Investigating the Role of BRCA2 in Telomere Replication and Homology-Directed Repair

ABSTRACT

The correct function of telomeres is necessary for genomic integrity and is dependent on their proper replication and their protection by the shelterin complex. Defects in shelterin cause telomeres to be recognized as double-stranded breaks (DSBs) leading to the activation of DSB repair pathways including the homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways. This can cause the formation of tumorigenic chromosome fusions. The breast cancer susceptibility protein BRCA2 is a necessary component of the HR pathway of DSB repair, but its role in HR at dysfunctional telomeres has remained largely unstudied. BCRA2 has additional roles in the stabilization and processing of stalled replication forks, although the mechanistic details of this are unclear. Telomeres present a major challenge to replication machinery due to their propensity to form complex secondary structures, and several HR proteins have been implicated in their proper replication. Here, we propose an investigation into the role of BRCA2 in HR at dysfunctional telomeres and in the proper replication of telomeric regions in response to replication stress. We hypothesize that BRCA2 depletion will inhibit the formation of HR-mediated chromosomal aberrations in the context of telomere dysfunction. We also predict that BRCA2 will be important in the restart of stalled replication forks at telomeres.

INTRODUCTION

The genome is constantly under attack by various sources of genotoxic stress, and as a result, eukaryotic life has evolved a variety of mechanisms that protect against DNA damage. The failure of these mechanisms can cause genomic instability leading to an increased mutation rate and the potential activation of proto-oncogenes and deactivation of tumor suppressors (Shen, 2011). One of the most important mechanisms that protects against genomic instability is the proper function of telomeres, nucleoprotein structures that protect the ends of all chromosomes. Mammalian telomeres consist of tandemly repeated TTAGGG sequences that associate with a core complex of telomere-binding proteins known as the shelterin complex. Shelterin protects telomeres from being recognized as sites of DNA damage and it includes the double-stranded DNA (dsDNA) binding proteins TRF1 and TRF2 as well as the single-stranded DNA (ssDNA) binding protein POT1 which is recruited by TPP1 (de Lange, 2018).

Defects in the shelterin complex can render telomeres dysfunctional causing them to be recognized as DNA double stranded breaks (DSBs) (Deng and Chang, 2009). This results in the activation of a DNA damage response (DDR) mediated by two central kinases, Ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), which promote the recruitment of DSB repair machinery leading to telomere end-to-end fusions and the formation of polycentric chromosomes (Guo et al. 2007; Deng and Chang, 2009). ATM and ATR also activate downstream effectors to trigger cellular senescence or apoptosis in order to prevent genomic instability (Wang et al. 2016). However, cancer cells that harbor checkpoint defects can bypass DNA damage-induced senescence and apoptosis, causing polycentric chromosomes to participate in breakage-fusion-bridge cycles and leading to widespread oncogenic genome instability (Maser and DePinho, 2002; Gu et al. 2017). Mammalian DSBs are repaired primarily through either error-prone non-homologous end joining (NHEJ) or error-free homologous recombination (HR) (Rai et al. 2010; Sfeir and de Lange, 2012). Illustrated in Figure 1 (Prakash et al. 2015), HR is a robust and tightly regulated pathway that starts with the 5' resection of DSBs (mainly mediated by BRCA1, CtIP and the MRN complex), followed by the coating of ssDNA regions with RPA, and then the displacement of RPA by RAD51 to form RAD51 filaments that participate in homology search, strand invasion and recombination (Prakash et al.

