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Novel insights into RAD51 activity and regulation during homologous recombination and DNA replication

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Abstract

In this review we focus on new insights that challenge our understanding of homologous recombination (HR) and Rad51 regulation. Recent advances using high resolution microscopy and single molecule techniques have broadened our knowledge of Rad51 filament formation and strand invasion at double-strand break (DSB) sites and at replication forks, which are one of most physiologically relevant forms of HR from yeast to humans. Rad51 filament formation and strand invasion is regulated by many mediator proteins such as the Rad51 paralogues and the Shu complex, consisting of a Shu2/SWS1 family member and additional Rad51 paralogues. Importantly, a novel RAD-51 paralogue was discovered in *C. elegans* and its *in vitro* characterization has demonstrated a new function for the worm RAD-51 paralogues during HR. Conservation of the human RAD51 paralogues function during HR and repair of replicative damage demonstrate how the RAD51 mediators play a critical role in human health and genomic integrity. Together, these new findings provide a framework for understanding RAD51 and its mediators in DNA repair during multiple cellular contexts.

Footnote

In this review, homologous recombination in multiple species is discussed. Where proteins from a specific species are referenced, the following prefixes are used: *Saccharomyces cerevisiae* (sc)*, Homo sapiens* (h), *Drosophila melanogaster* (dm), *Caenorhabditis elegans* (ce), *Schizosaccharomyces pombe* (sp). When general concepts or models from multiple species are referenced, such as Rad51 filament formation, we default to using the *Saccharomyces cerevisiae* nomenclature with no prefix.

Key words

RAD51, Rad51 paralogues, Homologous recombination, Shu Complex, DNA replication

Double-strand break repair by homologous recombination

DNA double-strand breaks (DSBs) are one of the most cytotoxic DNA lesions. One mechanism to repair DSBs is homologous recombination (HR), which uses a homologous template for repair and is therefore generally considered an error-free mechanism. Underscoring the importance of HR, mutations in HR genes are found in many cancer-associated diseases including Bloom syndrome, Werner syndrome, Fanconi anemia, and ataxia telangiectasia (Bernstein et al. 2010; Ellis et al. 1995; Kitao et al. 1998; Puranam and Blackshear 1994; Savitsky et al. 1995; Seki et al. 1994; Shiloh 1997; Yu et al. 1996). After a DSB occurs and HR is engaged, the DSB ends are resected giving rise to 3' single-stranded DNA overhangs (ssDNA; Figure 1). DNA end resection is first initiated by the Mre11-Rad50-Xrs2 (MRX) complex in the budding yeast *Saccharomyces cerevisiae* or the MRE11-RAD50-NBS1 (MRN) complex in human cells (Bernstein and

Rothstein 2009; Cejka 2015; Mimitou and Symington 2011; Paull 2010; Stracker and Petrini 2011; Takeda et al. 2007; Williams et al. 2010). After the MRX/MRN (yeast proteins are prefaced with sc while human proteins are indicated with an h herein) complex binds to the broken DSB ends, the endonuclease scSae2/hCtIP further stimulates resection in the 5' to 3' direction to generate 3' ssDNA overhangs [Figure 1 and 2; (Clerici et al. 2005; Huertas et al. 2008; Kim et al. 2008; Lengsfeld et al. 2007)]. The short-range resection by scSae2/hCtIP and the MRX/MRN complex is expanded by the redundant activities of the 5'-3' exonuclease scExo1/hEXO1 and the scSgs1/hBLM helicase in conjunction with the endonuclease scDna2/hDNA2 (Fiorentini et al. 1997; Huang and Symington 1993; Mimitou and Symington 2008; Zhu et al. 2008). The ssDNA that is generated is immediately coated by the ssDNA binding protein complex replication protein A (RPA). RPA-coated ssDNA protects the DSB ends from further degradation and signals to the cell the presence of unrepaired DNA damage (Ghospurkar et al. 2015; Manfrini et al. 2015; Sung and Klein 2006; Wold 1997).

Following ssDNA formation, RPA is displaced by formation of a Rad51 filament on the ssDNA, which is essential for the homology search and strand exchange steps that define HR in all eukaryotes [(Heyer et al. 2010); Figure 1]. Rad51 filament formation, also referred to as a presynaptic filament, is tightly regulated to prevent excessive recombination and genomic instability. The first barrier to Rad51 filament formation is the presence of RPA, which blocks Rad51 binding to ssDNA (Heyer et al. 2010). The inhibition of RPA is overcome by the activity of Rad51's regulators. In budding yeast, these include the Rad51 mediators, scRad52 and two Rad51 paralogues, scRad55 and scRad57, which form an obligate heterodimer *in vitro* (Krogh and Symington 2004;

Lisby et al. 2004; Sung 1997; Sung and Klein 2006; West 2003). Rad51 paralogues are proteins that structurally resemble Rad51's ATPase core, and are conserved throughout eukaryotes (Figure 2). Classically, Rad51 paralogues physically associate with each other (such as yeast scRad55 with scRad57) to regulate scRad51's function (Miller et al. 2002; Sung 1997; Wiese et al. 2002). In humans, the RAD51 paralogues form three discrete complexes- including the BCDX2 complex comprised of hRAD51B, hRAD51C, hRAD51D and hXRCC2, the CX3 complex containing hRAD51C and hXRCC3 [(Liu et al. 2011b; Miller et al. 2002; Wiese et al. 2002); Figure 2]. Additionally, the human Shu complex is comprised of the RAD51 paralogue, hSWSAP1, and the SWIM domain containing protein hSWS1, which is discussed in detail below [(Liu et al. 2011b; Miller et al. 2002; Wiese et al. 2002); Figure 2]. The human RAD51 paralogues are predicted to promote HR and regulate hRAD51, but their exact mechanism of action is still under investigation.

In yeast where the Rad51 mediator proteins are the most extensively characterized, they are thought to function by forming nucleation sites for Rad51 to bind to RPA-coated ssDNA. Subsequently, the Rad51 mediators promote the elongation of Rad51 filaments by directing incoming Rad51 monomers to bind to RPA-coated ssDNA (Gibb et al. 2014). The mechanism of how RPA is removed from the ssDNA ends is not well understood but occurs, at least in part, by continuous microscopic dissociation (Gibb et al. 2014). scRad52 is functionally analogous to human BRCA2 during Rad51 filament formation (Chen et al. 1999; Davies et al. 2001; Jensen et al. 2010; Jeyasekharan et al. 2013; Liu et al. 2010; Schlacher et al. 2012; Thorslund et al. 2010). A surprising new study has revealed that scRad52 binds to RPA-coated ssDNA and suppresses its turnover

where these RPA-ssDNA clusters remain interspersed along the presynaptic filament (Gibb et al. 2014). scRad51 then assembles onto the ssDNA where scRad51 filament sites are nucleated and extended. Why scRad52-RPA-ssDNA clusters remain along the pre-synaptic filament is puzzling. It has been proposed that they are important for mediating second end capture and/or stabilization of the displaced strand during strand exchange (Gibb et al. 2014). To prevent unnecessary HR, the activities of scRad52 and scRad55-Rad57 are controlled by post-translational modifications that are regulated by DNA damage checkpoint signaling (Bashkirov et al. 2000; Herzberg et al. 2006; Sacher et al. 2006; Torres-Rosell et al. 2007).

