# RPA HYPERPHOSPHORYLATION FACILITATES HUMAN RAD52 FUNCTION IN HOMOLOGOUS RECOMBINATION

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# RPA HYPERPHOSPHORYLATION FACILITATES HUMAN RAD52 FUNCTION IN HOMOLOGOUS RECOMBINATION

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RAD52 deficiency is synthetically lethal in BRCA1 and BRCA2 deficient tumors. RAD52 is therefore a potential therapeutic target for breast cancer patients with BRCA mutations, but not much is known about its role in humans. RAD52 and the BRCA proteins are involved in the homologous recombination (HR) pathway of DNA double-strand break (DSB) repair. In HR, DSBs are processed to generate single-stranded DNA (ssDNA) overhangs, which are then bound by the RPA complex. RAD51 is then recruited and performs homology search and strand invasion. *S. cerevisiae* RAD52 and hBRCA2 mediate the exchange of RPA for RAD51 and stimulate RAD51 strand invasion. Recent publications show that hRAD52 provides an alternative mediator pathway in cells that lack the BRCA pathway. RPA hyperphosphorylation and dephosphoprylation after DNA damage are important for HR, but its effect on RAD52 function is not well understood. Here, we show that phosphorylation of RPA is important for the alternative RAD52 pathway. Using BRCA2-depleted human cells, in which the only available mediator pathway is RAD52-dependent, expressing non-phosphorylatable (RPA2-A) and mock phosphorylated (RPA2-D) RPA2, we show that HR is reduced in the RPA2-phosphomutant cells compared to RPA2-WT cells, measured by the DR-GFP recombination assay and RAD51 focus formation. Furthermore, RPA-phosphomutant cells have reduced association of RAD52 and RAD51 by colocalization. Interestingly, there is no

effect of RPA phosphorylation on RAD52 recruitment to repair foci in RPA-mutant cells after treatment with camptothecin. However, the RPA-phosphomutants do not colocalize with RAD52 as well as the RPA-WT protein and more RAD52 immunoprecipitates with RPA-WT than RPA-A after camptothecin treatment. Finally, using biochemical assays we show that RPA phosphorylation does not affect RAD51 strand exchange, RAD52-mediation of RAD51 strand exchange, and RAD52-dependent ssDNA annealing, suggesting there are factors in cells not present in these assays that allow RPA phosphorylation to promote RAD52 function, or that cycling of phosphorylation and dephosphorylation is needed. Thus, although RAD52 is able to be recruited regardless of RPA phosphorylation status, RPA phosphorylation improves RAD52's association with RPA, and subsequently promotes RAD52-HR. RPA phosphorylation is therefore important for both BRCA2-directed and RAD52-directed HR.

## BIOGRAPHICAL SKETCH

Alison Crystal Carley was raised in Babylon Village on Long Island, NY. She attended New York University and graduated in May of 2007, summa cum laude, with a BA in biology and a minor in mathematics. She worked at Mount Sinai Medical Center from 2007-2009 in the lab of Dr. Goutham Narla, where she studied an oncogenic splice variant of the tumor suppressor gene KLF6. In 2009, she began her studies at Weill Cornell Graduate School of Medical Sciences, in the BCMB program. She joined the laboratory of Dr. Simon Powell at Memorial Sloan Kettering Cancer Center in 2010, where she worked on the homologous recombination DNA repair pathway.

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9-1-1 complex: Rad9-Hus1-Rad1 complex

γH2AX: phosphorylated histone H2AX

ADP: adenosine diphosphate

a-RPA: alternative RPA

alt-NHEJ: alternative NHEJ

ATP: adenosine triphosphate

BER: Base excision repair

BIR: break-induced replication

BGS: Bovine growth serum

bp: base pair

BrdU: bromodeoxyuridine

CaCL2: Calcium Chloride

CDK: cyclin-dependent kinase

CO: crossover

Co-IP/IP: Co-immunoprecipitation

CPT: Camptothecin (topoisomerase poison)

C-terminus: carboxy protein terminus

DBD: DNA binding domain

DDR: DNA damage response

dHJ: double Holliday Junction

D-loop: displacement loop

DMP: dimethyl pimelimidate

DNA: Deoxyribonucleic Acid

DSB: Double strand break

dsDNA: double-stranded DNA

DR-GFP: direct repeat green fluorescent protein

EDTA: Ethylenediaminetetraacetic acid

ETO: etoposide (topoisomerase poison)

FRET: Fluorescence resonance energy transfer

GFP: Green fluorescent protein

HR: Homologous recombination

HRP: horse radish peroxidase

HU: hydroxyurea (causes replication stress by nucleotide depletion)

hypRPA: hyperphosphorylated RPA

ICL: interstrand cross-link

IR: irradiation

IRIF: Irradiation induced foci

kDa: kilodalton

LOH: Loss of heterozygosity

MgCl2: Magnesium Chloride

MMC: mitomycin C (DNA crosslinker)

MMEJ: Microhomology-mediated end joining

MMR: Mismatch repair

MMS: Methyl methanesulfonate (DNA alkylating agent)

MRN: Mre11-RAD50-NBS1 complex

NCO: non-crossover

NER: Nucleotide excision repair

NLS: nuclear localization signal

NHEJ: Non-homologous end joining

NT: non-target

nt: nucleotide

N-terminus: Amino protein terminus

OB fold: are oligonucleotide/oligosaccharide-binding folds

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PIKK: phosphatidylinositol 3-related kinases

pRPA: phosphorylated RPA

PRR: Post-replication repair

RNA: Ribonucleic acid

RPA: replication protein A

RPA-A: RPA-alanine mutant

RPA-D: RPA-aspartate mutant

RPA-WT: WT RPA

SCE: sister chromatid exchange

SD: standard deviation

SDSA: synthesis-dependent strand annealing

SEM: standard error of the mean

siRNA: small interfering RNA

SSA: Single strand annealing

SSB: single strand break, single strand binding protein

ssDNA: single-stranded DNA

TBS, TBS-T: Tris buffered saline – tween

UT: untreated

UV: ultraviolet radiation

WT: wild-type

# CHAPTER ONE

# INTRODUCTION

## DNA DOUBLE STRAND BREAK REPAIR

Double strand breaks (DSBs) are the most deleterious type of DNA damage, arising during DNA replication or after exposure to ionizing radiation and other DNA damaging agents. DSBs can cause cell death or chromosomal rearrangements when they are not repaired or repaired incorrectly[1-3.] DSBs are resolved by two main pathways, homologous recombination (HR) or nonhomologous end joining (NHEJ). HR uses a homologous template to fill in breaks and is considered error free; it is active during the S and $G_2$ phases of the cell cycle when sister chromatids are available as a template. NHEJ can be mutagenic and functions throughout the cell cycle, ligating DNA ends without homology. Studies in mammalian cells have shown that NHEJ-deficient cells have reduced repair in all cell cycle stages while HR-deficient cells have only a minor defect in $G_1$ and a more clear repair defect in S, $G_2$ and M phases [2,4]. Evidence of competition between HR and NHEJ to repair DSBs is further supported by observations that HR is increased in NHEJ mutant lines[5], and that HR mutants are rescued by deficiencies in certain NHEJ factors[6,7]. HR and NHEJ double mutant mice have more severe phenotypes than single mutants, supporting their roles repairing differing DNA DSBs[8,9].

NHEJ involves alignment of DNA ends with minor processing to clear damage, and then DNA gap filling and end relegation. At clean breaks with compatible overhangs, religatable 5’ phosphates and 3’ overhangs, NHEJ is usually accurate. “Dirty” breaks with chemically damaged ends repaired by

NHEJ, and ligation between different chromosomes, can lead to mutations and translocations.

Homologous recombination begins with resection to generate 3' single-stranded DNA (ssDNA) overhangs. These overhangs are eventually coated by the RAD51 recombinase, which performs homology search and strand invasion of a complementary duplex. It is less error prone due to the fact that it repairs using the sister chromatid. HR also plays a role in regulating and repairing damage due to replication stress.

Other less well-characterized pathways of DSB repair include alternative-NHEJ (alt-NHEJ) and single strand annealing (SSA). Alt-NHEJ functions as a backup to canonical NHEJ and uses micro-homology to anneal ends; it is also referred to as microhomology mediated end joining (MMEJ), as it can ligate ends with small regions of homology. Single strand annealing (SSA) is a sub-pathway of HR that also begins with resection but then reanneals broken DNA ends at tandem repeats[3,10]. How a cell determines which pathway to utilize in order to repair DSBs is not well understood.

This thesis focuses on the RPA and RAD52 proteins, two important players in the HR pathway. Following in this chapter is a more detailed discussion of the HR pathway and when it is used. An in depth review of the RPA complex, which is an ssDNA-binding protein complex that binds the resected DNA ends in HR, is included. RPA is hyperphosphorylated in response to DNA damage, and the regulation and importance of this phosphorylation is described in detail. Finally, we discuss recombination mediators, including the BRCA2 and RAD52 proteins. Recombination mediators facilitate RPA replacement from ssDNA by RAD51 filaments and promote RAD51 homology search and strand invasion. BRCA2 mediates the

primary HR pathway in human cells, with RAD52 functioning in a backup pathway. The factors needed for the RAD52 pathway are not currently known. This thesis focuses on the potential role of RPA hyperphosphorylation in the RAD52 pathway.

**Replication stress**

DNA replication is highly coordinated in eukaryotes. The replisome is composed of polymerases, helicases, nucleases, and ligases. Replication requires replisomes to unwind chromatin, move bidirectionally, terminate synthesis, and repackage DNA. It is divided into three steps: initiation, elongation, and termination[11]. Errors in replication and replication stress can lead to genomic instability[11].

Replication stress can be caused by a number of factors, including the replisome encountering DNA lesions, conflicts with transcription or DNA-RNA hybrids, DNA secondary structure, proteins bound to the DNA template, topological strain, overexpression or constitutive activation of oncogenes, and chromosomal regions that are difficult to replicate like fragile sites, repetitive sequences, telomeres, and non-B form DNA[12]. Cells have a number of ways of dealing with replication stalling, including bypass of lesions or HR, though in eukaryotes the existence of multiple origins of replication allows some stalled forks to be offset by activating other replisomes[12,13].

Replisomes that are paused for too long lose activity and may result in fork collapse and a DSB, thought to be mediated by nuclease cleavage by the MUS81/EME1 complex[14,15]. In addition, when a replisome encounters a break in the phosphodiester backbone, the fork structure is lost, causing a DSB end. These breaks can be due to a failure to ligate Okazaki fragments, to repair breaks, or to topoisomerases failing to reseal breaks[12,16].

ATR is essential for DNA replication and is activated during S phase to regulate origin firing, to stabilize forks, and to promote repair and restart of damaged forks, ensuring complete DNA synthesis prior to mitosis[17]. It is recruited by ATRIP bound to RPA-ssDNA. RPA also stimulates binding and activation of RAD17-RFC clamp loader, which loads the PCNA related 9-1-1 complex and stimulates ATR kinase activity through TOPBP1[1,17-21]. In cells with extensive replication stress, the replication checkpoint is needed to prevent new origin firing and RPA exhaustion[22].

A cell can deal with replication blocks in a number of ways. Stalled forks can be cleared by translesion synthesis through polymerase switching, bypassing the lesion, or template switching, which can involve fork regression or strand invasion of the sister chromatid by HR. Fork regression involves reannealing the parental strands and is believed to be mediated by BLM, WRN, FANCM, HLTF, or SMARCAL1, generating a “chicken foot” which could allow restart of DNA synthesis[1,23-26]. The choice of which pathway is used is best characterized in yeast, where it is mediated through posttranslational modifications of PCNA[1,11,27-38]. There is evidence that this is also the case in humans[11].

The HR machinery has several roles at replication forks. It seals ssDNA gaps after replication, known as post-replication repair (PRR). It can protect nascent forks from extensive resection. Finally, recombination proteins can rebuild the replisome after collapse, as DSBs are generated when replication forks collapse and can then be repaired by HR. RAD51, BRCA2, MRE11, XRCC3, FANCA and FANCD2 have all been implicated in fork restart or stabilization—if defective, DSBs accumulate when cells are challenged with replication inhibitors or even during normal replication[39-41].

Fork protection by HR proteins helps prevent degradation of the nascent strand by nucleases, such as MRE11. RAD51 filaments and BRCA2 dependent stabilization of RAD51 filaments inhibits this nucleolytic degradation of nascent DNA[42-45]. Forks are also stabilized by other mechanisms, including preventing dissociation of replication proteins, regulation of HR proteins through phosphorylation, promotion of sister chromatid cohesion, upregulation of helicases that remodel forks to promote restart, downregulation of nucleases that may damage the stalled fork, regulation of chromatin modifications and histone supply, and targeting nuclear pore components that tether transcribed genes to release topological strain on replicating DNA[11].