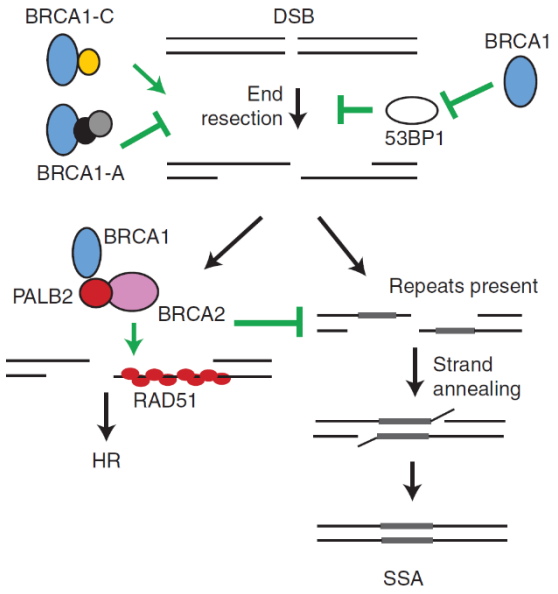

Figure 1: Schematic View of HR Initiation (obtained from Prakash et al. 2015). HR-mediated DSB repair begins with 5' resection promoted by BRCA1 followed by Rad51 filament formation mediated by BRCA2, which is recruited to BRCA1 through interaction with PALB2.

2015; Jasin and Rothstein, 2013). A critical step of the HR pathway is the displacement of RPA and the recruitment of RAD51 to 3' ssDNA overhangs. This process is dependent on BRCA2, which was originally identified as a tumor suppressor that protects against breast cancer (Wooster et al. 1995; Fradet-Turcotte et al. 2016). BRCA2 recruits RAD51 monomers via its BRC repeat motifs (Wong et al. 1997; Chen et al. 1998) and is also involved in stabilizing the RAD51 filament subsequent to its formation through interaction with the BRCA2 C-terminal TR2 domain (Davies and Pellegrini, 2007; Esashi et al. 2007). Deletion of BRCA2 abolishes the formation RAD51 foci and prevents HR, leading to chromosomal instability, increased susceptibility to DNA crosslinking agents and germline inviability in mice (Sharan et al. 1997, Fradet-Turcotte et al. 2016, Donoho et al. 2003).

In addition to DSB repair, BRCA2 has known roles in the maintenance of genomic stability under replication stress. During DNA replication, replication fork progression can be hampered by various DNA lesions or secondary structures. Depending on the nature of stress, forks can either collapse or can be stabilized and restarted (Meng and Zhao, 2017). BRCA2 is a known stabilizer of replication forks and has been found to co-localize with RAD51 and RPA at replication stress-induced foci (Buisson et al. 2014). It has also been shown to protect the nascent strand from degradation by MRE11 (Schlacher et al. 2011, Ying et al. 2012). It is thought that BRCA2's role in replication fork stability involves facilitating the recruitment of Rad51 to nascent ssDNA regions (Yata et al 2014) and stabilizing the RAD51 filament via the TR2 domain (Schlacher et al. 2011). The restart of stabilized forks primarily occurs through either fork repriming or fork regression (Berti & Vindigni, 2016) (Figure 2, Meng and Zhao, 2017), and there have been several reports that support a role of RAD51 and BRCA2 in fork regression (Petermann et al. 2010, Raghunandan et al. 2015, Zellweger et al. 2015).

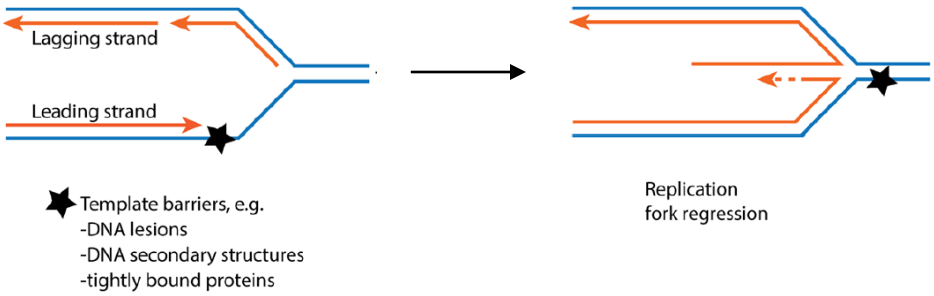

Figure 2: Replication fork regression (Adapted from Meng and Zhao, 2017). Fork regression involves the reversal of the fork and the re-annealing of the template DNA region surrounding the lesion. This allows the newly synthesized strands to anneal and for the incomplete nascent strand to be replicated using the other newly synthesized strand (template switching). It is thought that Rad51 formation is required to stabilize the involved ssDNA structures (Meng and Zhao, 2017, Petermann et al. 2010). Following template switching, various outcomes are possible, including bypass of the lesion.