In addition to the scRad51 mediators, there are additional proteins, such as the anti-recombinase scSrs2 in budding yeast, that prevent illegitimate HR (Krejci et al. 2003; Veaute et al. 2003). scRad51, which contains Walker A and B ATP binding motifs, will only bind to ssDNA when it is ATP bound. scSrs2 functions by stimulating scRad51 to hydrolyze ATP to ADP, which releases scRad51 from ssDNA (Krejci et al. 2003; Veaute et al. 2003). In humans, numerous proteins carry out anti-recombinase functions to negatively regulate hRAD51 at both pre- and post-synaptic recombination steps, such as hRTEL, hPARI, hFBH1, and hRECQL5, although their mechanisms of action may differ from scSrs2 (Branzei and Foiani 2007; Bugreev et al. 2007; Chu et al. 2015; Hu et al. 2007; Karpenshif and Bernstein 2012; Mankouri et al. 2012; Moldovan et al. 2012). These redundant regulatory systems ensure that functional Rad51 filaments form only at sites of damage and not at other, undamaged portions of the genome.

In human cells, analogous to scRad52, hBRCA2 mediates hRAD51 binding to DSB sites (Chen et al. 1999; Davies et al. 2001; Jensen et al. 2010; Jeyasekharan et al. 2013; Liu et al. 2010; Schlacher et al. 2012; Thorslund et al. 2010). Using live cell imaging of fluorescently-tagged hBRCA2, it was observed that hBRCA2 forms oligomeric clusters (2-5 monomers) and has decreased mobility upon DNA damage [ionizing radiation (IR), hydroxyurea (HU), and mitomycin C (MMC)] suggesting that hBRCA2 is being recruited to damage sites (Reuter et al. 2014). Despite the size difference between hRAD51 and hBRCA2, hRAD51 displays a similar diffusive behavior to hBRCA2 (Reuter et al. 2014). Therefore, the interaction between hBRCA2 and hRAD51 is not limited to the initial hRAD51 nucleation steps of HR (Reuter et al. 2014). These results are consistent with EM studies finding hBRCA2 forming dimers (Shahid et al. 2014). It is possible that positioning hBRCA2 and hRAD51 in a similar orientation will help to mediate second end capture or to attach to ssDNA independent of the initial orientation that the DNA is bound (Shahid et al. 2014).

Once a Rad51 filament forms, it is stimulated to invade duplex DNA by the Swi/Snf translocase scRad54/hRAD54 where it searches for a homologous stretch of DNA (Heyer et al. 2006; Petukhova et al. 1998). Pre-synapsis, the yeast scRad51-ssDNA samples dsDNA for 8 nucleotide tracts of homology (Qi et al. 2015). Although 8 nucleotides of contiguous homology tracts are the minimum requirement of scRad51 for dsDNA sampling, increasing the homology tract length leads to reduced dissociation rates with a minimal threshold length of 15 nucleotides (Qi et al. 2015). The 15 nucleotide threshold suggests that this is the point when the scRad51 nucleoprotein is no longer capable of sampling another dsDNA for homology and likely represents a

commitment step to HR. New insights using a single molecule approach show that the strand exchange activity of scRad51 during the homology search on a complementary dsDNA occurs in three-nucleotide steps (Lee et al. 2015; Qi et al. 2015). Interestingly, a single mismatch within the three-nucleotide sequence can abolish the triplet but scRad51 has the potential to step over mismatches (Lee et al. 2015). After scRad51-mediated strand exchange, scRad51-coated ssDNA filaments are disassembled by scRad54. This allows DNA polymerases to access the heteroduplex DNA and extend the invading ssDNA by an average of 1,700 base pairs (Lo et al. 2006; Solinger et al. 2002). Using total internal reflection fluorescence (TIRF) and scanning force microscopy, the Wyman group has shown that human hRAD54 can integrate into the hRAD51 filament, both at the ends and internally under native conditions (Sanchez et al. 2013). These results support a model in which hRAD54 can both direct RAD51 strand invasion as well as dissemble hRAD51 from the invading ssDNA end to enable polymerases to access the ssDNA (Sanchez et al. 2013).

Once the strand invasion product is extended, it is disassembled through a multi-step process that can result in either a non-crossover or crossover product (Figure 1). Recent *in vitro* experiments using yeast and human purified proteins has revealed a novel role for scTop3/hTopoIIIα in dissolving scRad51/hRAD51-scRad54/hRAD54 D-loops in a topoisomerase-dependent manner (Fasching et al. 2015). This scTop3/hTopoIIIα activity is only observed on protein coated D-loops and is stimulated by scRmi1/hRMI1-RMI2 (Fasching et al. 2015). No direct interactions between scTop3-Rmi1 and scRad51-Rad54 were observed (Fasching et al. 2015). However, one can imagine the need for topoisomerase-mediated decatenation in D-loop resolution, especially in the context of

chromatin-bound DNA. In the simplest form of repair is called synthesis dependent strand annealing (SDSA). During SDSA, the heteroduplex is unwound, the extended ssDNA end invades the other side of the DSB, and the remaining gaps are then filled in and ligated, forming a fully intact DNA helix without a crossover [(Heyer et al. 2010), Figure 1]. Alternatively, the second end of a DSB can be captured, forming a double Holliday junction (dHJ). HJs are resolved through the activity of the scSgs1-Top3-Rmi1/hBLM-TOP3α-RMI1/2 resolvases or through the activities of various nucleases, such as scMus81-Mms4/hMUS81-EME1 and scYen1/hGEN1. These proteins act to cleave the dHJ and generate either crossover or non-crossover HR products (Boddy et al. 2001; Fricke and Brill 2003; Heyer et al. 2010; Ip et al. 2008; Ira et al. 2003; Schwartz and Heyer 2011). If the second end of a DSB is not captured, an alternative form of HR called break-induced replication (BIR) can occur (Figure 1). During BIR, the strand invasion product becomes a full-fledged replication fork and can replicate the template DNA for hundreds of kilobases, potentially to the end of a chromosome (Anand et al. 2013; Costantino et al. 2014). This form of HR can lead to widespread loss of heterozygosity if the donor template is not a sister chromatid.

Novel insights into the function of homologous recombination and RAD51 at damaged replication forks

The HR pathway has been extensively studied at direct clean-ended DSBs, such as those induced by endonucleases. A primary example is the HO cut-site at the mating-type locus in budding yeast or in meiosis at scSpo11-induced DSBs (Haber 1995; Keeney

et al. 1997; Weiffenbach and Haber 1985). However, in a normal replicating cell, stalled or broken replication forks are likely the major substrates acted on by the HR machinery (Li and Heyer 2008). Numerous lesions can initiate HR at a replication fork by stalling or blocking replication fork progression. For example, abasic sites produced during processing of base damage as well as bulky DNA adducts such as photoproducts can block replication fork progression. ssDNA breaks can cause replication forks to collapse into one-ended DSBs. Additionally, inter-strand crosslinks (ICLs) which prevent DNA unwinding and are repaired and bypassed, in part, by the HR pathway (Li and Heyer 2008). Each of these lesions is repaired by different mechanisms, but our primary focus here will be on HR at a stalled replication fork (Figure 3).