**DNA Damage Response signaling**

There is a network of interacting pathways that mediate the cellular response to DNA damage, known as the DNA damage response (DDR). After breaks, four main sensors that can detect DSBs: PARP, Ku70/Ku80, MRN, and RPA[1]. Three central kinases mediate the DDR: DNA-PKcs, ATM, and ATR, members of the phosphatidylinositol-3-kinase-related kinase (PIKK) family[46]. DNA-PKcs primarily targets proteins involved in NHEJ and is recruited through Ku70/Ku80[47]. ATM and ATR also activate more phosphorylation through the CHK1 and CHK2 kinases[1,17]. ATM is primarily activated by DSBs through the MRN complex[48], while ATR responds to a broader spectrum of damage including DSBs through RPA-ssDNA[17]. PARP is thought to promote NHEJ, to mediate the accumulation of MRN, and to facilitate ATM activation[49,50]. These kinases have overlapping roles in the DDR, and coordinate the response, acting to regulate DNA repair enzymes through

post-translational modifications (PTMs), modifying chromatin around the damage and in the nucleus or cell to allow for repair.

The DDR can activate cell cycle checkpoints, including the $G_1$/S, intra-S, $G_2$/M checkpoints[1,11,18]. Detection of DNA damage during these points in the cell cycle will prevent cell cycle progression while the damage is repaired, through the PIKKs. Checkpoints regulate DNA replication at the initiation, fork progression, and fork stability steps, and also involve the decision between cell cycle arrest, apoptosis, or senescence, through post-translational modification signaling and transcriptional regulation[1,11].

The PIKKs have several mechanisms of dealing with DNA damage. ATR activation occurs through recruitment to RPA. RPA bound to ssDNA at damage sites and replication stress centers recruits ATRIP, which recruits ATR[20]. RPA also recruits RAD17, which loads the Rad9-Hus1-Rad1 complex (9-1-1 complex); the 9-1-1 complex then loads TopBP1, which activates ATR[17,19-21]. Activated ATR activates Chk1, which can inhibit new replication origin firing to allow for repair[22]. ATR also directly targets replication and recombination proteins, which are necessary for fork restart, to regulate checkpoints[17]. Another mechanism proposed is that prevention of origin firing prevents “exhaustion” of nuclear RPA levels—increasing amounts of ssDNA would occur if dormant origins fired during replication stress, and ssDNA unbound by RPA due to RPA exhaustion would be converted to DSBs[22,51]. ATM may also function in the response to replication stress by activating the HR pathway, though some models suggest that ATM is only important in replication stress once DSBs are formed. Another important protein in the DDR, p53, is regulated by ATM and CHK2 in response to DSBs[1]. P53 induces

cell cycle arrest, apoptosis, or senescence in response to DNA damage through transcriptional regulation[52].

A major hallmark of damage is phosphorylation of histone H2AX at S139, which spreads megabases in mammals flanking the DSB; this phosphorylated form is referred to as γH2AX[53-55]. Phosphorylation of H2AX is likely through ATM, and recruits MDC1. Phosphorylated MDC1 then recruits the E3 ligase RNF8[56,57], which is mediated through the RNF8 FHA domain[56-58]. RNF8 with UBC13 then ubiquitinates histones H2A and H2AX. RNF168, which is also an E3 ligase, is then recruited and with UBC13 amplifies the ubiquitin signal via lysine 63 linked chains of ubiquitin on H2A and H2AX[59,60]. These polyubiquitinated histones recruit RAP80 through its ubiquitin interacting motif, and RAP80 then recruits BRCA1 through Abraxas[58,61-68]. 53BP1 is also recruited through MDC1 and RNF168, and binds dimethylated histone H4 (H4K20me2)[69]. Besides ubiquitination signaling, sumoylation by PIAS1 and PIAS4 is also important for assembly of BRCA1, 53BP1, and RNF168, at damage sites[70,71]. After this signaling pathway leads to recruitment of BRCA1 and 53BP1, these factors compete and coordinate to determine which DNA repair pathway is used to repair the break; this will be discussed in more detail later. If resection is activated, it is thought to induce an ATM to ATR switch, as the ssDNA leads to ATR activation while ATM activity is attenuated[72]. ATR then activates Chk1, which phosphorylates RAD51 and promotes repair of break through HR after DSBs at stalled forks[73].

Besides the repair of DSBs, cells have other pathways to repair simpler damage to DNA. Mismatch Repair (MMR) and base excision repair (BER) can repair mismatches and small chemical damage, involving excision of the damaged base. Nucleotide excision repair (NER) removes more complex,

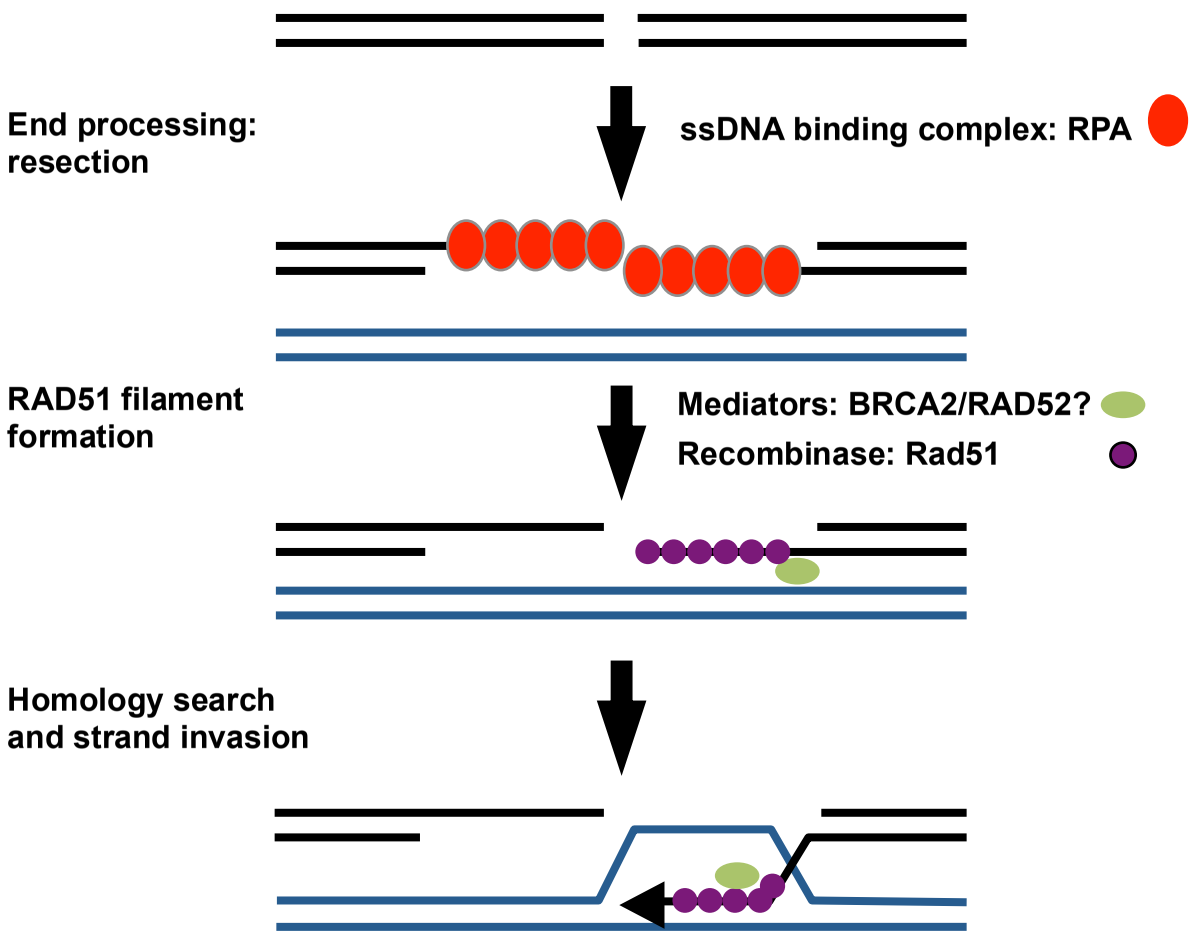
bulky lesions, like pyrimidine dimers, and removes a stretch of oligonucleotides around the damage around 30 nucleotides. Intrastrand-crosslink repair (ICL) occurs by the Fanconi Anemia pathway, which requires many HR factors[46].

**Homologous recombination (Figure 1)**

Studies in yeast have shown that in somatic cells there is a bias toward recombination using the sister chromatid rather than the homologous chromosome[74], though there is evidence of HR between homologs in $G_1$[75]. Cohesion of sister chromatids ensures proximity of the recombination template and stabilizes interactions between them. HR repairs DSBs and replication associated breaks in the S and $G_2$ phases of the cell cycle; ssDNA nicks and gaps can also be repaired by HR. Homologous recombination in meiosis involves similar mechanisms to mitotic HR and occurs after Spo11 initiates DSBs[76]. Though functional HR promotes genome stability, it can also cause genomic instability when it acts inappropriately or in an unregulated manner[77,78]. Proteins recruited to repair DSBs can be visualized by the formation of repair foci at lesions[79].

Mutations in genes involved in DNA repair have been implicated in human disease. For example, BRCA1 and BRCA2 are important factors in the homologous recombination pathway, and mutations in these proteins are associated with increased risk of cancer. Hereditary breast cancer from heterozygous germline BRCA mutations accounts for 5-7% of all cases of breast cancer, and patients with BRCA mutations have 50-80% risk of breast cancer and 30-50% risk for ovarian cancer, as well as a slightly increased risk for prostate and pancreatic cancers[80]. It is generally thought that BRCA tumors have loss of heterozygosity (LOH) or reduction in BRCA expression,

**Figure 1: Homologous Recombination pathway.** After a DNA DSB, DNA ends are resected to generate 3' ssDNA overhangs that are then bound by RPA. In order for HR to proceed, mediators such as BRCA2 and RAD52 are needed to promote RAD51 filament formation and strand invasion. The RAD51 recombinase then performs homology search, essential to HR, forming a D-loop intermediate that can be processed by several pathways.


End processing:
resection
ssDNA binding complex: RPA
RAD51 filament
formation
Mediators: BRCA2/RAD52?
Recombinase: Rad51
Homology search
and strand invasion

but LOH does not always happen in tumors[81] and it is possible there is haploinsufficiency. Biallelic BRCA2, BRCA1, and PALB2 mutations are rare and cause Fanconi Anemia, which is associated with defects in interstrand crosslink (ICL) repair[82-86]. Other diseases caused by HR defects include Nijmegen breakage syndrome (due to NBS1 mutation), Fanconi Anemia (ICL repair protein mutations)[87], Bloom's syndrome (BLM mutation)[88], ataxia telangiectasia (ATM mutation)[89], ataxia telangiectasia-like disorder (Mre11 mutations)[90], Seckel syndrome (ATR and CtIP mutation)[91,92], Werner syndrome (WRN mutation)[23], and Rothmund-Thomson Syndrome (RECQL4 mutation)[18,23,90-92]. Thus, HR is important to DNA repair function.

An outline of the initial steps of the homologous recombination pathway of DNA repair can be found in figure 1.

*BRCA1*

BRCA1 is a key player in DNA repair through HR. SSA and HDR are reduced by mutation of BRCA1, suggesting it is important upstream in these homology-directed pathways[93]. BRCA1 forms several different complexes with other proteins, and is involved not only in DSB repair but transcription regulation and cell-cycle checkpoints. BRCA1 has RING domain and nuclear localization signal (NLS) at its N-terminus, a coiled-coil domain, and BRCT repeats at the C-terminus[80]. The RING interacts with BARD1, and is important in BRCA1's function as an E3 ubiquitin ligase[94]. The coiled-coil domain mediates BRCA1's interaction with PALB2[95-97]. The C terminal BRCT repeats interact with phosphopeptides, mutually exclusively with Abraxas, BRIP1, and CtIP. BRCA1 forms complexes labeled A, B, and C, based on these BRCT interactions. The BRCA1-A complex forms through BRCA1 interaction with Abraxas. This complex ubiquitinates targets at DSBs through UBC13 and also

contains RAP80, which recognizes polyubiquitinated histones like γH2AX to target the complex to damage sites[62,66,68]. The BRCA1-B complex includes the BRIP1 helicase, which is required for checkpoint activation when replication forks are stalled or collapsed[98,99]. Finally, the BRCA1-C complex includes CtIP and MRN, and may play a role in resection[100-102]. The BRCA1-A complex has surprising anti-resection activity—depleting RAP80 results in hyper-recombination phenotype, mediated through CtIP resection[103]. Because BRCA1 complexes are mutually exclusive, this may be a result of BRCA1 that would be in BRCA1-A instead forming BRCA1-B or -C complexes[83].

Another complex forms through the BRCA1 interaction with PALB2, which connects BRCA1 to BRCA2[95-97]. BRCA1 disruption reduces PALB2, BRCA2, and RAD51 foci; PALB2 disruption reduces BRCA2 and RAD51 foci; BRCA2 disruption only prevents RAD51 foci. These observations suggest a pathway for HR, with BRCA1 is recruited upstream, and then recruits the BRCA2 mediator of RAD51 through PALB2[83].

*Resection*

Initiation of homologous recombination involves nucleolytic resection of the 5' end at DSB sites to generate 3' ssDNA overhangs[10,104-107]. Mammalian DSB resection and its regulation *in vivo* are not well understand, but it is believed to be an important mechanism in DSB repair pathway choice, as it inhibits NHEJ and promotes HR[104,108,109]. Other DSB repair pathways require resection as well: SSA begins similarly to HR, with fairly extensive resection, and MMEJ (or alt-NHEJ) also requires limited resection.