Telomeres are known hotspots of replication stress due to their propensity for the formation of G-quadruplex structures and the lack of origins of replication within telomeric regions (Martinez and Blasco, 2015). Indeed, BRCA2 has been shown to contribute to telomere integrity, and it is thought that this is due to the fact that BRCA2 facilitates telomere replication through Rad51 loading at telomeres (Badie et al. 2010, Min et al. 2012, Zimmer et al. 2016). However, the exact mechanism of BRCA2 activity in telomere replication remains unclear, and DNA fiber analysis could elucidate this mechanism. We wish to test the model that BRCA2 facilitates telomere replication by promoting Rad51 filament formation and stability to promote fork regression.

Although the role of BRCA2 in HR throughout the genome has been studied extensively, fewer studies have investigated its specific activities at telomeres. HR at telomeres can be triggered by loss of POT1 and can cause the formation of mutagenic chromosome end-to-end fusions (Gu et al. 2017). In absence of exogenous telomere dysfunction, BRCA2 has been shown to contribute to the formation of spontaneous chromosomal aberrations (Min et al. 2012). However, the effects of BRCA2 depletion in the context of telomere dysfunction due to shelterin defects has not been studied extensively. We predict that BRCA2 depletion will inhibit the formation of HR-mediated chromosomal aberrations if HR-promoting telomere dysfunction is induced.

We propose an investigation to clarify the role of BRCA2 in HR at dysfunctional telomeres and elucidate its mechanistic role in telomere replication. **We hypothesize that BRCA2 depletion will inhibit the recruitment of RAD51 to dysfunctional telomeres and decrease the frequency of HR-mediated chromosomal aberrations. We also expect BRCA2-depleted cells to exhibit sensitivity to replication stress-inducing agents, reduced fork regression, and delayed fork restart.**

Our findings could potentially reveal two context-specific roles for BRCA2 in oncogenesis: as a promoter of genome instability that mediates chromosome fusion when shelterin is defective, and as a guardian of chromosome stability that prevents telomere replication defects.

Aim 1: Investigating the Role of BRCA2 in Homologous Recombination at Dysfunctional Telomeres

Rationale: BRCA2 is known to be required for the recruitment of Rad51 and the stabilization of the Rad51 filament at genomic DSBs. This is a critical step for HR-mediated repair of DSBs. We expect that BRCA2 will play a similar role in promoting aberrant HR at dysfunctional telomeres with shelterin defects.

We will deplete BRCA2 expression in an HCT116 human colorectal carcinoma cell line by stably expressing a short-hairpin RNA (shRNA) against BRCA2 using retroviral transduction. We will then induce telomere dysfunction by overexpressing $TPP1^{\Delta RD}$, which is a dominant-negative defective shelterin component that cannot recruit POT1, leading to ssDNA damage signaling at telomeres that causes robust HR activity (Guo et al. 2007).

We will then use Immunofluorescence-Fluorescence *in-situ* hybridization (IF-FISH) to evaluate the telomeric localization of BARD1, BRCA2, and RAD51. BARD1 is the stabilizing binding partner of BRCA1, which is involved in the 5' resection stage of HR upstream of BRCA2 and RAD51. It therefore serves as an internal control that should not be affected by BRCA2 depletion. We expect the shBRCA2-expressing groups to exhibit lower localization of RAD51 due to the decreased BRCA2 expression levels. Telomeres will be visualized using a telomeric peptide nucleic acid (PNA) probe.