Visualization of replication fork intermediates at specific replicating origins has been best characterized using two-dimensional (2D) gel electrophoresis (Brewer and Fangman 1987). It has long been suspected that stalled replication forks lead to the formation of recombination intermediates. These intermediates are observed as cruciform structures by 2D gel electrophoresis after exposure to the DNA alkylating agent methylmethane sulfonate (MMS) (Liberi et al. 2005). If the replication fork encounters a MMS-induced DNA lesion, the HR machinery can be used to repair the replication fork damage. In support of HR occurring at damaged replication forks, X-structure formation is sc*RAD51* dependent and X-structures accumulate in the absence of sc*SGS1* (Liberi et al. 2005). This genetic evidence strongly supports a model in which stalled replication forks are able to bypass the blocking lesions by HR (Figure 3). New evidence has shown that the X-structures observed by electron microscopy (EM) at MMS-damaged replication forks are indeed HR intermediates (Giannattasio et al. 2014). By directly

purifying the X-structures at MMS-damaged replication forks, the Branzei group visualized these structures by EM. Importantly, these damaged forks contained numerous HR intermediates, including dHJs and the hemicatenanes proposed to form during scSgs1-mediated dissolution of dHJs (Giannattasio et al. 2014). Interestingly, in the context of this MMS damage, HR appears to occur at gapped ssDNA (Gap invasion, Figure 3), and not free ssDNA ends (Strand invasion, Figure 3). This work represents some of the strongest evidence to date in support of HR occurring at replicative damage and being initiated by the ssDNA that is produced during blocked replication (Giannattasio et al. 2014; Hashimoto et al. 2010).

Beyond the direct role HR plays in initiating bypass of lesions at stalled replication forks, two groups have independently proposed entirely novel roles for human hRAD51 in protecting these replication forks (Chen et al. 2015; Wang et al. 2015). In a study directed by the Smogorzewska group, a patient who is heterozygous for a single point mutation in the Walker A motif of hRAD51 (hRAD51-T131P) was identified to have Fanconi anemia-like symptoms and a general sensitivity to crosslinking agents (Wang et al. 2015). These findings have resulted in hRAD51 being classified as hFANCR (Wang et al. 2015). Interestingly, *in vitro,* purified hRAD51-T131P mutant proteins have reduced hRAD51 filament formation when co-incubated with wild-type hRAD51, suggesting that hRAD51-T131P is a co-dominant allele (Wang et al. 2015). However, when integrated into cells by CRISPR, this threshold activity of hRAD51-T131P is sufficient for HR to occur at IR-induced DNA damage sites but not at ICLs (Wang et al. 2015). These findings raise the possibility that hRAD51's role in repair of ICLs, and perhaps replication forks in general, may be distinct or differentially regulated

than at IR-induced DSBs. Consistent with an alternative role for hRAD51 during replication-associated repair, an exciting new study by the Kupfer group has reported an HR-independent mechanism for hRAD51 at HU-stalled replication forks (Chen et al. 2015). They find that hRAD51 physically associates with hRAD18 and hFANCD2 specifically following HU, but not MMC treatment. hRAD18 is an E3 Ub-ligase that monoubiquitinates PCNA during replicative stress to initiate bypass of stalled lesions by translesion synthesis (TLS; Figure 3). hFANCD2 is a central protein in the ICL repair pathway that is required to initiate HR at an ICL. Furthermore, the complex of hRAD51 with hRAD18 and hFANCD2 is critical for the monoubiquitination of PCNA and subsequent recruitment of hPol η, which promotes survival in response to HU. This role for hRAD51 in translesion synthesis (TLS) at a stalled replication fork is independent of HR as it occurs in h*brca2-/-* cells and when hRAD51's activity is pharmacologically inhibited with B02, a small molecule that inhibits hRAD51's nucleoprotein filament formation. Together, these studies suggest that hRAD51 has a novel role in replication-associated repair which is likely distinct from its canonical role in HR.

Links between mutations in the RAD51 regulators, cancer predisposition and Fanconi anemia

Mutation in hRAD51 itself has recently been associated with a Fanconi anemia-like disorder (hFANCR) (Ameziane et al. 2015; Wang et al. 2015). Consistently, the RAD51 mediators, such as hBRCA2 (hFANCD1) and the hBRCA2 regulator hPALB2 (hFANCN), have long been associated with Fanconi anemia (D'Andrea and Grompe

2003; Xia et al. 2007). In addition to elevated cancer risk, bi-allelic mutations in hRAD51C have been found to cause a Fanconi anemia-like disorder, leading to the classification of hRAD51C as hFANCO (Vaz et al. 2010). The multiple phenotypic consequences of hRAD51C mutations, where some defects lead to cancer predisposition and others lead to Fanconi anemia, suggest that unique functions of hRAD51C may be impaired leading to different disease outcomes. In addition to hRAD51C and hRAD51 being classified as unique Fanconi anemia complementation groups, a patient with a Fanconi anemia-like disorder was recently uncovered to have a truncation in hXRCC2 which is now classified as a Fanconi anemia-associated gene (Shamseldin et al. 2012).

In addition to mutations in hRAD51 metabolism being linked to Fanconi anemia-like disorders, heritable point mutations in hRAD51D, hRAD51C, hXRCC2, and hXRCC3 have become strongly implicated in non-hBRCA2 associated breast, ovarian, and colorectal cancer families (Blanco et al. 2014; Clague et al. 2011; Meindl et al. 2010; Michalska et al. 2015; Namazi et al. 2015; Osorio et al. 2012; Pelttari et al. 2015; Thompson et al. 2012). Understanding how these mutant alleles negatively impact hRAD51 paralogue function and protein interactions is essential for both determining how the hRAD51 paralogues normally function and how alterations in their normal biology can lead to cancer development.

Function of the human RAD51 paralogues in DSB repair and replication

Significant technical barriers have limited our understanding of the human hRAD51 paralogues function both *in vitro* and *in vivo*. For example, the low abundance and insolubility of individual hRAD51 paralogues has created a technical barrier for *in vitro* characterization of their function in human cells (Liu et al. 2011b; Masson et al. 2001). In addition, siRNA depletion of individual hRAD51 paralogues leads to instability of its interacting partners (Chun et al. 2013; Liu et al. 2011b). However, using yeast-2-hybrid and yeast-3-hybrid approaches, two hRAD51 paralogue containing complexes have been described including the BCDX2 (hRAD51B, hRAD51C, hRAD51D, hXRCC2) and CX3 (hRAD51C, hXRCC3) complexes (Liu et al. 2011b; Miller et al. 2002; Wiese et al. 2002). Formation of the BCDX2 and CX3 complexes was later confirmed using proteins purified from *E. coli* and insect cells (Compton et al. 2010; Yokoyama et al. 2004). Unlike human cells, where the hRAD51 paralogues are essential, in other model systems such as hamster (CHO) and chicken (DT40) cells the RAD51 paralogues could be deleted by homologous recombination (Johnson et al. 1999; Pierce et al. 1999; Takata et al. 2000; Takata et al. 2001). Unfortunately, mouse knockout models have resulted in embryonic lethality making our understanding of mammalian RAD51 paralogues restricted to specific cell lines (Deans et al. 2000; Kuznetsov et al. 2009; Pittman and Schimenti 2000; Prakash et al. 2015; Shu et al. 1999).