Resection is initiated by the nucleases MRN and CtIP. The MRN complex, composed of MRE11, RAD50, and NBS1, recognizes and binds DSB ends. RAD50 and MRE11 stabilize the break and tether the DNA ends,

while NBS1 interacts with the ATM kinase, which phosphorylates and regulates many DNA repair proteins[90,110,111]. MRE11 has ssDNA endonuclease, and 3'-5' exonuclease activities (though 5'-3'resection occurs *in vivo*); its endonuclease activity is believed to be important in resection[111]. MRN interacts with CtIP, which promotes resection[106,107,112,113]. CtIP has a 5' flap endonuclease activity independent of MRN, and its phosphorylation at numerous sites in response to DNA damage by cyclin-dependent kinase (CDK), ATM, and ATR, is believed to regulate its function[114]. HR at complex breaks, like those with topoisomerase adducts or generated by IR, requires CtIP nuclease activity, while this nuclease activity is dispensable for CtIP's role in HR repair of endonuclease breaks[114]. CtIP has also been shown to enhance the nuclease activity of MRE11 *in vitro*, and is important for ssDNA formation *in vivo*[113]. It has been suggested that MRN and CtIP may clip Ku from DNA ends similar to way they clip Spo11 off in meiosis[115].

A generally accepted model of resection is that MRN and CtIP initiate end resection by endonucleolytic cleavage of 5' ends internal to break ends, releasing oligonucleotides[116]. Biochemical studies with human proteins and studies in yeast suggest that there are two routes for more extensive resection, stimulated by RPA and MRN, and requiring either the DNA2 nuclease in complex with BLM-TOPIIIα-RMI1 or the EXO1 nuclease[117-120]. Resection varies in length from a few hundred nucleotides to tens of kilobases, depending on the availability and location of homologous template[116].

BRCA1 colocalizes and interacts with MRN and CtIP after damage[101,106,107,112,113,121,122]. It promotes HR and SSA while it inhibits canonical NHEJ[93,108,123], as does CtIP[113,124] suggesting BRCA1 may also be involved in resection[93]. Nevertheless, there is controversy over the role of

BRCA1 in resection, as there is conflicting evidence over the role of BRCA1 and its interaction with MRN-CtIP in resection, with some groups showing no role at all for BRCA1 in resection[100,102,125-130]. One model is that BRCA1 recruits CDK-phosphorylated CtIP to DSB sites, as CtIP phosphorylation promotes the CtIP-BRCA2 interaction, but a CtIP mutant mouse that does not interact with BRCA1 has normal HR, disputing this[131]. Thus, the role of BRCA1 in resection and interacting with MRN-CtIP needs to be better defined.

Using a quantitative method to measure ssDNA intermediates in human cells at endonuclease-generated break sites, it was determined that DSBs are resected up to 3.5 kb in a cell cycle-dependent manner[132]. Depletion of CtIP, Mre11, Exo1, or SOSS1 blocked resection, while depletion of 53BP1, Ku or DNA-PKcs leads to increased resection[132]. Interestingly, they found no role for BRCA1 in this regulation[132]. Using this direct measurement in human cells and *in vitro* assays, DNA-PKcs inhibited resection by blocking the recruitment of resection enzymes such as EXO1, while ATM and MRN promoted resection[133]. MRN stimulated resection in presence of Ku and DNA-PKcs by recruiting Exo1 and inhibited DNA ligase IV/XRCC4-mediated end rejoining[133]. Another study found that the CDK and PIKK sites of phosphorylation in CtIP were also essential for the recruitment of Exo1 and BLM to sites of laser-induced damage[134].

Limiting resection to the S and $G_2$ phases of the cell cycle in turn limits HR to these phases. Reduced resection in $G_1$ has been attributed to Ku binding DNA ends, NHEJ, and low CDK activity[116]. Cell cycle regulation is also achieved by CtIP degradation by the proteasome in $G_1$, and through CDK phosphorylation of CtIP at S327 in S and $G_2$, promoting its interaction with BRCA1 and MRN[126,135]. Limited resection in $G_1$, likely by CtIP, promotes alt-

NHEJ or MMEJ, as CtIP promotes alt-NHEJ[102,106,124,136,137]. CDK also phosphorylates Exo1, which contributes to this cell cycle specificity[138].

53BP1 is another important protein in the DNA damage response, and has been shown to limit resection. After DSB formation, 53BP1 rapidly localizes to discrete foci that co-localize with γH2AX and BRCA1; it has a key role in DNA repair response and checkpoint control. 53BP1 inhibition on its own increases levels of HR[105,139,140], likely by relieving 53BP1 inhibition of end resection at DSBs[6,7]. 53BP1 binds and protects ends in $G_1$, and competes with BRCA1 in S/ $G_2$; BRCA1 spatially excludes 53BP1 from DSBs and 53BP1 negatively regulates resection in $G_1$[141,142]. BRCA1 loss leads to 53BP1 foci in $G_2$, while 53BP1 loss leads to BRCA1 foci in $G_1$[127], suggesting the structures to recruit BRCA1 and 53BP1 are present in each cell cycle phase, but these protein mutually block one another. 53BP1 functions through effector proteins RIF1[127,143-147] and PTIP[148]. In addition to preventing resection, 53BP1 plays a role in NHEJ[139,149], partially by increasing stability and mobility of DSBs to find each other for productive ligation[150,151].

Interestingly, 53BP1 loss reverses much of the phenotype associated with BRCA1 loss, potentially through resection. 53BP1 loss rescues the embryonic lethality, tumor susceptibility, and premature aging in *Brca1*$^{\Delta 11/\Delta 11}$ mice, and rescues homology-directed repair in *Brca1*$^{\Delta 11/\Delta 11}$ mouse cells without fully eliminating chromosome instability[152]. It reduces asymmetric radial chromosome structures in *Brca*$^{\Delta 11/\Delta 11}$ cells, increases RPA phosphorylation, reduces chromosome aberrations and reverses cisplatin sensitivity[7], and reduces checkpoint activation elicited by unrepaired DNA damage due to Brca1 inactivation[6]. These data support a model where 53BP1 prevents resection, and removing this inhibition rescues BRCA1-depleted cells.

53BP1 loss does not rescue PALB2 or BRCA2 mutants, so the synthetic viability between 53BP1 and BRCA1 is likely due to BRCA1's role in resection[153]. Ku depletion does not also rescue BRCA1 loss[154], so this effect specific to 53BP1 and not just due to NHEJ deficiency. Interestingly, depletion of 53BP1 or RIF1 depletion restores resection in BRCA1-deficient cells but not those lacking CtIP, supporting a function for CtIP in resection other than 53BP1 removal[61].

*After Resection—strand invasion or SSA*

After resection, the heterotrimeric ssDNA binding complex RPA binds the 3' ssDNA overhangs[1-3,72]. For HR to proceed, RPA needs to be exchanged for filaments of the RAD51 recombinase, which performs the essential homology search and strand invasion steps of HR. The RAD51-ssDNA nucleprotein filament is called the presynaptic filament. When the 3' ssDNA invades a DNA duplex, it base pairs to a complementary strand and displaces the other strand of the duplex, resulting in the formation of a displacement loop, or D-loop. *S. cerevisiae* RAD52 mediates the exchange of RPA for RAD51, promoting RAD51 filament formation on RPA-coated ssDNA and stimulating RAD51 strand invasion[155,156]. BRCA2 performs this mediator function in humans, leaving the role of human RAD52 unclear[157,158]. Moreover, RAD52 mouse knockouts show little phenotype nor sensitivity to DSB-inducing agents, hRAD52 is inefficient at displacing RPA and stimulating strand exchange *in vitro*, and hRAD52 is not essential for RAD51 function or HR[157,159,160].

Nevertheless, recent evidence suggests that hRAD52 provides an alternative mediator pathway to BRCA2[161,162]. In BRCA2-deficient human cancer cell lines depletion of RAD52 reduced cell survival and proliferation, thus there is a synthetic lethal relationship between the two proteins[162].

RAD52 functions independently of BRCA2, since its localization to damage was not affected by the presence of the BRCA2 protein and RAD52 interacts with RAD51 independently of BRCA2[162]. Additionally, RAD52 was necessary for RAD51-mediated HR in BRCA2-deficient cells[162]. These results suggest that RAD52 provides a backup HR pathway in human cells: while BRCA2 is present, RAD52 has little effect on HR and viability, whereas in BRCA2-deficient cells, RAD52 is important for viability and for HR[162]. A more in depth discussion of RAD51 and recombination mediators is presented later on.

The SSA pathway also begins with resection, but is independent of RAD51 and is mutagenic. Rather than invasion of the resected strand of the sister chromatin, the break is repaired through annealing with downstream repeats. This pathway, in contrast to HR, has been shown to be dependent on RAD52[93].

*Completing HR*

After RAD51-mediated strand invasion and D-loop formation, there are several ways repair can be completed. How a cell decides which pathway it uses to resolve this intermediate is not well understood, but likely depends upon the point in the cell cycle, the type of damage being repaired, and the DNA structure present. RAD51 D-loops can result in crossovers or noncrossovers (CO or NCO) depending on which pathway is chosen—a CO results in the loss or exchange of the chromosome arm surrounding the break, an NCO does not. COs can lead to loss of heterozygosity (LOH) in mitotic cells, which can lead to tumor formation depending on the genetic material lost. A CO between repeats can lead to copy number variation. The balance between COs and NCOs is different between mitotic and meiotic cells, as crossovers are needed in meiosis to allow exchange of genetic material and to

pair the homologous chromosomes[163,164]. D-loops can lead to break-induced replication (BIR), synthesis-dependent strand annealing (SDSA), or the formation of double Holliday Junctions (dHJ).

BIR involves pairing between the resected 3' ssDNA and the homologous duplex region of the sister chromatid, with no second end capture of the other end of the break due to loss or inaccessibility of the second end. The invading strand then replicates off of the duplex it invaded, allowing replisome assembly and initiation of leading and lagging strand synthesis. The 3' overhang can invade the sister chromatid, the homologous chromosome, or a homologous region of another chromosome to initiate DNA synthesis, but use of templates other than the sister chromatid leads to LOH. This pathway is disfavored in mitotic recombination over SDSA[165].

SDSA, in contrast to BIR, does involve annealing to the other end of the break. In this pathway, the extended D-loop is disrupted, and the invading strand dissociates to anneal to the other 3' overhang on the other side of the DNA break. HR is then completed by filling in the gap and ligation[166,167]. This pathway can only result in noncrossovers. Antirecombinases promote this activity which reduces sister chromatid exchanges; helicases believed to promote SDSA include RECQ5[168], RTEL1[169]. Mph1, and Fml1.

Second end capture of the displaced strand in the D-loop, by annealing to the second 3' end of the break, leads to the formation of a dHJ. dHJs can result in CO or NCO depending on how they are processed. In mitotic cells, dHJs can be processed by dissolution by the BLM/TOP3α/RMI1/RMI2 complex, which involves branch migration and topoisomerase activity leading to a NCO[170]. The DSB repair (or DSBR) pathway involves dHJ resolution to form CO or NCO, depending on where the strands are cut. This is performed

by different resolvases: MUS81/EME1, GEN1, or SLX1/SLX4[171-174]. In meiosis, there is a bias toward DSBR by CO, while COs are suppressed in mitotic HR[175]. Second end capture to generate dHJs are mediated by RAD52 in yeast and humans[176-180], while the *U. maydis* Brh2 is capable of promoted second end capture when RAD52 annealing is inhibited[181].

Antirecombinases function to disrupt RAD51-ssDNA filaments or D-loop intermediates. In yeast, Srs2 and Sgs2 have been shown to function as antirecombinases. In humans, a number of proteins have been implicated, including FBH1, PARI, BLM, FANCJ, FANCM, RECQ5, RTEL1 and RECQ1, and mutations in some of these genes have been shown to increase crossovers and sister chromatid exchanges (SCEs)[3,27,170,182]. Their role may be to reduce excessive HR or to promote SDSA over dHJ formation and/or noncrossovers over crossovers through dHJ, but their exact function and mechanisms are not well understood. Similarly, the mismatch repair (MMR) machinery recognizes mismatches in HR intermediates and helps disrupt D-loops of mismatched duplexes, potentially through Msh2 interacting with BLM or WRN[183-186]. The complexity of the regulation of the RAD51-filament has been suggested to be due to another activity of human RAD51 filaments beyond homology search and strand exchange: at stalled replication forks RAD51 filaments protect ssDNA from Mre11 degradation[42], which may be regulated through BRCA2[27,44].

In summary, homologous recombination is important in the repair of DNA double-strand breaks, including those resulting from errors in DNA replication. How cells choose which pathway of DSB repair to use is under complex regulation. Mutations in proteins involved in homologous recombination lead to an increased risk in certain cancer types, highlighting

the importance of this pathway. In the next section, we describe the RPA protein complex and its phosphorylation. This protein is involved in many DNA processes in cells, including DSB repair through HR.

