and TLS are established by interactions and the employment of common ubiquitin-modifying enzymes (for reviews, see Kim and D'Andrea 2012; Kim et al. 2012; Fu et al. 2013; Tian et al. 2013; Boisvert and Howlett 2014; Choe and Moldovan 2017). Notably the pathways are also genetically linked. Biallelic inactivation of Rev7(FANCV), the regulatory subunit of the TLS polymerase Pol ζ , underlies the genetic defect in the FA-V complementation group I (Bluteau et al. 2016). One could speculate that TLS defects in *BRCA-FA*-deficient cells selects for compensatory mechanisms that activate TLS downstream from the BRCA-FA pathway. This could underlie how BRCA-FA cells overcome proliferation defects due to endogenous lesions such as aldehyde-induced damage and R-loops, and/or how bone marrow failure transforms into leukemia in FA patients (García-Rubio et al. 2015). Moreover, a gain in TLS could eliminate mitotic DNA synthesis that compensates for under-

replication (Bhowmick et al. 2016) especially in *BRCA2*-deficient cells (Lai et al. 2017). Indeed, an investigation of the mutational signature in cells deficient in *BRCA1* suggests a compensatory up-regulation of TLS (Zamborszky et al. 2017).

Although it may be easier to tip the balance toward TLS in BRCA-FA cancer cells, the rewired replisome may take this a step further. In particular, TLS could suffice to replace replicative polymerases during replication if gaps are very short, which could be possible if nuclease-dependent resection at the fork is blocked or limited. In addition, the simultaneous loss of RAD51 and associated Pol α (Kolinjivadi et al. 2017b) could enable other gap-filling pathways to compensate as the replisome stalls. Indeed, monoUb-PCNA and RAD51 have nonredundant functions in gap filling (Hashimoto et al. 2010). A switch to TLS through a series of modifications on PCNA (Kannouche and Lehmann 2004) and increased chromatin access to stalled forks may underlie the mechanism by which CHD4 loss confers therapy resistance. Indeed, CHD4 loss in *BRCA2*-mutant cells reduced chromatin bound MRE11 and RAD51, while also elevating focal accumulation of the E3 ubiquitin ligase RAD18 that is necessary for PCNA ubiquitination (Guillemette et al. 2015; Chaudhuri et al. 2016). Notably, chromatin-bound RAD51 in *BRCA2*-mutant cells is already very low, but CHD4 depletion reduces this further (Guillemette et al. 2015). Thus, by disrupting the residual RAD51 that operates independent of BRCA2 to promote fork reversal (Kolinjivadi et al. 2017b) and/or that captures ssDNA for gap-filling reactions postreplication (González-Prieto et al. 2013), CHD4 loss could block fork reversal and degradation as well as liberate ssDNA for TLS reactions at the fork. Other routes to elevated TLS through changes in chromatin access or RAD18 complexes have been described (Kim et al. 2014; Yamada et al. 2014). We also found that the FANCD1 DNA helicase has the ability to aberrantly promote TLS and suppress HR when its interaction with BRCA1 or its carboxy-terminal acetylation are disrupted (Cantor and Nayak 2016; Xie et al. 2010a, 2012). Conceivably, when not properly regulated by BRCA1, FANCD1 disrupts fork-remodeling pathways and/or improves TLS efficiency by unfolding DNA secondary structures that interfere with replication. Interestingly, the mismatch repair protein MSH2, which binds secondary structures formed at stalled replication forks, blocks TLS pathways in FANCD1-deficient cells (Peng et al. 2014).

Further characterization will be required to decipher the contribution of TLS to chemoresistance and rewired fork protection in BRCA cancers. Nevertheless, it is an important therapeutic target (Yamanaka et al. 2017) given the growing body of evidence illustrating a causative role for TLS in the development of chemoresistance (Doles et al. 2010; Xie et al. 2010b; Srivastava et al. 2015). Importantly, significant progress has been made in the development of TLS inhibitors (Actis et al. 2013; Korzhnev and Hadden 2016; Sail et al. 2017; Sanders et al. 2017). TLS inhibitors may be efficacious as a first-line combination chemotherapy or prevent chemoresistance via restored fork protection when used in combination with cisplatin

or PARPi. It will also be important to determine whether alternative strategies to block HR and/or fork protection mechanisms of chemoresistance such as inhibitors of ATR (Yazinski et al. 2017) disrupt permissive replication mediated by TLS or other pathways at the replication fork.

CONCLUSION

The BRCA-FA pathway displays indispensable roles in maintaining genome stability, suppressing tumors, and mediating chemoresistance. It does this, not only through integral roles in DNA repair, but growing evidence indicates the BRCA/FA pathway contributes to the maintenance of replication forks during times of replication stress. In the absence of replication fork maintenance, *BRCA1/2*-mutant cancers are capable of “rewiring” the replication fork to allow replication to proceed. We propose that the TLS bypass pathway plays an important part in the mechanism by which replication forks proceed through replication stress. The role of the FA-BRCA pathway in maintaining replication forks and the newly established role for rewired replication forks in chemoresistance provide exciting new possibilities for the development of new chemotherapeutic interventions.

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