Consistent with a role for the RAD51 paralogues in HR, DT40 T lymphocytes with RAD51 paralogue disruption exhibit decreases in integration of DNA fragments, spontaneous and MMC-induced SCEs rates, and IR-induced RAD51 foci (Takata et al. 2000; Takata et al. 2001). Furthermore, RAD51 paralogue disruption results in decreased cell viability, chromosomal instability, and sensitivity to MMC and cisplatin (Takata et

al. 2000; Takata et al. 2001). These data strongly suggest a role for the RAD51 paralogues during HR but what the specific functions of the BCDX2 and CX3 complexes are is an ongoing area of investigation.

Recent work in human cell lines (breast cancer cell line MCF7 and osteosarcoma cell line U2OS) has suggested separation-of-function between the two human hRAD51 paralogue sub-complexes (Chun et al. 2013). For example, transient knockdown of the BCDX2 complex by siRNA (specifically *hXRCC2* or *hRAD51D*) reduces hRAD51 foci formation, but knockdown of *hXRCC3*, a unique component of the CX3 complex, does not (Chun et al. 2013). These results suggest that the BCDX2 complex is important for early steps during hRAD51 filament formation. Simultaneously depleting members from both hRAD51 paralogue complexes have no additive effect on hRAD51 foci levels (Chun et al. 2013). These findings support the notion that only BCDX2 facilitates early hRAD51 foci formation in response to IR (Chun et al. 2013). Despite the unique roles the hRAD51 paralogues may play in RAD51 regulation, depletion of either hRAD51 paralogue complex results in decreased HR at an inducible I-*Sce*I endonuclease cut-site (Chun et al. 2013).

Where do the human RAD51 paralogue complexes function in relation to the other known hRAD51 mediator proteins such as hBRCA2? Current evidence from the Powell group suggest that recruitment of hBRCA2 to DNA damage sites following IR is independent of both the BCDX2 and CX3 complexes (Chun et al. 2013). Furthermore, hBRCA2 is epistatic to the hRAD51 paralogues as co-depletion of hBRCA2 with individual hRAD51 paralogues resembles the HR frequency observed with siBRCA2

alone (Chun et al. 2013). These results are consistent with hBRCA2 functioning as a nucleator of hRAD51 filament formation on ssDNA as has been shown for the human protein as well as other species (Carreira et al. 2009; Shahid et al. 2014; Yang et al. 2005). Intriguingly, the hRAD51 paralogues are not epistatic with human RAD52, which has a distinct function from hBRCA2 (Chun et al. 2013). Consistently, co-depletion of hRAD52 and hXRCC3 in DT40 and U2OS cells causes synthetic lethality and decreased HR rates, suggesting that hRAD52 and the hRAD51 paralogues function in discrete pathways (Chun et al. 2013; Fujimori et al. 2001).

Similar to what has been shown with hRAD51, the hRAD51 paralogues likely have dual roles in canonical HR as well as in repair of damaged replication forks. While the BCDX2 complex is important for repair of DSBs induced by the I-*Sce*I endonuclease or IR (Chun et al. 2013), other complex lesions, such as ICLs or damaged replication forks, may require the activity of the CX3 complex. The Nagaraju group, using a DNA fiber spreading technique in chinese hamster ovary (CHO) cells, found that at HU-stalled replication forks, the CX3 complex promotes replication restart by disassembling RAD51 filaments (Somyajit et al. 2015b). They found that the hamster RAD51 paralogues localize to nascent DNA synthesized during replication and at fragile sites upon HU-induced fork stalling, where the RAD51 paralogues protect replication forks from degradation (Somyajit et al. 2015b). For example, knocking out RAD51C, XRCC2, or XRCC3 in CHO cells results in significantly shorter nascent DNA tract lengths and more parental ssDNA (Somyajit et al. 2015b). These findings are consistent with the nascent DNA around the fork being resected and thus exposing the parental ssDNA. Furthermore, disruption of the RAD51 paralogues results in elevated 53BP1 foci suggesting replication

fork collapse. This work provides evidence that the RAD51 paralogues actively respond to replication fork damage where they 1) protect nascent ssDNA at stalled forks from resection, 2) prevent replication fork collapse, and 3) facilitate replication restart. Further investigation of lesion specificity of the RAD51 paralogues will shed light on their mechanistic roles in diverse repair contexts.

The Shu complex interacts with scRad52 and scRad55-Rad57 to stimulate scRad51 filament formation

Recently the budding yeast Shu complex has been uncovered to be a new scRad51 regulator and evidence suggests that this complex is conserved throughout eukaryotes (Gaines et al. 2015; Godin et al. 2015; Liu et al. 2011b; Mankouri et al. 2007; Martin et al. 2006; Shor et al. 2005). The Shu complex is an obligate heterotetramer comprised of scShu2, a SWIM domain-containing protein, in complex with scShu1, scCsm2, and scPsy3 (Ito et al. 2001; Shor et al. 2005). The Shu complex was first characterized a decade ago in budding yeast where it was found to generate the complex HR intermediates acted on by scSgs1-Top3-Rmi1 (Shor et al. 2005). Disruption of the Shu complex members results in sensitivity to replication fork stalling caused by base damage, such as by MMS, but not to direct damage induced by IR (Ball et al. 2009; Shor et al. 2005). Puzzlingly, Shu complex disruption causes no sensitivity to HU, which reduces nucleotide pools and causes replication fork stalling. However, disruption of the Shu complex strongly suppresses an *sgs1*Δ mutant's HU sensitivity, suggesting a nuanced role for the Shu complex at stalled replication forks. Therefore, the Shu complex likely

performs a specialized function to promote HR in the context of replication-associated DNA damage (Ball et al. 2009; Xu et al. 2013). Recent work found that the Shu complex likely acts upstream of scRad54, indicating a potential role for the Shu complex in promoting scRad51 filament formation (Mankouri et al. 2007). However, how the Shu complex acts to promote scRad51 filament formation remains an unanswered question.

Several groups have crystalized two of the Shu complex subunits, scCsm2 and scPsy3, and found that these proteins mediate the DNA binding activity of the Shu complex and are structural scRad51 paralogues, despite sharing little sequence homology with scRad51 (Sasanuma et al. 2013; She et al. 2012; Tao et al. 2012). Like budding yeast, fission and humans contain a SWIM-domain protein called spSws1/hSWS1 that is also associated with the RAD51 paralogues [(Godin et al. 2013; Godin et al. 2015; Liu et al. 2011b; Martin et al. 2006) Figure 2]. Recently, scShu2/hSWS1 orthologues have been identified in every eukaryotic lineage by PSI-BLAST (Godin et al. 2015). This includes the early, divergent eukaryotic species *Giardia lamblia,* plant lineages such as *Arabidopsis thaliana,* and in all major model systems such as *D. melanogaster* and *C. elegans* (Godin et al. 2015). In all of these species the canonical SWIM domain of scShu2/hSWS1, a zinc-finger-like motif defined by CXC…X_n…CXHXXA, is invariant (Godin et al. 2015). Importantly, mutations in the SWIM domain impair budding yeast and human scShu2/hSWS1 interactions with their divergent Rad51 paralogue interacting partners (Godin et al. 2015). For example, in fission yeast Sws1 forms a complex with the spRad51 paralogues spRlp1 and spRdl1 while in human cells hSWS1 forms an obligate heterodimer with the newly identified SWS associated protein 1, hSWSAP1, which is a novel hRAD51 paralogue (Liu et al. 2011b; Martin et al. 2006). Both hSWS1 and

hSWSAP1 are important for hRAD51 foci formation, HR at an inducible I-*Sce*I cut-site, and for MMS resistance (Liu et al. 2011b; Martin et al. 2006). These results suggest that the Shu complex function is likely conserved from yeast to humans.