**REPLICATION PROTEIN A (RPA):**

Replication Protein A (RPA) is a heterotrimeric protein complex composed of subunits RPA1, RPA2, and RPA3. RPA is the primary eukaryotic single-stranded DNA binding protein (SSB), which are found in both prokaryotes and eukaryotes. ssDNA is generated during many DNA metabolic processes and as a result of damage to DNA; SSBs bind this ssDNA, protecting it from endonuclease degradation and preventing the formation of DNA secondary structure to allow DNA processes to proceed. In addition to this role, RPA also is involved in a number of protein-protein interactions that allow it to regulate DNA metabolic processes including DNA replication, repair, and recombination. RPA is also involved in transcription, cell cycle and DNA damage checkpoints, and telomere maintenance[187-191].

SSBs, including RPA, are essential to cellular survival. While there is no strong homology between prokaryotic SSBs and RPA, there is a high level of homology between eukaryotic species in RPA, particularly in RPA1 and RPA2. RPA was originally isolated as a factor required for SV40 DNA replication[192], and it is involved in the initiation and elongation steps of DNA replication. Loss of any of the three RPA subunits is lethal, and non-lethal mutations have been found to cause DNA repair defects and genome instability. RPA is found in the nucleus, and localizes to DNA in foci during replication and DNA repair. An abundant protein at a constant level through the cell cycle[193-195], RPA binds ssDNA with high affinity, so as a result ssDNA in cells is rapidly bound by RPA.

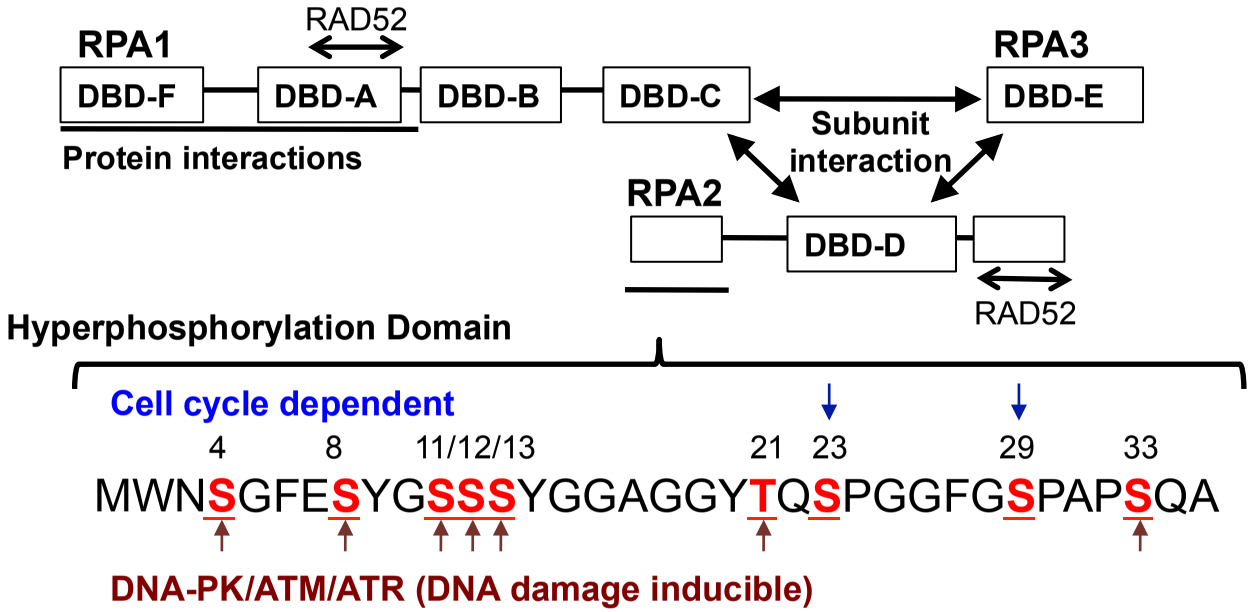
**RPA structure**

RPA's subunits RPA1, RPA2, and RPA3, are 70, 32, and 14 kDa, respectively (Figure 2). Between these three subunits there are six DNA binding domains (DBD) labeled A through F, which are oligonucleotide/oligosaccharide-binding folds (OB folds), common to DNA binding proteins. The OB folds of RPA are composed of five beta sheets that form beta barrels that can wrap around ssDNA[196,197]. To form the trimeric complex, each subunit contributes an OB fold to the trimerization core, consisting of DBD-C, DBD-D, and DBD-E, from RPA1, RPA2, and RPA3, respectively. Outside of the trimerization core, the other domains of RPA extend on flexible unstructured linkers, which allow for dynamic binding to DNA and protein partners, and multiple conformations[198].

When subunits of RPA are expressed individually they are insoluble or aggregate with other proteins, but with all three expressed a large portion of each peptide forms a soluble complex. Interestingly, RPA2 and RPA3 were shown to form a soluble complex when coexpressed without RPA1, but this complex is not functional and is believed to be a precursor to the heterotrimeric complex[199].

RPA1 contains four OB fold domains: DBD-A, DBD-B, DBD-C, and DBD-F. The high affinity DNA binding of RPA is attributable to DBD-A and DBD-B. These domains have a short linker between them, allowing for cooperative binding. DBD-C also binds ssDNA in some conformations, and contains zinc finger motif that influences DNA binding[200,201]. DBD-F contains a basic cleft at the N-terminus of RPA1 and though it can bind with ssDNA, it is thought to primarily act in protein-protein interactions[202]. Some models

**Figure 2: RPA domains and phosphorylation sites.** Top, the domains of the three RPA subunits, RPA1, RPA2, and RPA3. DBD A-F are composed of OB folds. Protein interaction domains are located on RPA1 and RPA2, which both interact with RAD52. DBD-C, DBD-D, and DBD-F form the trimerization core. Bottom, the hyperphosphorylation domain of RPA2 at the N-terminus is phosphorylated by CDKs in a cell cycle dependent manner at S23 and S29, and by DNA-PK, ATM, and ATR, in a DNA damage inducible manner at S4, S8, S11, S12, S13, T21, and S33.


RAD52
RPA1
RPA3
DBD-F
DBD-A
DBD-B
DBD-C
DBD-E
Subunit interaction
Protein interactions
RPA2
DBD-D
RAD52
Hyperphosphorylation Domain
Cell cycle dependent
4
8
11/12/13
21
23
29
33
MWNSGFESYGSSSYGGAGGYTQSPGGFGSPAPSQA
DNA-PK/ATM/ATR (DNA damage inducible)

suggest that proteins and DNA are in direct competition for the binding site in DBD-F[202].

The other two subunits of RPA also play a role in DNA binding, inter-subunit interactions, and protein-protein interactions with factors that allow RPA to regulate DNA processes. RPA2 has an unstructured N-terminal phosphorylation domain, which is hyperphosphorylated in response to DNA damage and during certain phases of the cell cycle. This phosphorylation is important in regulating not only RPA conformation and possibly DNA binding activity, but for RPA's role in DNA repair and replication. There is extensive literature on RPA phosphorylation that will be discussed in more detail[20,112,189,191,195,199,203-268]. RPA2 also has a central DNA binding domain, DBD-D, which is also part of the trimerization core, and a C-terminal winged helix domain involved in protein-protein interactions. RPA3 is mostly important for heterotrimer stability, containing DBD-E that is part of the trimerization core. Though structural results suggest that it can bind ssDNA, it has not been observed[269,270].

RPA plays a role in processes that generate ssDNA, and its function in the cell is mediated through protein partners. Many proteins have been identified that interact with RPA1, including p53, ATRIP, MRE11, RAD9, RAD17, WRN, FANCJ, BLM, and XPG[187,189,271]. Many of these interact through an acidic alpha helical domain that bind a basic cleft of the N-terminus of RPA1 and are involved in DNA repair or the DDR. RPA also interacts with replication factors polymerase alpha, PCNA, and DNA2, the NER protein XPA, the BER protein Ung2, DSB repair proteins RAD52, RAD51, BRCA1, and plays a role in MMR and telomere maintenance[187,189,271].

**RPA-DNA binding**

Human RPA binding DNA has been extensively studied. RPA binds ssDNA with much higher affinity than dsDNA or RNA (at least three orders of magnitude greater), with 5'-3' polarity[188,190,191,196,272-274] and an association constant of $10^{9}$-$10^{11}$ $M^{-1}$ [275,276]. It has a 10-50 fold preference for polypyrimidine sequences over polypurine sequences[276,277], and binds with a length dependence such that it binds more strongly to 30 nucleotide oligos compared to 10 nucleotide oligos[271]. RPA protein binds ssDNA with low cooperativity, and the major ssDNA binding affinity is attributed to DBD-A and DBD-B in RPA1, with a short linker between them allowing greater binding affinity[278].

A model for RPA binding suggests that RPA binds ssDNA sequentially, 5'-3', alphabetically through DBDs A-D, with the complex becoming more stable as more DNA is bound[188-191,271]. RPA1's DBD-A and DBD-B bind first, with a binding footprint of eight nucleotides. This eight nucleotide binding footprint has primarily been observed with glutaraldehyde crosslinking, suggesting it is unstable and likely a precursor to longer binding modes[190,275]. RPA1's DBD-C then binds, to make the total segment 12-23 nucleotides. Finally RPA2's DBD-D binds, bringing the footprint 25-30 nucleotides. This 30 nucleotide segment is the most well characterized and stable[191].

More recent studies suggest that RPA binding is more dynamic than this sequential model. Thermodynamic analyses suggested that there are only 18-20 and 28-30 nucleotide binding modes, likely representing three of four of the DBDs bound, and that the smaller binding modes are unstable and temporary[271,279,280]. Studying yeast RPA, Gibb et al[281] found that without other unbound proteins present, RPA remains stably bound to ssDNA for long periods of time and rarely dissociates. In contrast, RPA rapidly dissociates and

is exchanged when there is unbound RPA, scRAD51, or E. coli SSB, present in solution. They proposed that RPA dissociation involves a partially dissociated intermediate that exposes a small section of ssDNA to allow other proteins access, which could then carry out DNA metabolic processes like recombination or repair. Another study[280] found that human RPA rapidly diffuses along ssDNA, allowing other proteins access and also productively promoting the destabilization of hairpins adjacent to ssDNA.

Though RPA binds ssDNA with much greater affinity, it does bind dsDNA. When RPA binds dsDNA, it can destabilize the duplex, disrupting the bonds between two strands[282-284]. RPA also has a preference for ultraviolet radiation (UV) and cisplatin damaged dsDNA compared to undamaged dsDNA, likely due to this destabilizing activity[285-289]. Conversely, RPA has a reduced affinity for damaged ssDNA compared to undamaged ssDNA[283,290].

**RPA and the DNA Damage Response**

Due to its role as an ssDNA binding protein, and its interactions with other proteins involved in DNA metabolism, RPA has been identified as an important player in the DDR. In addition to its role in the DNA repair pathways themselves, RPA is involved in DNA damage and cell cycle checkpoints, which delay the cell cycle so that DNA can be repaired, or induce cell death in cells with irreparable damage. Failure of the DDR leads to genomic instability.

As described previously, RPA interacts with important players in the DDR. When ssDNA is generated during replication stress or other kinds of DNA damage, RPA binds the ssDNA and then recruits ATR through ATRIP[20]. The RAD17-RFC complex, which requires RPA-ssDNA for its recruitment, loads the 9-1-1 sliding clamp complex onto DNA[17,19-21]. 9-1-1 has structural similarity to PCNA and plays a role in DNA repair. ATR becomes active

through interaction with TopBP1, which is recruited to damage through binding Rad9 of the 9-1-1 complex[17,19-21]. Thus, RPA is important in recruitment and activation of ATR[20]. RPA also colocalizes and interacts with MRN, which may connect it to the ATM kinase as well.

A recent study by Toledo et al[22] elucidated part of the role of RPA in genome stability. Stalled forks in ATR-deficient cells were found to have nucleus-wide breakage due to the excess of ssDNA generated by unscheduled origin firing that exhausts the pool of RPA. The authors suggest that ATR suppresses dormant origins, which protects against this breakage by preventing the exhaustion of RPA. If there is too much ssDNA during replication stress the nuclear pool of RPA is exhausted, which leads to DSBs. Cells with less RPA are less tolerant of replication stress, and cells with overexpressed RPA are more tolerant. The ATR damage checkpoint was found to help prevent this exhaustion by inhibiting origin firing, which would generate more ssDNA. RPA normally acts to stabilize stalled forks and activate damage checkpoints through ATR, and after damage allows restart of replication[20,205,233,291].

**RPA-RAD52 interaction**

The RPA1 and RPA2 subunits are thought to be responsible for its interaction with RAD52[292-294]—thus connecting the central proteins of this thesis. The RPA2 subunit contains a C-terminal winged helix domain that interacts with a positively charged alpha helix in RAD52 (residues 257-274 of RAD52). This helix contains an Arg-Gln-Lys sequence similar to sequences in SMARCAL1, Tipin, Ung2, and XPA, that also interact with the RPA2 C-terminus[189,294,295]. In addition to the RPA2 interaction, human RAD52 is thought to interact with DBD-A and DBD-B of RPA1[292,294,296]. Budding yeast

contain an additional acidic helix domain in RAD52 not found in humans, which is crucial for RAD52's RPA interaction through RPA1 and recruitment to repair centers[297].