In addition, we will use chromosome-orientation FISH (CO-FISH) on metaphase spreads to look at the effects of BRCA2 depletion on the frequency and nature of chromosomal aberrations in the $TPP1^{\Delta RD}$-expressing cells. We expect the BRCA2-depleted cells to exhibit a lower frequency of chromosome end-to-end fusions due to decreased HR activity. In addition, we predict that BRCA2 depletion will increase the frequency of the Multiple Telomeric Signals (MTS) phenotype. MTS are indicative of telomere fragility due to spontaneous replication stress (Sfeir et al. 2010). Due to its putative role in replication fork stability, we expect BRCA2 to suppress this phenotype. Finally, we predict the BRCA2-depleted cells to exhibit a lower frequency of telomere sister-chromatid exchanges (T-SCEs), which are indicative of crossover HR events at telomeres.

Potential Pitfalls and Alternative Strategies: There is a possibility that the BRCA2 depletion may be lethal to the cells and cause rapid apoptosis before $Tpp1^{\Delta RD}$ can be expressed. We do not expect this to be the case since BRCA2-knockout cell lines are routinely used in the literature (Fradet-Turcotte *et al.* 2016). If this occurs, however, we could attempt to use a cell line other than HCT116, or to use a conditional knockout BRCA2 cell line and induce BRCA2 knockdown after $Tpp1^{\Delta RD}$ expression. In addition, we may experience double-labelling issues in the CO-FISH experiment that prevent chromosome orientation from being distinguished. If this is the case, a different cell line could be used (such as U2OS). Alternatively, a PNA-FISH could be used that allows chromosomes and telomeres to be visualized but would not provide data on T-SCEs.

Aim 2: Investigating the Role of BRCA2 in Replication Fork Progression at Telomeres

Rationale: Badie et al. (2010) have shown that BRCA2-deficient cells display telomere replication defects and that BRCA2 facilitates telomere replication through Rad51 loading, presumably allowing the restart of stalled replication forks by HR-like mechanisms. Zimmer et al. (2016) have shown that such replication defects seem to be due to BRCA2's role in restart of forks that are stalled due to G-quadruplex structures at telomeres. However, the mechanistic details of how BRCA2 and Rad51 filament formation assist in the restart of replication forks at telomeres remains unclear.

First, we will evaluate the sensitivity of the BRCA2-depleted cell lines to the DNA replication stress-inducing agent aphidicolin (APH). We expect BRCA2-depleted cell lines to be more sensitive to APH treatment, as measured via a crystal violet growth assay. In addition, we will perform a PNA-FISH on metaphase spreads from APH-treated WT or BRCA2-depleted cells, and

we expect to see a dramatically higher frequency of MTS in the BRCA2-depleted cells, indicating telomere replication defects.

Telomere fiber analysis is potentially a useful method to gain mechanistic insight into the role of BRCA2 in telomere replication fork stability and restart (Sfeir et al. 2009). Reviewed in Quinet et al. (2017), DNA fiber-FISH involves pulse-labelling replication forks *in vivo* to track fork progression and monitor fork regression. BRCA2-depleted and WT cell lines will be treated with APH and DNA fiber-FISH will be performed, using a PNA probe to identify telomeric regions. We expect the BRCA2-depleted cells to exhibit delayed replication fork restart and lower levels of fork regression, which has been shown to depend on Rad51 (Petermann et al. 2010).

Potential Pitfalls and Alternative Strategies: it is possible that the BRCA2-depleted cells are hypersensitive to APH treatment and not enough cells will be available for the telomere Fiber-FISH analysis. If this is the case, the concentration of APH could be reduced, or an alternative agent such as 4-hydroxyurea could be used. Should we experience difficulties in implementing the DNA fiber-FISH assay, we could perform replication forks restart experiments using flow cytometry, as described in Jiang et al. (2015). The cells will be pulse labeled with BrdU, exposed to HU treatment and then labeled again with EdU. The ratio of $EdU^+/BrdU^+$ cells will be directly proportional to the replication fork restart efficiency.

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