While it has long been known that the yeast Rad51 paralogues, scRad55-Rad57, stimulate scRad51 filament formation (Sung 1997), mechanistic insight into the function of the Shu complex has only recently been uncovered (Gaines et al. 2015). For example, we and others have shown that the budding yeast Shu complex physically associates with scRad51 and the other Rad51 mediators including scRad52 and scRad55-Rad57 by yeast-two-hybrid (Y2H) and *in vitro* pull downs (Gaines et al. 2015; Godin et al. 2013; Xu et al. 2013). Critically, the interaction between the Shu complex and scRad55-Rad57 is mediated by the Shu complex member, scCsm2. Furthermore scRad55-Rad57 bridges an interaction between the Shu complex and the scRad51 filament machinery (Gaines et al. 2015; Godin et al. 2013; Xu et al. 2013). New *in vitro* data demonstrates that the Shu complex stimulates scRad51 filament formation two- to three-fold, but only if scRad52 and scRad55-Rad57 are present (Gaines et al. 2015; Godin et al. 2013; Shor et al. 2005; Sung 1997; Xu et al. 2013). Excitingly, these results show a direct role for the Shu complex as a stimulatory co-factor for scRad51 filament formation (Gaines et al. 2015).

Rad51 paralogues may suppress inhibitors of Rad51 filament formation

In addition to the Rad51 paralogues' role during Rad51 filament formation, the Rad51 paralogues also inhibit proteins that antagonize Rad51 [Reviewed in(Karpenshif

and Bernstein 2012)]. The most studied inhibitor of Rad51 filaments is the budding yeast helicase scSrs2 (Krejci et al. 2003; Veaute et al. 2003). scSrs2 interacts with scRad51's ATPase domain, causing scRad51 to hydrolyze ATP and lose its DNA binding affinity (Krejci et al. 2003; Veaute et al. 2003). For many years after its discovery, how scSrs2 would interact with other regulators of scRad51 filament formation was of particular interest, especially since scSrs2 interacts with Shu2 in both budding and fission yeast by Y2H (Ito et al. 2001; Martin et al. 2006). In addition to a physical interaction, deletion of fission yeast *spSWS1* suppresses many of *srs2*Δ's defects such as its camptothecin sensitivity. Additionally, disruption of the budding yeast Shu complex increases scSrs2 focus formation at fluorescently labeled DSB sites by fluorescent microscopy (Bernstein et al. 2011; Martin et al. 2006). However, the most striking example of the interplay between scSrs2 and the scRad51 paralogues is the finding that scRad55-Rad57 can directly inhibit scSrs2's ability to destabilize Rad51 filaments *in vitro* (Liu et al. 2011a). This work complements genetic observations that budding yeast *rad55*Δ's hypersensitivity to IR is rescued by further deletion of *scSRS2 (Liu et al. 2011a).* While these findings are clear with IR damage, deletion of sc*SRS2* does not rescue the MMS sensitivity of either a sc*RAD55* or a Shu complex-disrupted cell. Indeed, for MMS-treated cells, an *srs2*Δ*, rad55*Δ*,* and *srs2*Δ *rad55*Δ cells exhibit the same MMS sensitivity, suggesting that these genes all act in the same pathway to promote repair in budding yeast (Xu et al. 2013). As of now, the functional significance of the physical interaction between Srs2 and the budding and fission yeast Shu complexes remains unknown. Moreover, further studies are needed to understand the interplay between scRad55 and scSrs2 at different lesions, e.g. IR-induced DSBs and MMS-induced replicative stress.

The worm RAD-51 paralogues, RIP-1 and RFS-1, reveal a novel mechanism in promoting RAD-51-dependent HR

Recently, the ceRAD-51 paralogue family in *C. elegans* grew to incorporate a novel, divergent ceRAD-51 paralogue, ceRIP-1 (Taylor et al. 2015). This novel ceRAD-51 paralogue physically associates with the only known ceRAD-51 paralogue in *C. elegans,* ceRFS-1, forming the first known ceRAD-51 paralogue complex in nematodes (Taylor et al. 2015; Ward et al. 2010). Recently, we identified ceRIP-1 to directly interact with the worm scShu2 orthologue, ceSWS-1 by Y2H and to bridge an interaction between ce-SWS-1 with ceRFS-1 by Y3H (McClendon et al. 2016). Our results show that ceSWS-1 interaction with ceRIP-1 is mediated by the SWIM domain of ce-SWS-1 and the Walker B motif of ceRIP-1 (McClendon et al. 2016). Using CRISPR to create a null *sws-1* allele, *sws-1* worms exhibit defects consistent with a role in HR such as DNA damage sensitivity, increased males, increased mutation rates, and fewer mitotic ceRAD-51 foci (McClendon et al. 2016). Together these results suggest that the Shu complex is conserved in higher eukaryotes.

The *C. elegans* ceRAD-51 paralogues share several features with the yeast and human proteins, in that they bind to ceRAD-51-coated ssDNA *in vitro* and their disruption *in vivo* inhibits normal HR (Taylor et al. 2015). However, unlike the budding yeast Rad51 paralogues, the ceRIP-1/RFS-1 heterodimer does not stimulate ceRAD-51 filament formation *in vitro*. Instead, the *C. elegans* ceRAD-51 paralogues promote ceRAD-51-dependent strand exchange (Taylor et al. 2015). The Krejci and Boulton groups furnish

evidence that this stimulation is due to ceRIP-1/RFS-1 remodeling the ceRAD-51 filament into a more "open" configuration that is more accessible by various proteins, as shown by the ability of nucleases to access the DNA. This "open" configuration is hypothesized to be more amenable to the strand exchange and homology search steps that define HR (Taylor et al. 2015). These exciting findings open many new possibilities for how ceRAD-51 paralogues may function outside of *C. elegans*. For instance, it would be important to determine if the other eukaryotic Rad51 paralogues also stimulate Rad51 filaments to adopt a more open configuration as shown for the *C. elegans* proteins.

Future Directions:

Clinical implications: Recently hRAD51C and hXRCC3 were found to assemble into an additional complex that is scaffolded by hPALB2, termed the "HR complex" comprised of hPALB2, hRAD51, hRAD51C, hBRCA2 and hXRCC3 (Park et al. 2014a). Mechanistically, this complex may promote hRAD51 filament formation although further investigation of this unique complex will be necessary to define its function. Clinically, this new complex is extremely intriguing for further study as heterozygous mutations in *hPALB2*, *hBRCA2*, and *hRAD51C* are associated with hereditary breast and ovarian cancers that arise when the WT copy is lost (Reviewed in Prakash et al. 2015). Furthermore, inherited homozygous mutations in *hPALB2* (*FANCN*), *hBRCA2* (*FANCD1*), and *hRAD51C* (*FANCO*) can cause FA [reviewed in (Levy-Lahad 2010; Park et al. 2014b)]. Moving forward, it will be essential to determine how different mutations in the same genes give rise to unique diseases.