The interaction between RPA and RAD52 has been shown to be functionally significant. For example, Park et al[298] found that RAD52 mutants that lack an interaction domain (amino acids 221-280 of RAD52) fail to enhance HR in monkey cells, while overexpressed RAD52 containing this segment does enhance HR. Grimme et al[295] found using FRET ssDNA annealing assays that the RAD52-RPA interaction facilitated binding of hRAD52 to RPA-ssDNA, and counteracts RPA's helix destabilizing activity. Another study by Jackson et al[292] showed that RPA-RPA52 complexes have a five to 18-fold higher affinity for ssDNA than either protein alone, even using a RAD52 mutant lacking its own DNA binding domain. Nevertheless, although human RAD52 clearly interacts with RPA, a central player in DNA repair, the function of RAD52 in humans and other vertebrates is still not well defined.

**Other human SSBs**

There is a homolog to RPA2, RPA4, which replaces the canonical RPA2 in a form of RPA known as alternative–RPA (a-RPA)[299]. RPA4 interacts with RPA3 and RPA1, but not RPA2[299,300], and only has complete coding sequences in primates and horse, with other mammals containing a pseudogene or related sequences[301]. Like RPA2, RPA4 contains an N-terminal putative phosphorylation domain[300]. RPA4 is expressed in quiescent cells and in all normal human tissues, but at different levels in different tissues, and its expression is reduced in cancerous tissues and cell lines[299,302]

a-RPA has similar ssDNA binding properties to RPA; it binds ssDNA with high affinity and low cooperativity, and forms similar solution structures to

RPA[199,303,304]. While a-RPA has only a slightly reduced binding affinity than RPA for undamaged DNA, it has a higher affinity than RPA for damaged DNA and shows a ten-fold greater preference for damaged DNA than RPA. a-RPA is not functional in replication, and acts as a dominant negative, inhibiting replication by canonical RPA. RPA4 does not support proliferation or S phase progression[301].

While a-RPA does not support replication, it has been shown to be capable of supporting DNA repair, as it is functional in both NER and recombination[300-302]. RPA4 localizes to foci after treatment with camptothcin (CPT), colocalizing with γ-H2AX and p-CHK2. CPT is a topoisomerase poison that causes replication stress and replication-associated breaks. It also interacts with RAD51 and RAD52, and supports RAD51 *in vitro* strand exchange[301,302].

Two other SSB proteins, hSSB1 and hSSB2, have also recently been described in humans[305-321]. These proteins have more structural similarity to archael SSBs than to RPA. They form SOSS complexes with two other proteins, INTS3, and a previously uncharacterized protein. hSSB1 forms foci in response to DSBs but does not form replication foci, and is phosphorylated by ATM[313]. hSSB1/2 and their SOSS partners have been shown to play a role in HR repair and ATM checkpoint signaling, as cells defective in these proteins have hypersensitivity to damaging agents, chromosomal instability, reduced HR and RAD51 recruitment, and reduced ATM-dependent phosphorylation[306,308,311,313]. hSSB1 has also been suggested to regulate resection through promoting MRN and Exo1 nuclease activity and recruitment[309,310,318].

hSSB1 and other SOSS complex proteins have also been suggested to regulate checkpoints, similar to RPA. hSSB1 has been found to bind p21 and to affect the $G_1$/S and $G_2$/M transitions potentially through p21[314]; it also interacts with p53, stabilizing it and allowing p53-dependent p21 transcription[317]. hSSB1 relocates to damaged replication forks[305] and facilitates repair and restart stalled forks, through promotion of ATR and CHK1 activity and Mre11 and Rad51 recruitment[305]; in RPA-depleted cells hSSB1 can recruit and activate ATR-ATRIP[307]. Finally, deletion of the hSSB homologs in mice leads to repair and growth defects and lethality[315], and these mouse homologs were shown to play a role in telomere protection[316].

The identification of RPA4 and the SOSS complex demonstrates that cells have a complicated network of regulations to DNA damage. How these proteins function in cells and their role and competition with canonical RPA is not well understood. In addition to these potential damage-specific SSBs in humans, another mechanism of regulation of SSBs response to DNA damage that is not yet well understood is hyperphosphorylation of the N-terminus of RPA2.

**RPA Phosphorylation**

The N terminus of RPA2 in humans is phosphorylated in response to DNA damage and at certain points in the cell cycle[20,112,189,191,195,199,203-268]. A common strategy for studying this phosphorylation involves mutation of putative phosphorylation sites to either alanine, which would act as a non-phosphorylatable protein, or mutation of the sites to aspartate, which would add an acidic residue believed to mimic a phosphate group. While this study focuses on human RPA phosphorylation, yeast RPA has also been observed

to be phosphorylated in response to DNA damage on both the RPA1 and RPA2 subunits, which is dependent on the ATR homolog MEC1[212,238,260,263].

Two sites specifically on the RPA2 N-terminus are phosphorylated in a cell cycle-dependent manner during the $G_1$/S transition and M phase by CDKs: S23 and S29[195,218-221]. S29 is phosphorylated in mitosis, and S23 is phosphorylated in the S and M cell cycle phases[195,219,220,237,242,250,256]. S23 and S29 are CDK consensus sites containing an S/T-P motif, and are phosphorylated by cyclin A-Cdk2 and Cyclin B-Cdk1 during DNA replication and mitosis, respectively[189,199,219,220,234,242]. As a result, the phopsho-S23/S29 form of RPA is specific to mitosis[195,204,220,240,250,257]. The S phase phosphorylated form has been found to be associated with the replication initiation complex[221]. Dephosphorylation occurs during progression into $G_1$ at mitotic exit, as RPA is relocated to the nucleus[222,250]. This may function to help remove RPA from chromosomes, since RPA is excluded from the chromosomes during mitosis[250] and a decrease in dsDNA binding and duplex destabilizing activity is observed when RPA is phosphorylated at these sites[240].

Besides the two CDK-dependent phosphorylation sites, seven other sites have been identified as phosphorylation targets in the RPA2 N-terminus: S4, S8, S11, S12, S13, T21, and S33[237,256]. The specific kinase involved and which residues that kinase phosphorylates at what time point varies depending on which damaging agent is used and what phase of the cell cycle cells are in[20,204,205,209,211,213,215,223,225,228,230,231,233,234,237-239,241,242,246-252,254,256,257,259,261,262,264]. Three kinases phosphorylate these sites: DNA-PK, ATM, and ATR. Although early studies indicated that ATR and ATM respond to replication stress and replication-independent DSBs, respectively, and DNA-PK functions in DSB repair by NHEJ, it is now clear that PIKKs have

overlapping roles and display crosstalk in various DNA damage response pathways[205]. There are four to nine identifiable isomers of hyperphosphorylated RPA after damge[211]. Evidence suggests that RPA phosphorylation is dependent upon RPA binding to chromatin and on replication, as treatment with aphidicolin (which arrests DNA replication) inhibits CPT-induced RPA hyperphosphorylation[209,237,239,248]. RPA binding to DNA is believed to cause a conformational change that promotes efficient phosphorylation.

DNA-PK, ATM, and ATR each have been implicated in RPA hyperphosphorylation, though which kinase is responsible may differ depending on the type of damage. The pattern of hyperphosphorylation differs depending on damaging agent used[238,244,256]. There has been controversy over whether RPA is hyperphosphorylated in response to IR, with some studies observing this phosphorylation[203,217,250] and others suggesting that the hyperphosphorylation response is limited to agents that generate larger amounts of ssDNA[204,234,250]. DNA-PK, which has been implicated in both NHEJ and HR repair, contributes to RPA hyperphosphorylation[237,242]. DNA-PK has been observed to phosphorylate *in vitro* each phosphorylation site in the N-terminus of RPA2[237,256], and to phosphorylate RPA in cells at multiple sites after treatment with CPT, etoposide (ETO, a topoisomerase poison), IR, and UV[204,205,209,231,233,247,248,250,254]. It has been observed to interact *in vitro* with RPA1[248]. Cells with DNA-PK defects have similar phenotypes to those with RPA phospho-mutants, further demonstrating its important role in RPA phosphorylation[205]. ATM is also believed to contribute to RPA hyperphosphorylation, including after IR, UV, and ETO[209,234,239,250,257], but possibly not after CPT treatment[247]. Defects in RPA phosphorylation have

been observed in A-T cells, which are deficient in ATM, supporting a role for ATM in RPA phosphorylation[234,239,261]. Finally, ATR is not only recruited by RPA, but plays a role in RPA hyperphosphorylation[20]. Phosphorylation of RPA by ATR is dependent on ssDNA and stimulated by ATRIP[251,262]. ATR has been implicated in RPA hyperphosphorylation after IR, CPT, UV, and hydroxyurea (HU, which depletes nucleotide pools thereby causing replication stress) treatment[233,241,247,252,262,264]. Interestingly, ATR kinase activity has been suggested to be needed for ATR and RPA foci formation after IR[262]. While many studies have been published on RPA hyperphosphorylation by these kinases, the relative contributions of each kinase after different damaging agents, and the role of each phosphorylation site, are not well defined.

Beyond these nine better-characterized sites of phosphorylation in the RPA2 N-terminus, additional residues on RPA may be phosphorylated. Phosphorylation of RPA1 has been observed in yeast[260,263] and humans[238]. ATR was observed to phosphorylate the S52, S72, and S174, S/Q consensus sites on RPA2 *in vitro*, but the contribution of these sites *in vivo* was not determined[241]. Another study found five new potential phosphorylation sites on RPA2, including T98, and five sites on RPA1[238], while another suggested there are up to eight unidentified phosphorylation sites on RPA2[211]. Finally, the PHOSIDA and PhosphoSitePlus databases show human RPA1, RPA2 and RPA3 contain 28, 19 and 4 potential phosphorylation sites, respectively, that were measured at least once by mass spectrometry[225,228].

Phosphorylation at certain residues on RPA2 may be primed by phosphorylation at others. For example, hyperphosphorylation has been suggested to be dependent on CDK phosphorylation at S23 and S29 in some cases[204,234,242]. However, other studies did not see this effect of S23 and S29

on hyperphosphorylation[250,259], which may be due to different damaging agents and systems used. S33 phosphorylation by ATR may also stimulate subsequent phosphorylation at other sites[204,241,252]. In another example, S23A/S29A or T21A/S33A mutants both inhibit S4/S8 phosphorylation after CPT, though 21/33 does so to a greater extent[204]. Additionally, S4/S8 and T21 phosphorylation may exhibit reciprocal priming effects[204,233], while S33 phosphorylation regulates S4/S8 phosphorylation[204], but not vice versa[233]. In contrast, other studies have shown that S4/S8 phosphorylation does not depend on other sites, and that T21 and S33 do not trigger RPA2 phosphorylation after CPT[247]. Interestingly, one study found that phosphorylation could be primed in trans: phosphorylation of one RPA promoted phosphorylation on another[204]. Thus, while it is likely that priming at some sites does occur, the exact relationships are still unclear.

*Effect on hyperphosphorylation of RPA-DNA binding*

There is evidence for a relationship between phosphorylation of RPA and its DNA binding activity, though it is not well-defined and there have been some contradictory results[207,223,240,244]. In one study, no difference in hyperphosphorylated and non-phosphorylated RPA binding to pyrimidine-rich ssDNA sequences was observed, but hyperphosphorylated RPA (hypRPA) had decreased binding to purine-rich ssDNA and dsDNA[244]. Simarlarly, IR-induced hypRPA exhibited decreased binding to ssDNA compared to non-phosphorylated RPA in crude mouse cell extracts[223] and a lower affinity to DNA with 5’ or 3’ tails has been observed for phosphorylated RPA compared to RPA[235]. In contrast, using a mixed sequence 25-nucleotide oligomer, a relative increase in ssDNA binding by hypRPA over RPA was observed[216]. Other studies described little effect of RPA phosphorylation on ssDNA binding:

the mitotic phosphorylated form and mutations of mitotic sites shows no difference in ssDNA binding between pRPA and RPA[240,259], and deletion of the N-terminus (amino acids 1-40) of RPA2 or mutations of the phosphorylation sites to alanine or aspartate each had only modest effect ssDNA binding activity[265]. Thus, while it is possible that phosphorylation of RPA may regulate its DNA binding, how it does so is still unclear and may be dependent on the length and sequences of nucleotides used. It has been suggested that a decrease in ssDNA binding, may facilitate binding of other repair proteins to the ssDNA, but this decrease in binding has not been observed consistently.

Studies investigating phosphorylated RPA binding to dsDNA have been more consistent, demonstrating a reduction in RPA-dsDNA binding upon phosphorylation. Mitotically phosphorylated RPA binds less efficiently to dsDNA compared to non-phosphorylated RPA[240]. This was proposed to facilitate removal of RPA from DNA in mitosis, and reduce RPA duplex destabilization. Similar to this finding, the addition of multiple negative charges to the RPA2 phosphorylation domain reduces RPA-dsDNA duplex destabilization, while deletion of the phosphorylation domain or mutation to alanine did not effect helix destabilization[206]. This duplex destabilization defect was also observed with deletion of RPA1 N-terminal domain, suggesting a functional link between these domains[206]. Upon damage to dsDNA this effect was shown to be reversed: a higher affinity for cisplatin-damaged duplex DNA binding has been found for hypRPA compared to RPA, suggesting that hypRPA may signal DNA damage to other repair proteins[244].

*RPA recruitment and phosphorylation*

There is some evidence that RPA phosphorylation affects RPA foci formation, though the published data is unclear. RPA replication foci, in

general, show no difference between RPA2-WT and RPA2-alanine mutants, while RPA2-aspartate mutants have been observed to have defects. For example, an RPA2-A (S8/S11/S12/S13/T21/S23/S29/S33) mutant was shown to have normal distribution and to associate with replication centers in untreated cells, while an RPA2-D mutant at the same sites did not associated with replication centers, despite still immunoprecipitating with RPA1 and being able to support DNA replication in an SV40 *in vitro* reaction[253], Similarly, other RPA2-D mutants (S4D/S8D/S11D/S12D/S13D/T21D/S33D and T21D/S33D) had defective RPA2 S-phase-specific foci, while a S4D/S8D/S11D/S12D/S13D mutant had a more minor defect in forming normal S-phase foci[241]. Mutation of these sites to alanine did not result in a defect in association with replication centers[241]. In agreement with these results, in untreated cells no difference between RPA2-WT and an S23A/S29A[204] mutant or T21A/S33A[252] mutant foci was observed[204,252]. Looking at endogenous protein, hypRPA does not appear to localize to chromatin in untreated cells: no S4/S8 foci are observed in untreated cells[249], mitotically phosphorylated RPA do not localize to chromosomes[250] and an antibody specific to p-S4/S8 did not show phosphorylated RPA at replication centers[253]. Complementing these studies, depletion of ATR, DNA-PK, ATM, or treatment with caffeine did not affect RPA2 foci formation in untreated cells[247]. In sum, these data suggest that in untreated cells RPA can be recruited without phosphorylation and that phosphorylation prevents RPA recruitment to replication foci.

In cells with DNA damage, both phospho-mimic and unphosphorylatable RPA are recruited normally. Expressing a kinase-dead mutant of ATR does not affect RPA2 recruitment to chromatin after damage with UV or HU, though hyperphosphorylation is reduced[241,264], and

hyperphosphorylation has been observed to occur after initial localization of RPA to damage sites[247,249]. Expressing kinase inactive ATR or treatment with caffeine reduces RPA foci formation two hours after 50Gy IR, however, mutation in two ATR RPA phosphorylation sites does not affect RPA IR-induced foci (IRIF) so this effect may be an indirect effect of ATR inactivation and not specific to RPA phosphorylation, or the sites mutated were sufficient for the effect[262]. Looking at RPA mutants directly, after treatment with CPT, HU, or IR, there are normal levels of RPA foci in both RPA2-A and RPA2-D (S8, S11, S12, S13, T21, S23, S29, S33) mutants[253]. Similarly, an RPA2-A mutant containing alanine substitutions at all of the damage-inducible phosphorylation sites (S4A/S8A/S11A/S12A/S13A/T21A/S33A) did not result in a defect in association with damage foci after UV and HU, although this mutant did not slow replication as well as WT in response to UV, similar to ATR deficiency[241]. These observations support the model that RPA is phosphorylated before recruitment to DNA.

In seeming contrast to these findings, there is evidence that RPA phosphorylation affects RPA foci kinetics after damage. In one study, depletion of ATR or treatment with caffeine abrogated RPA2 foci formation after CPT, though there was normal RPA2 foci formation in DNA-PK and ATM deficient cells after CPT treatment[247]. Feng et al found that an RPA2-D mutant (T21D/S33D) formed foci normally after HU, but these foci persisted during recovery, which is likely due to unresolved damage, as γH2AX foci also persist[112]. Though some studies[241,253] show phosphorylation prevents association of RPA with the replication machinery after stress, they did not see a defect in T21D/33D association with BrdU replication centers[112]. In another study, RPA2-T21A/S33A mutant cells had normal DNA replication without

stress, but after stress had abnormally high levels of chromatin bound RPA, defective recovery from stress, and higher levels of ssDNA formation[252]. Lee et al found that depletion of PP4R2 (a phosphatase that targets RPA2) resulted in fewer RPA foci at early time points (up to 1.5 hours) after CPT treatment, but at later time points (four hours) there was no difference compared to control cells[230]. Consistent with this finding, an RPA2-D mutant (S8, S23, S29, S33) showed a significant delay in RPA2-focus formation after CPT[230]. One paper observed a similar fraction of cells with RPA2 foci, but those foci were of greater intensity immediately after and eight hours after treatment with CPT in a S23A/S29A mutant[204]. It's possible that the model that RPA is hyperphosphorylated independent of recruitment is still consistent with these findings, and that RPA hyperphosphorylation's effect on repair is responsible for differences seen. This is also consistent with an unaltered DNA binding affinity of hypRPA compared with RPA to ssDNA. RPA hyperphosphorylation promotes DNA repair, and as a result levels of ssDNA, which would alter RPA foci kinetics. Thus, it's unlikely for RPA phosphorylation mutants to have no effect at all on RPA foci kinetics.

*RPA phosphorylation, DNA repair, and replication stress*

Current models suggest that RPA phosphorylation and dephosphorylation are important in regulating RPA's role in the repair of damaged DNA. Defects in RPA phosphorylation alter the response to replication stress and lead to sustained damage. Several studies have shown that RPA phosphorylation defects lead to sensitivity to DNA damaging agents; for example, RPA2-D (S8/S23/S29/S33) mutants have increased CPT sensitivity[230] and HU and UV sensitivity[112], while RPA2-A mutants have increased chromosomal aberrations and decreased survival after HU[249].

RPA phosphorylation defects have also been shown to affect damage repair, resulting in persistent damage and ssDNA. For example, after treatment with CPT, RPA2-A (S23/S29) mutant cells had more intense RPA2 and γH2AX staining compared to RPA2-WT cells [204], RPA2-A (S4/S8) mutant cells have sustained H2AX phosphorylation after HU and enter mitosis with unrepaired damage[233], while an RPA2-D (T21/S33) mutant and PP2A-depleted cells also have persistent DNA damage foci of RPA and γH2AX in HU recovery[112]. Similarly, RPA2-A (S4/S8/S11/S12/S13/T21/S33) mutant cells have higher frequencies of chromatid and chromosome breaks, radial structures, and decreased cell survival after HU, and hypRPA associated with ssDNA after HU, supporting a role for RPA phosphorylation during replication stress and recovery[249]. In agreement with this, more DSBs were measured by the COMET assay in an RPA2-A (S4/S8/S11/S12/S13/T21/S33) mutant compared to RPA2-WT cells after CPT[268] and in RPA2-D (T21/S33) after HU[112]. RPA2-D and RPA2-A can associate with damage foci, as they are shown to co-localize with γH2AX[253], implying that although RPA can localize to breaks and sites of stress, RPA recruitment of downstream factors is impaired when the regulation of hyperphosphorylation is impaired, leading to persistent breaks. Curiously, one study found that RPA2-WT cells had more γH2AX by western blot after stress compared to an RPA-S33A or RPA-T21A/S33A mutants, which was argued to be the result of more breaks generated during replication during stress recovery[252]. This may be due to different assays used, as other studies looked at γH2AX foci, phosphorylation by western, or due to different timepoints, mutants, or cell backgrounds used.

Assays measuring homologous recombination more directly have shown a role for RPA phosphorylation in its regulation. RAD51 foci formation

is often reduced in RPA phosphorylation mutants. For example, compared to RPA2-WT cells, RPA2-A (S4/S8/S11/S12/S13/T21/S33) mutant cells have fewer RAD51 foci after HU, though not after IR, and hypRPA2 colocalizes with RAD51 in response to HU[249]. Dephosphorylation is also important for HR, as PP4R2-depleted or RPA2-D (S4/S23/S29/S33) mutant cells also have reduced RAD51 foci after CPT[230]. Notably, deficiency in RPA2 hyperphosphorylation has no obvious effect on the fraction of cells with RAD51 foci in untreated cells[249]. The DR-GFP assay has also been used to study the effect of RPA hyperphophorylation with similar results. Synchronized cells treated with HU in S phase have decreased DR-GFP HR in RPA2-A (4/8/11/12/13/21/33) mutant cells compared to WT, but phospho-mutant cells are normal in HR of I-SceI-induced breaks in this assay[249]. Another study did see an effect on endonuclease breaks, as cells depleted of PP4 phosphatase subunits or expressing an RPA2-D mutant (S8/S23/S29/S33) show lower HR induced by I-SceI compared to control cells[230]. Further, mutation in the p53 phosphorylation sites that regulate its interaction with phosphorylated RPA also reduced DRGFP-HR[268]. Interestingly, it was shown that aberrant HR was increased in RPA-phospho mutant cells, as measured by UV-induced sister chromatid exchanges (SCEs), which were increased in RPA2-A (S4/S8) mutant cells compared to RPA2-WT cells[231]. This effect has also been seen for DNA-PK-KD (kinase dead) but not DNA-PK null cells after HU[205].

The effect of RPA phosphorylation on unstressed DNA replication has also been studied. RPA2-D mutants and hypRPA have been suggested to be deficient in their association with replication centers in cells[241,253], and phosphorylation of RPA2 has been suggested to inhibit DNA replication[213,244]. HypRPA has also been shown to have deficient interactions with pol-alpha

primase[209,240], which would limit its role in replication. In contrast, other *in vivo* and *in vitro* studies have not seen deficient replication or localization of RPA to foci without damage[112,243,252,253,259]. While the effect of RPA phosphorylation on replication without damage is unclear, after DNA damage, it is generally accepted that RPA phosphorylation and dephosphorylation promote replication-associated repair[207].

RPA phosphorylation promotes RPA function in recovery from replication stress. HypRPA colocalizes with ssDNA after HU, supporting the role of RPA phosphorylation in replication stress recovery[249]. Further, phosphorylation mutants are deficient in recovery from stress. For example, RPA2-A (T21/S33) mutant cells are deficient in recovery from HU, with more RPA bound to chromatin, reduced BrdU incorporation, higher levels of ssDNA formation, and increased apoptosis compared to RPA2-WT cells[252]. Dephosphorylation is also needed, as DNA synthesis after IR is impaired in PP4R2-depleted, and RPA2-D (S8/S33 and S8/S23/S29/S33) mutant cells[230]. Additionally single molecule fiber analysis in RPA2-A (T21/S33 and S23/S29) mutant, RPA2-D (T21/S33) mutant, and PP4R2-depleted cells shows reduced synthesis at replication forks during HU-induced replication stress and recovery from stress compared to WT cells[236].

Some studies have shown different effects of RPA phosphorylation on replication recovery after stress. Feng et al found that RPA2-D (T21/S33) cells have normal RPA2 foci after damage, and have normal DNA replication levels after HU treatment, as measured by thymidine incorporation[112]; while there were elevated levels of radioactive thymidine incorporation after HU treatment in RPA2-A (T21/S33) cells[112]. This RPA2-A effect was argued to implicate RPA phosphorylation in the intra-S checkpoint. The discrepancy with other

studies listed above, which described reduced nucleotide analog incorporation (BrdU) in phospho-mutants, may be due to the use of a low concentration of HU, 0.2mM, compared to between 1.5 and 5 mM for the other studies. In a different study, Olson et al using flow cytometry analysis did not see a defect in replication resumption after 1mM HU release in RPA2-A mutant (S4/S8/S11/S12/S13/T21/S23/S29/S33) cells, though similar to Feng et al they saw an elevated levels of DNA synthesis by thymidine incorporation after UV treatment[241]. This may also be due to differing methods and treatment with UV as opposed to high concentrations of HU[241]. Another argument against the function of hypRPA in replication stress recovery could be made by the observation by Vassin et al, that an RPA pS4/S8 antibody did not colocalize to areas of BrdU incorporation after CPT treatment, but their analysis was not rigourous[253]. Nevertheless, it is clear that disruption of RPA phosphorylation and dephosphorylation does affect recovery from replication stress.

In line with defects in DSB repair and replication stress response, altered regulation of RPA phosphorylation leads to altered cell cycle distributions after DNA damage. Some groups have also implicated RPA phosphorylation directly in checkpoint responses, with phosphorylation mutants failing to stall the cell cycle to allow damage to be repaired. RPA2-A (S23/S29) mutant cells mutants have a delayed G2/M transition, delayed mitotic exit, and increased apoptosis[204,257]; RPA2-A mutant (S4/S8) cells have increased mitotic entry with unrepaired damage[231,233], and fail to arrest following replication stress, with defective G2/M arrest after ETO and cisplatin treatment, premature replication fork restart, failure to block late origin firing, and increased mitotic catastrophe[205]. PP4R2-silenced and RPA2-D mutant (S8/S23/S29/S33) cells have an extended G2/M checkpoint[230], though another

study found no effect of RPA phosphorylation on checkpoint and cell cycle distribution in RPA2-D (T21/S33) mutant cells[112]. In one report, replication-induced Chk1 phosphorylation was defective in DNA-PKcs and RPA2-A (S4/S8) mutants[233], which was argued to be because RPA2 S4/S8 phosphorylation regulates replication checkpoint signaling via MRE11 and TopBP1 phosphorylation, as an RPA2-A (S4/S8) mutant has reduced phosphorylation of MRE11 and TobBP1[233]. Other studies have found no effect on Chk1 phosphorylation in RPA2-A mutant cells, after HU or UV treatment[241].