Due to its clinical importance, many studies on the human RAD51 paralogues have focused on hRAD51C. hRAD51C was recently shown to promote HR as disruption of hRAD51C caused a compensatory increase in NHEJ after IR-induced DSBs (Somyajit et al. 2015a). Based on this finding, the Nagaraju group proposed a model to selectively target hRAD51C mutant cells with PARP inhibitors. Similar to the sensitivity of tumors with hBRCA1/2 mutations to PARP inhibitors, tumors with mutations in hRAD51 paralogues could likewise be targeted by PARP inhibition (Somyajit et al. 2015a). Combining hRAD51 mutations with PARP inhibitors leads to replication fork collapse and activation of the error-prone NHEJ pathway resulting in increased cell death (Somyajit et al. 2015a). Future studies will reveal whether this is a clinically viable treatment strategy.

Models of Rad51 paralogue function during HR: Although less is known about the human RAD51 mediators, new insights into the yeast proteins provide a framework for understanding how the Rad51 paralogues and the Shu complex may hypothetically contribute to repair by HR (Figure 4). After the DSB is resected and coated by RPA, then scRad52 is recruited where it stabilizes RPA-coated ssDNA in patches along the DNA (Gibb et al. 2014). Subsequently, scRad55-Rad57 and the Shu complex are assembled and enable scRad51 filament nucleation, extension and stabilization. The precise mechanism of how the scRad51 paralogues perform these functions is currently unknown. Here we propose three hypothetical models of how these mediators may function. In the first model, scRad52-RPA co-filaments are formed and scRad55-Rad57 and the Shu complex aid in the initial steps of scRad51 presynaptic filament assembly but are released from the filament (Figure 4, left model- Release). In the second model,

scRad52-RPA co-filaments form and scRad55-Rad57 and the Shu complex directly bind to the ssDNA and are integrated into the filament but exhibit no further interactions with scRad52-RPA (Figure 4, middle model- Integration). Lastly, it is possible that scRad55-Rad57 and the Shu complex interact with scRad52-RPA to coat the scRad51 filament as a co-filament (Figure 4, right model- Co-filament). Alternatively, the Shu complex could act to "cap" a scRad51 filament, and direct Rad51 filaments to expand unidirectionally, as was suggested for its meiotic function (Sasanuma et al. 2013). If the Shu complex remains associated with the scRad51 filament, it will be important to determine its impact in downstream HR steps, such as the recruitment of strand invasion factors, e.g. scRad54, scRdh54, or the inhibition of scSrs2 during scRad51 filament formation. Moving forward, the models proposed in Figure 4 will need to be tested by DNA curtain, TIRF microscopy, scanning force microscopy, and other single molecule approaches.

Alternative roles for the RAD51 mediators during DNA replication Collectively the evidence for a role for hRAD51 and the hRAD51 paralogues during repair of replicative damage raises important areas of future investigation. For example, if hRAD51's function in repair of HU-stalled replication forks is independently of HR, then will the hRAD51 mediators also exhibit HR-independent roles? Since mutations in the human hRAD51 paralogues are implicated in Fanconi anemia, do they contribute to hRAD51's HR-independent functions by regulating its activity? Different DNA intermediates may arise at replication forks depending upon DNA damaging agent that may require a unique set of RAD51 mediators for repair. Using the EM techniques pioneered by the Branzei group, it will be important to further evaluate the HR-dependent and independent roles of hRAD51 in repair of these lesions.

Concluding remarks Here we discuss recent advances in our understanding of Rad51 regulation during HR and how repair of replicative damage has opened many new areas of investigation and experimentation. For example, although the Rad51 paralogues were identified more than twenty years ago, our understanding of their mechanistic function of the Rad51 paralogues is still limited. In humans, the BCDX2 and HR complex (hPALB2, hBRCA2, hRAD51C, hXRCC3) regulate hRAD51 filaments, but the function of the CX3 and Shu complex (hSWS1-hSWSAP1) remains enigmatic. Perhaps, like *C. elegans*, certain human RAD51 paralogue complexes will regulate hRAD51 presynaptic filament assembly, while other hRAD51 paralogue complexes will regulate the activity of the pre- and post-synaptic filament. Moreover, since the budding yeast Rad51 paralogues, scRad55-Rad57, and the Shu complex interact with scRad52 to stimulate scRad51 filament formation, it remains unanswered whether the human RAD51 paralogues similarly function with hRAD52 or hBRCA2 to regulate hRAD51. As new findings emerge in one model system, it is imperative that we apply those paradigms to other models such as mammalian cells. By determining how well conserved the mechanistic function of the Rad51 paralogues are from yeast to humans, we will develop novel ways of investigating the human paralogues and their role in human disease.

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Figure legends:

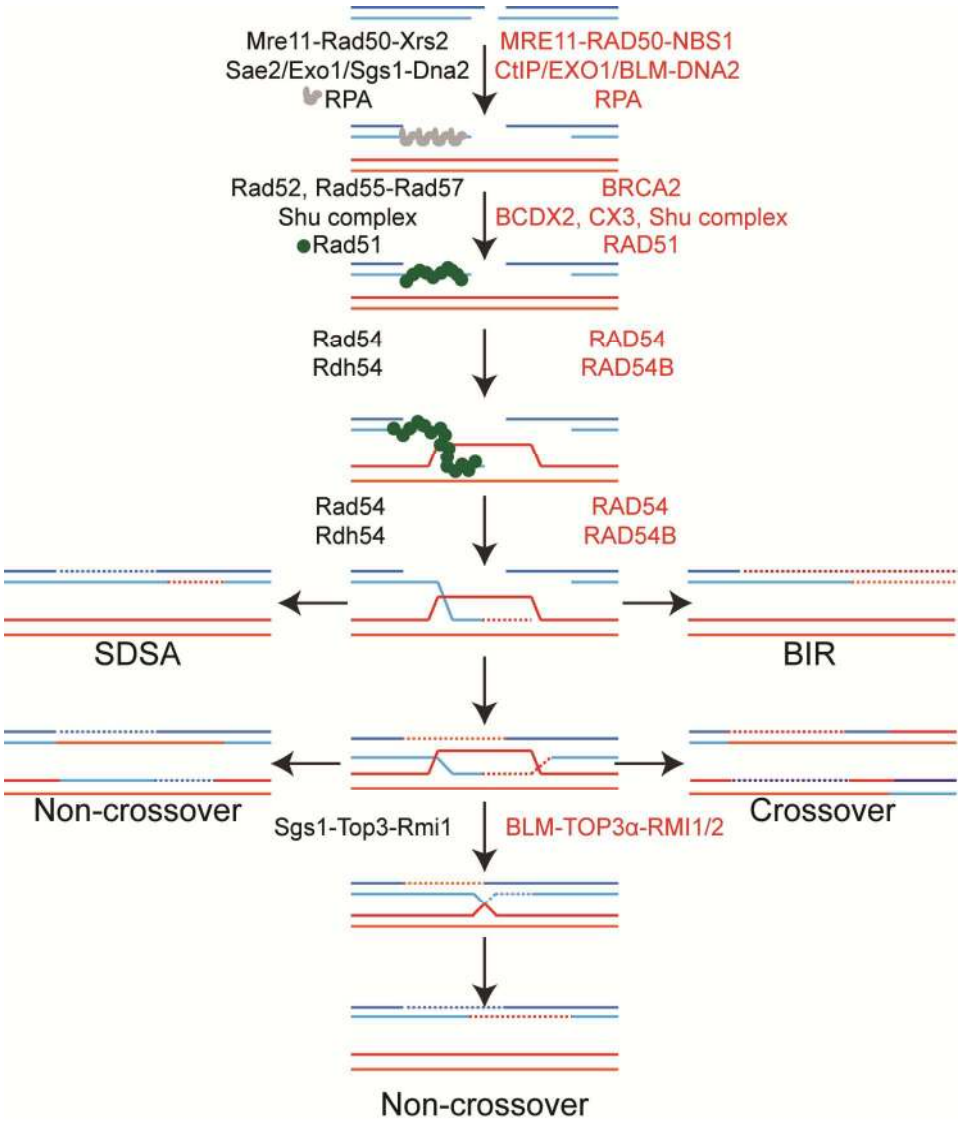

Figure 1: The different pathways utilized during homologous recombination. Protein names in black refer to budding yeast and are used in the legend, while red names refer to human proteins. All pathways begin with recognition of the DSB, followed by resection mediated by the MRX complex in conjunction with Sae2, Exo1, and Sgs1-Dna2 to generated extended 3' ssDNA overhangs. These overhangs are immediately coated by the ssDNA binding protein complex RPA. In order for HR to proceed, Rad51 filaments are induced to form on the ssDNA overhangs by the