RPA phosphorylation also regulates DNA repair by altering protein interactions. For example, RPA's interaction with the MRN complex is dependent on the regulation of its phosphorylation, although results of these studies are contradictory [245,267]. Robison et al found that RPA and MRE11 colocalize in response to HU and UV, both become phosphorylated, and a co-IP showed this interaction is abrogated by phosphatase treatment, suggesting that phosphorylation of these proteins promotes their interaction[245]. Oakley et al showed that purified RPA interacts with Mre11 and Nbs1 through the RPA1 N-terminus, but in this case a phosphomimetic RPA2 mutant is deficient in this interaction[267], while an alanine mutant had increased colocalization with MRN after etoposide[267]. The differences between these results may be due to the different damaging agents methods used, or because Robison et al used a phosphatase to look at the effect of phosphorylation which would be non-specific, while Oakley et al used purified proteins, phosphorylation mutants, and RPA bound to ssDNA for their IP. Thus, although the direction in which RPA phosphorylation affects its MRN interaction is unclear, it is likely that this interaction is somehow regulated by phosphorylation.

Another example of phosphorylation altering RPA-protein interactions involves the p53 tumor suppressor. Phosphorylation of RPA by DNA-PK and phosphorylation of p53 by ATR and ATM after DNA damage disrupts the p53-RPA complex, which is needed for HR repair[268]. Disruption of the release of the RPA-p53 complex reduces HR repair of DSBs, while phopshorylation of either protein alone shows no effect[268]. It was also shown that ssDNA and an RPA2-D mutant displace p53 in binding RPA1, leading to a model that after damage RPA2 phosphorylation results in the RPA2 N-terminus interacting with RPA1, displacing p53 and dissociating the p53-RPA complex[210].

Key to HR repair of DNA breaks are the RAD51 and RAD52 proteins, and phosphorylation also regulates RPA's interaction with these proteins. A co-IP showed that RPA's interaction with RAD51 and RAD52 after UV and CPT is promoted by hyperphosphorylation[255]. RAD51 and RAD52 have higher affinity for hypRPA in an *in vitro* binding assay as well[255]. hypRPA co-localizes with RAD52 in foci[255], while RAD52 focus formation is reduced in RPA2-A mutant cells[268]. An *in vitro* study further investigated the interaction of hypRPA and RAD52, and found that hyperphosphorylaiton of RPA promotes formation of a complex with monomeric RAD52 and causes transfer of ssDNA from RPA to RAD52[216]. The RPA-ssDNA-Rad52 complex was found to be more stable when it included hypRPA compared to unphosphorylated RPA, and more RAD52 crosslinks to ssDNA when the RPA in the complex is hyperphosphorylated[216]. Other studies have confirmed that hyperphosphorylation promotes RPA's interaction with RAD51: RAD51 preferentially co-immunoprecipitates with an RPA2-D mutant[230] and RAD51 *in vitro* preferentially pulls down hypRPA with little non-phosphorylated RPA2[255,268].

The HR protein PALB2 also interacts preferentially with hypRPA: hypRPA's ability to stimulate fork recovery has been suggested to be mediated through PALB2, and RPA phosphorylation increases localization of PALB2 and BRCA2 to RPA nuclear foci after replication stress[236]. hypRPA also recruited PALB2 to ssDNA *in vitro*[236]. Another protein involved in the regulation of repair, the ATR kinase, not only phosphorylates RPA but phosphorylation has been shown to improve its interaction with RPA: ATR binds more efficiently with hypRPA after damage, and hypRPA co-localizes with ATR in foci[255]. Thus, hyperphosphorylation of RPA regulates its role in DNA repair partially through regulating protein interactions.

A current model proposes that RPA undergoes a conformational change upon phosphorylation. Using scanning transmission electron microscopy, it was shown that formation of the extended RPA-30 nucleotide ssDNA complex correlates with increased phosphorylation[208]. Another study compared native and hypRPA using mass spectrometry, fluorescence spectroscopy, and limited proteolysis[235]. The authors found a conformational change upon hyperphosphorylation of RPA in which three residues in DBD-B of RPA1 were shielded in hypRPA,[235] leading to a model in whice the RPA2 N-terminus interacts with DBD-B of RPA1 in a phosphorylation dependent manner[235]. Other studies have supported this model that phosphorylation of the N-terminus of RPA2 results in RPA2 binding to RPA1, displacing RPA1 from ssDNA or other proteins. The binding of RPA2 to RPA1 would release the RPA1 N-terminus from ssDNA and then allow DNA damage response proteins to interact with RPA1 instead, or hypRPA2 may compete for binding of RPA1 with damage repair proteins to allow them to bind to other proteins. Supporting this hypothesis, the interactions with some proteins that interact

with RPA1 are altered after RPA is phosphorylated, including MRN, RAD9[322], p53, ATRIP and DNA-PK[210,240,245,248,267,322]. P53 binds to the N-terminus of RPA-1, and p53 is displaced by ssDNA and RPA2-D mutant protein, supporting this hypothesis[210]. A functional link between these two RPA domains was also demonstrated, as a phosphomimetic RPA2 mutant shares a similar defect in DNA duplex destabilization as a mutant with the RPA1 N-terminus deleted[206]. Further, NMR studies showed evidence for a direct interaction between the RPA1 N-terminus and phosphomimetic mutant RPA2 mutant[206].

*RPA regulation by phosphatases*

In addition to regulation of phosphorylation by kinases, dephosphorylation of RPA by phosphatases is also important for RPA regulation and DNA repair, as mentioned previously. The PP2A phosphatase has numerous substrates involved in DNA repair, replication and cell cycle progression[323-325]. Feng et al showed that RPA is dephosphorylated by PP2A phosphatase during recovery from HU[112]; the catalytic subunit of PP2A binds to RPA2 after damage and can dephosphorylate RPA2 *in vitro*[112]. Further, RPA2-T21/S33 phosphomimetic mutant cells had increased HU and UV sensitivity and persistent DNA damage foci of RPA and γH2AX[112]

The PP4 phosphatase also dephosphorylates RPA2. PP4C–PP4R2–PP4R3β forms the PP4 heterotrimeric complex, which is involved in the DSB response; PP4C and PP4R2 form a heterodimeric complex *in vivo* and *in vitro*, which then recruits PP4R3α or PP4R3β. Lee et al showed that PP4R2, the regulatory subunit, mediates RPA2 dephosphorylation by recruiting the PP4C catalytic subunit[230]. PP4 dephosphorylates RPA2 *in vitro,* and depletion of PP4R2 in cells alters the pattern of RPA2 phosphorylation, inhibits HR,

reduces RAD51 foci formation, extends the $G_2$/M checkpoint, and causes hypersensitivity to DNA damage[230]. Further, cells expressing a phosphomimetic RPA2-D mutant cells (S8/S33/S23/S29) are comparable in these defects to those depleted of PP4[230]. RPA2 interacts with PP4C and PP4R2 (not with PP4R3β) after treatment with CPT[230]. Silencing PP4C and PP4R2 increases RPA phosphorylation after CPT and IR, which is not dependent on H2AX, another target of PP4C[230], and these effects are not due to kinase activity of ATR or DNA-PK[230]. These data are consistent with a role of the PP4 phosphatase in regulating RPA2 phosphorylation. In agreement with this study, Murphy et al found that after treatment with CPT, cells deficient in PP4R2 showed an increase in levels of hyperphosphorylated RPA, with slowed replication fork movement during stress and recovery[236]. Thus, regulation not only by kinases but also by phosphatases through dephosphorylation is important in regulating RPA2 phosphorylation and DNA damage.

These findings are in line with similar studies that show that phosphatases regulate other proteins involved in DNA repair, replication, and cell cycle progression. PP4C dephosphorylates other critical DNA repair factors, including H2AX, KAP-1, and 53BP1[230,232,326-329]. PP4 has also been shown to be involved DSB repair through both HR and NHEJ[232].

Recent unpublished data from our lab suggest that BRCA1 controls RPA2 phosphorylation through phosphatases in response to replication stress. BRCA1-deficient cells have a marked reduction and delay in phosphorylated RPA2 expression after treatment with hydroxyurea, and a consequent reduction in the recruitment of pRPA2 to DNA damage foci. A constitutive association between BRCA1 and the catalytic subunit of PP2A *in vivo*

dissociates after DNA damage. Thus, RPA2 phosphorylation status is regulated by a BRCA1-PP2A complex that dissociates upon damage to promote RPA2 phosphorylation and coordinate timely HR. Taken together, these data suggest that regulation of RPA phosphorylation is regulated both by phosphorylation and dephosphorylation, and this switch is important in the regulation of DNA repair.

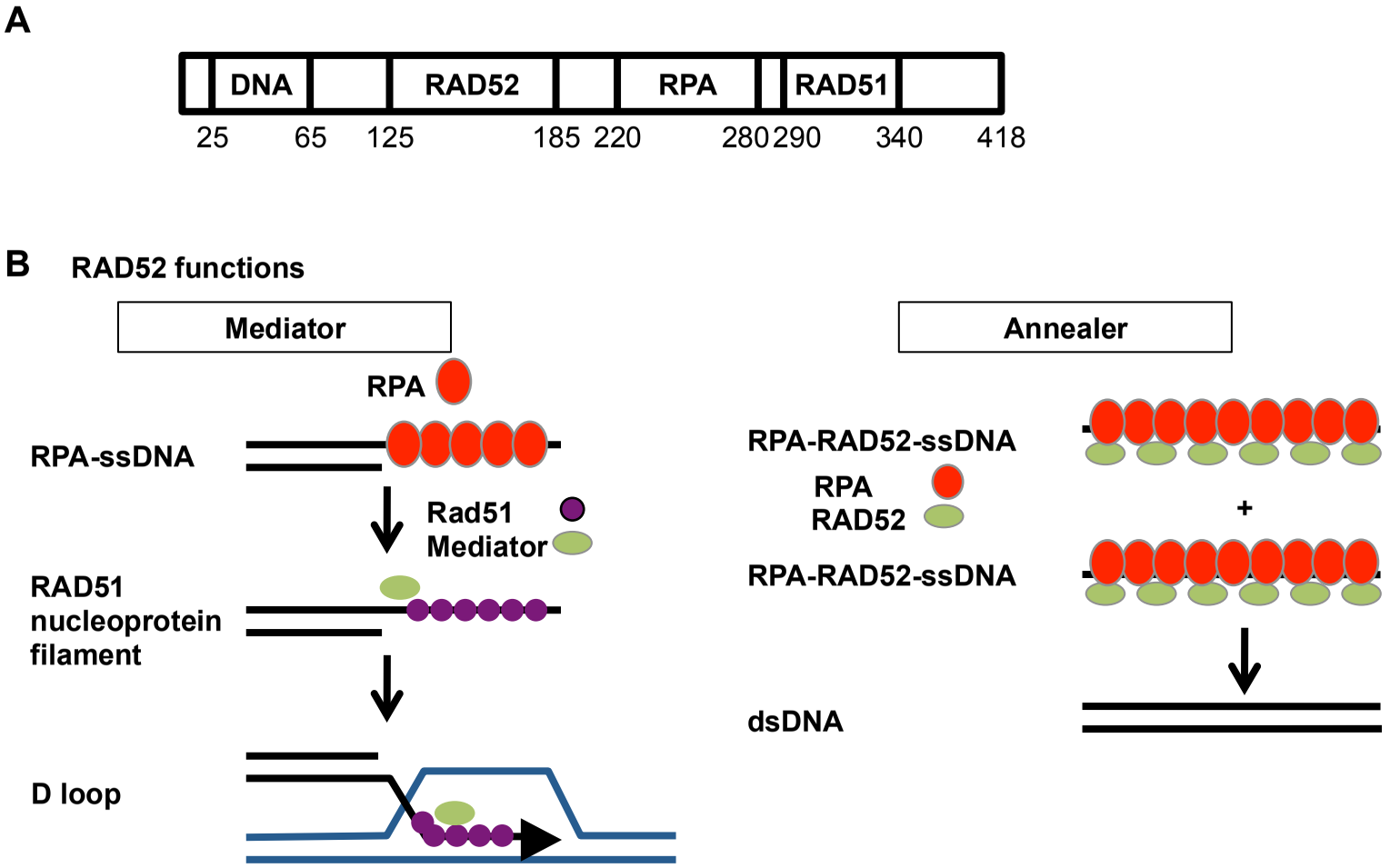
In summary, RPA is an essential protein in eukaryotes, as ssDNA is produced through numerous cellular processes. RPA plays a key role in the repair of DNA damage, and its interactions with both DNA and repair proteins allow it to regulate repair processes. RPA is itself regulated by phosphorylation, which is clearly important in the repair of DNA damage, but the mechanisms and precise regulation of RPA phosphorylation requires further clarification. Of interest to this thesis, is how RPA phosphorylation affects mediators of RAD51-homologous recombination. In the next section, we describe in more detail the RAD51 and the RAD51 mediators.

## RAD52 AND MEDIATIORS OF RECOMBINATION

RAD52 has two main functions (Figure 3). One is as an ssDNA annealer, base-pairing complementary ssDNAs. The other is as a mediater, facilitationg formation of RAD51 filaments on RPA bound ssDNA and promoting RAD51-exchange. While both human and yeast RAD52 proteins clearly anneal ssDNA *in vivo* and *in vitro*, RAD52's role as a mediator in humans and other vertebrates has been called into question. Following is a discussion of the key players in mediation in vertebrates: RAD51, BRCA2, and RAD52.