activity of Rad52, the Rad51 paralogues, and the Shu complex. Rad51 filaments, in conjunction with Rad54 and Rdh54, carry out the critical homology search and strand invasion steps of HR. The strand invasion step is the last common step for all the HR pathways modeled here. During SDSA, the strand invasion product is extended past the break-site before being disassembled and reannealed to the other side of the break, generating a non-crossover repair product. In BIR, the strand invasion product becomes a full replication fork and can progress to the end of the chromosome, creating widespread loss-of-heterozygosity. Alternatively, the second end of the DSB can be captured to generate a double Holliday junction (Center). This double Holliday junction can be cleaved by various nucleases to generate both non-crossover and crossover events as depicted on the left and right respectively. The double Holliday junction can also be dissolved by Sgs1-Top3-Rmi1 to yield non-crossover events. Dashed lines refer to DNA synthesized during HR.

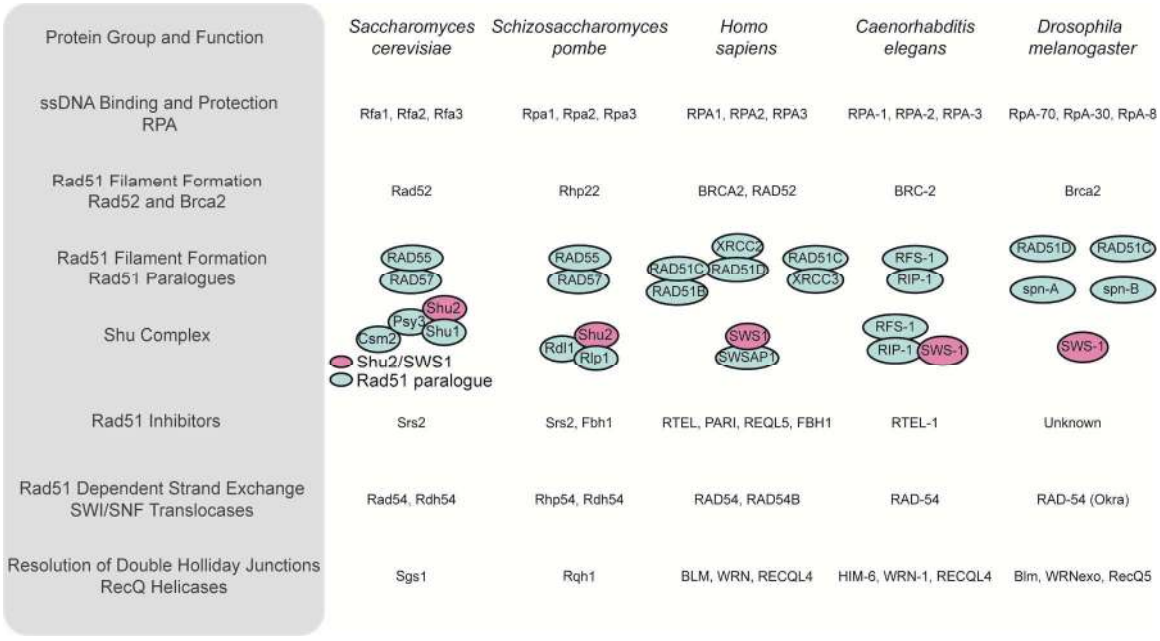

Figure 2: A schematic of the major HR proteins discussed. Homologues in five eukaryotic lineages are shown, and the Rad51 paralogues and the Shu complex, which are discussed in detail in this review, are drawn to show the known complex members in each species.

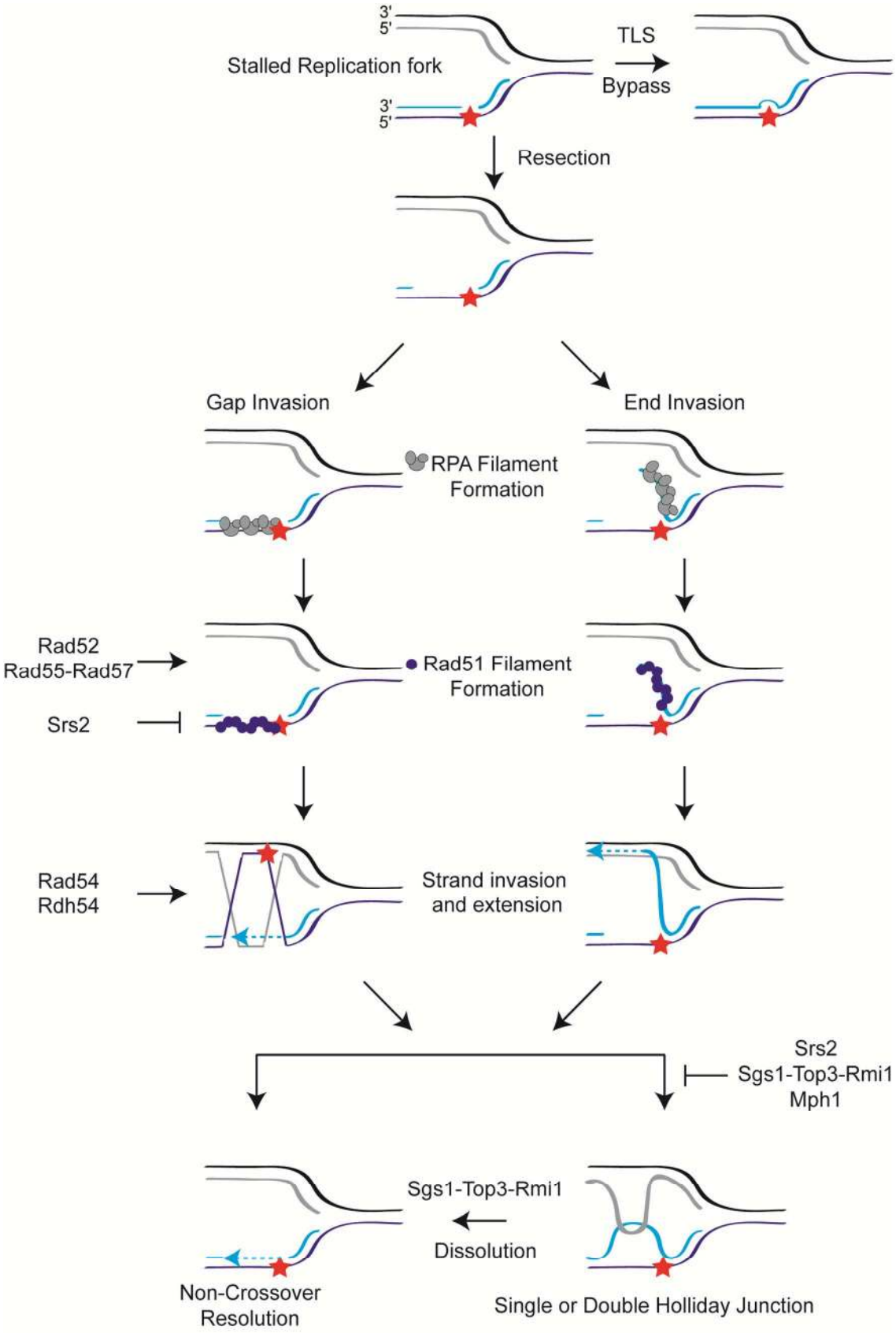
3'
5'
Stalled Replication fork
3'
5'
TLS
Bypass
Resection
Gap Invasion
End Invasion
RPA Filament
Formation
Rad52
Rad55-Rad57
Srs2
Rad51 Filament
Formation
Rad54
Rdh54
Strand invasion
and extension
Srs2
Sgs1-Top3-Rmi1
Mph1
Sgs1-Top3-Rmi1
Dissolution
Non-Crossover
Resolution
Single or Double Holliday Junction

Figure 3: A schematic highlighting three potential methods to restart a stalled replication fork in budding yeast. In this model, a blocking lesion, indicated by a red star, stalls replication behind the replication fork. This lesion can be bypassed by error-prone translesion polymerases (Top right; TLS), or it can be bypassed using high-fidelity HR (Bottom). Two potential models of how HR can progress at a damaged replication fork are shown. In the model on the left, the ssDNA gap at the replication fork serves as the template for HR. In the right-hand model, the newly synthesized, blocked DNA strand serves as the template. In both pathways, ssDNA is RPA-coated before Rad51 filaments are formed by the activities of Rad52, Rad55-Rad57, and the Shu complex. At the same time, illegitimate HR is inhibited by the anti-recombinase Srs2. Rad51-coated DNA is able to invade the undamaged, newly-synthesized sister chromatid by the activity of Rad54 and Rdh54. This allows extension by different DNA polymerases past the blocking lesion. Finally, both the gap invasion and end invasion pathways can either be dissolved into a non-crossover product (Bottom left) or result in the formation of a single or double Holliday junction (Bottom right), which is then dissolved by Sgs1-Top3-Rmi1.

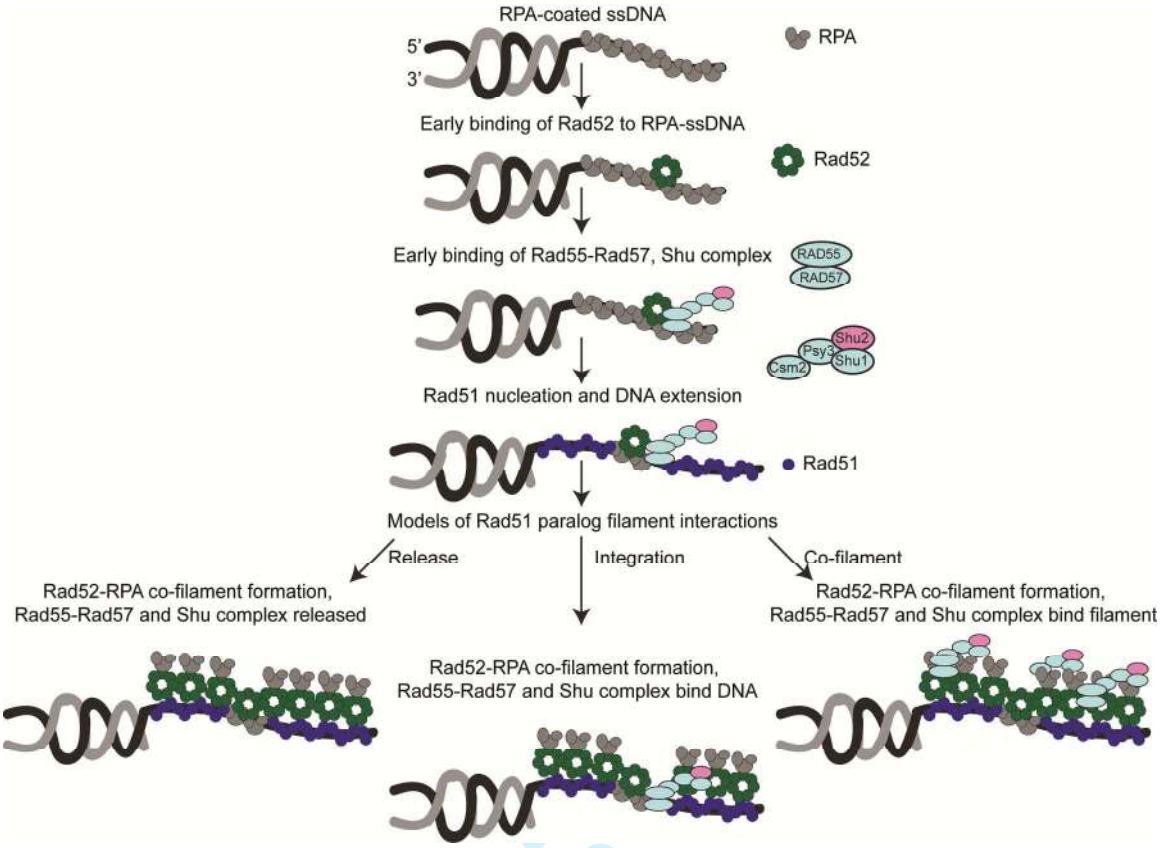

Figure 4: Hypothetical models for how the yeast Rad51 paralogues could associate with the Rad51-Rad52-RPA filament during HR [adapted from (Gibb et al. 2014)]. A DSB is recognized and resected to generate 3' ssDNA overhangs, which are immediately coated by the ssDNA binding complex RPA. Rad52 then binds the RPA-coated ssDNA and stabilizes a patch of RPA on the ssDNA. Next, the Rad51 paralogues, Rad55-Rad57, form a higher order ensemble with Rad52 and the Shu complex to stimulate Rad51 filament formation. Although filaments of Rad52-RPA coat the Rad51 nucleoprotein filament, whether Rad55-Rad57 and the Shu complex exhibit similar interactions with the Rad51 nucleoprotein filament remains unknown. Three hypothetical models for how the Rad51 paralogues and the Shu complex may interact with the Rad52-RPA-Rad51 nucleoprotein filament are

shown. On the left, Rad55-Rad57 and the Shu complex are released after Rad51 filaments form and are not associated with the Rad52-RPA-Rad51 filament. In the middle model, Rad55-Rad57 and the Shu complex are integrated into the Rad51 nucleoprotein filament but have no additional interactions with Rad52-RPA. On the right, Rad55-Rad57 and the Shu complex interact with Rad52-RPA and together coat the Rad51 filament as a co-filament. Importantly, the middle and right-hand models are not mutually exclusive, and Rad55-Rad57 and the Shu complex could both integrate into the Rad51 filament as well as form a co-filament with Rad52-RPA.