Two main *in vitro* assays are used to assess RAD51 function: the strand exchange assay and the D-loop assay. The strand exchange assay

**Figure 3: RAD52 domains and function. a.** RAD52 domains. In the N-terminal half of the protein, RAD52 interacts with DNA and itself to form higher order structures. In the C-terminal half, RAD52 interacts with RPA and RAD51, pointing to its role as a mediator. **b.** RAD52 functions. RAD52 acts as a mediator, promoting RAD51 filament formation on RPA-ssDNA, and strand invasion. RAD52 also acts as an annealer, base-pairing complementary ssDNA bound by RPA. **c.** Proposed annealing pathways. RAD52 annealing function may be important to Single-Strand Annealing (SSA), Synthesis-Dependent Strand Annealing (SDSA), or Second End Capture.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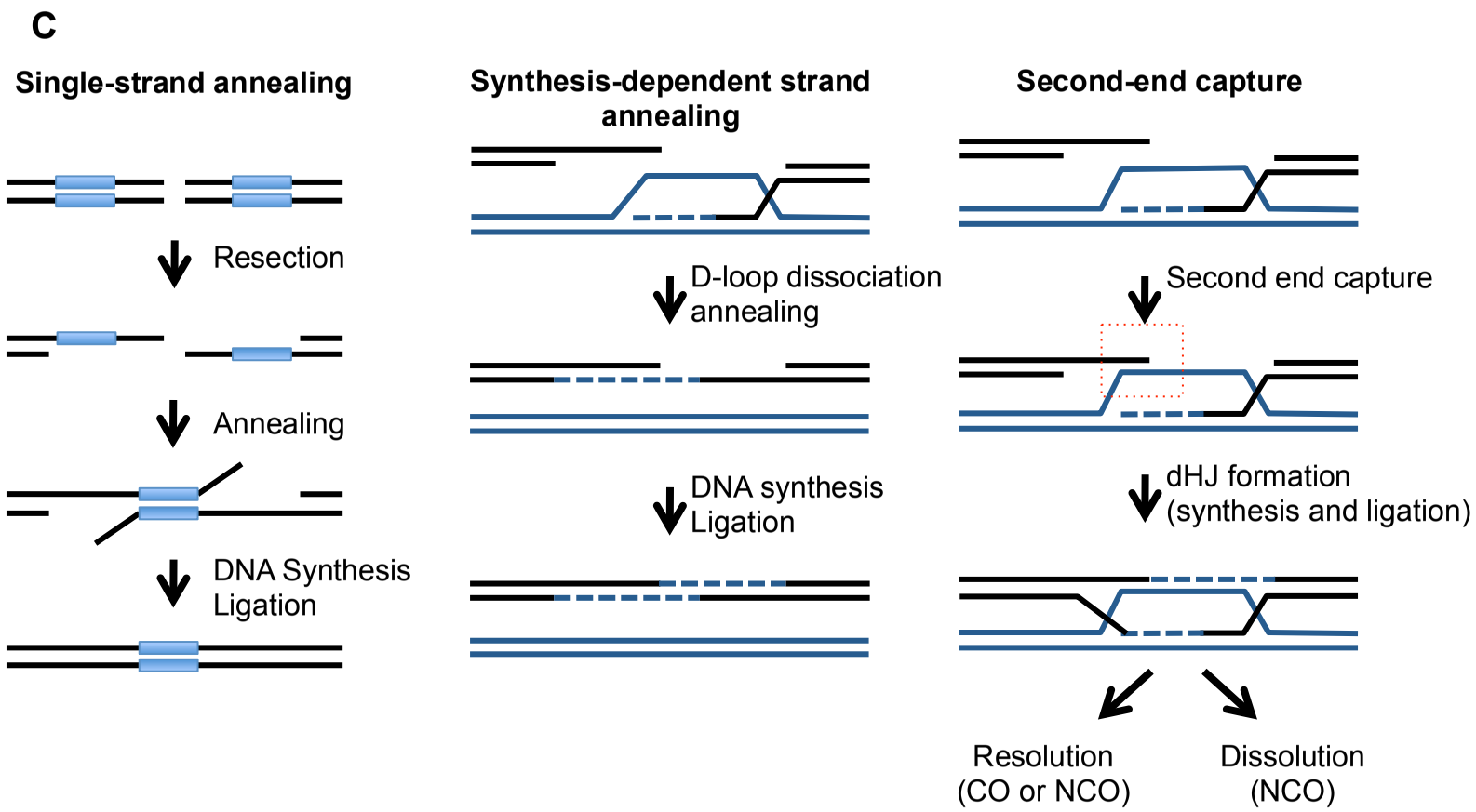



**Figure 3 (continued)**

takes ssDNA, or a duplex with an ssDNA overhang, and incubates it with a dsDNA duplex that contains a strand homologous to the ssDNA. Strand exchange, catalyzed by a recombinase, involves the ssDNA or overhang invading the homologous dsDNA duplex and displacing the bound complementary strand. Similarly, in the D-loop assay, ssDNA or an ssDNA overhang is added to a plasmid containing a region of homology to the ssDNA. D-loops are formed when the ssDNA invades the duplex, base pairing to the complementary region of the plasmid, and displacing the other strands.

**RAD51**

An essential protein for HR, the RAD51 recombinase is a homolog of RecA from bacteria. These recombinases form nucleoprotein filaments on DNA and catalyze homology search and strand exchange. RAD51 is needed for both mitotic and meiotic recombination, while DMC1, the other eukaryotic recombinase, is involved only in meiotic recombination. RAD51 loss is lethal early in mouse embryogenesis[40,330,331] likely due to its role in DNA repair in replicating cells through HR, and disruption of RAD51 in vertebrate cells leads to increases in chromosome aberrations. In cells, RAD51 forms repair foci at ssDNA sites in response to DNA damage[332]. Formation of a RAD51 filament leads to repair by HR and suppresses SSA and alt-NHEJ; SSA is demonstrably increased while HR is reduced in RAD51- and BRCA2-deficient cells[93,333,334].

The crystal structure of RecA filaments shed light on how strand exchange works[335] and this mechanism is likely conserved in eukaryotes. RecA-ssDNA with ATP forms a helical filament that binds to dsDNA, searches for homology, and then catalyzes the exchange of the complementary strand, producing a new heteroduplex. Chen et al found that ssDNA bound with

RAD51 has units of three nucleotides that are B-form in structure, and between these triplets the DNA is stretched. Homology search in the duplex is believed to occur by random collisions, and the complementary strand in the dsDNA interacts primarily through base pairing, so that heteroduplex formation is solely dependent on base-complementarity. ATP hydrolysis promotes the dissociation of the new heteroduplex DNA and the displaced strand. DNA in yeast and human RAD51 filaments is also extended significantly compared to B form DNA, which is thought to facilitate homology sampling, though the polarity of pairing and invasion are opposite that of RecA.

RAD51, like RecA, contains ATPase activity, though its ATP hydrolysis rate is slower. In comparison to RecA, hRAD51 protein also exhibits lower strand-exchange activity *in vitro*. For homology search and strand exchange, RAD51 ATP binding but not hydrolysis is required[336]. ATP hydrolysis is needed for dissociation of the recombinase from newly formed heteroduplex DNA. As a consequence, the use of a nonhydrolyzable nucleotide analogue[337], calcium ions[338], or a RAD51 mutants lacking ATPase activity[337], leads to the stabilization of the presynaptic filament and also enhanced recombinase activity *in vitro*. Turnover from ATP hydrolysis could promote recycling of recombinases within the cell, and make the primer in the D-loop available for DNA repair synthesis[339]. Mouse ES cells with an ATPase-dead (K133R) mutation of RAD51 have increased sensitivity to MMC and IR, reduced SCE efficiency, and defective HR[340]. Expression of an ATP-binding mutant of RAD51 causes a greater than 90-fold shift to SSA over HR, and expression of an ATP hydrolysis mutant of RAD51 resulted in more extensive gene conversion, which increases genetic loss during HDR[93]. These studies

demonstrate that although *in vitro* ATPase activity is not required for exchange activity, it is likely important for functional HR in cells.

Certain factors have been shown to affect RAD51 exchange activity. The effect of $Ca^{2+}$ of modulating its ATPase activity is unique to human RAD51[338]. When $Mg^{2+}$ is included in *in vitro* reactions, the hRAD51-ATP-ssDNA filament is quickly converted to an inactive hRAD51-ADP-ssDNA form, due to relatively rapid ATP hydrolysis and slow dissociation of ADP, while inclusion of $Ca^{2+}$ maintains the active hRAD51-ATP-ssDNA filament by reducing the ATP hydrolysis rate. The efficiency of *in vitro* recombination by hRAD51 protein can also be significantly enhanced by reducing RAD51 binding to dsDNA by addition of 100 mM ammonium sulfate[341]. Finally, RAD51 activity is stimulated by the presence of high salt, which favors co-aggregation of the nucleoprotein filaments with dsDNA, destabilizes RAD51 interactions with dsDNA, and causing a RAD51 conformational change that leads to extended filaments[342].

The formation of the RAD51 filament on ssDNA and D-loop intermediates are both reversible, through antirecombination and anticrossover pathways. The RAD51-ssDNA filament is reversible through yeast Srs2, and human proteins implicated in this function include FBH1, PARI, BLM, FANCJ, RECQ5. Nascent D-loops can be reversed through mismatch repair proteins, which reject D-loops when heterology is detected by these proteins. Extended D-loops can also be reversed, which promotes SDSA and NCOs. Yeast Srs2 and human RECQ5 have been implicated in this process, as have human RTEL1, FANCM, and BLM (yeast Sgs1).

RPA plays a role in the strand exchange reaction as well, both inhibiting and promoting the reaction. It is believed to prevent secondary structure

formation in ssDNA, and to bind the displaced strand preventing reversal of the reaction. However, in *in vitro* biochemical reactions, RPA added prior to RAD51 inhibits exchange, by inhibiting formation of RAD51 filaments on ssDNA. This can be overcome by mediators[157]. There are several mediators of RAD51 exchange in eukaryotes, including BRCA2 and RAD52.

**BRCA2**

BRCA2 is the primary mediator of RAD51 filament formation in humans and other vertebrates[157,158]. Mutations in BRCA2 cause a predisposition to cancer, meiotic defects, DNA damage sensitivity, defective replication and checkpoints, and increased choromosomal instability, evidenced by increased chromosomal breaks, translocations, exchanges and abnormal structures[39,343-346]. Hypomorphic biallelic mutations in BRCA2 cause Fanconi Anemia—BRCA2 is also known as FANCD1. Early studies showed that BRCA2 interacts with RAD51, is important for RAD51 recruitment to foci, and is important in HR[333,344,347-350]. Like loss of RAD51, deficiency in BRCA2 leads to reduced HDR and increased SSA[93]. BRCA2 also functions to stabilize stalled replication forks through RAD51[39].

BRCA2 is a large protein of 3418 amino acids[80]. At the N-terminus, BRCA2 interacts with PALB2. Between amino acids 1009 and 2083, BRCA2 contains eight BRC repeats, which are important for its interaction with RAD51. Closer toward the C-terminus lies its DNA-binding domain, consisting of a helical domain, three OB folds, and a tower domain, which facilitates BRCA2 binding to both ssDNA and dsDNA and interacts with DSS1. At the C-terminus is BRCA2's nuclear localization signal, as well as a CDK phosphorylation site that mediates its RAD51 interaction[80].

Protein partners of BRCA2 are important for its function in HR. PALB2 functions to facilitate BRCA2 mediation, as it stabilizes RAD51 binding to ssDNA and promotes the formation of D-loops[351,352]. PALB2 mutations are also associated with breast cancer, and biallelic mutations result in Fanconi Anemia. BRCA2 also relies on the DSS1 protein: BRCA2 mutants that do not bind DSS1 have HR defects, and DSS1 is thought to promote BRCA2 loading of RAD51 and BRCA2 stability[43,158,353].

The BRC repeats of BRCA2 mediate its interaction with RAD51. There is great variability in the number of BRC repeats between species. There is also poor sequence identity between BRC repeats within a species, and different BRC repeats are thought to have diverging functions. BRC1-4 bind free RAD51 at high affinity, reduce RAD51 ATPase activity, target RAD51 to ssDNA instead of dsDNA, promote RAD51 nucleation, and stimulate exchange[354]. The second set of BRC repeats, BRC5-8, lacks these activities, binding free RAD51 with low affinity but the RAD51-ssDNA filament with high affinity, stabilizing the filament and facilitating filament extension[354]. Interestingly, fusions of BRCA2 BRC repeats to RPA1 in BRCA2-mutant cells improved HDR and suppressed mutagenic repair through SSA, and also restored RAD51 foci formation[355]. This supports the idea that the main function of BRCA2 is in HR, specifically bringing RAD51 to DNA.

The C-terminal interaction of BRCA2 with RAD51 is regulated by CDK phosphorylation at S3291 at the $G_2$/M transition[356]. This interaction stabilizes RAD51 filaments. Interestingly, mutation of the S3291 site shows little or no DNA damage sensitivity or HR defect[44,357,358]. However, this site is thought to be important for BRCA2 to stabilize nascent DNA: RAD51 binding to this site

has been implicated in the protection of nascent strands at the replication fork[44].

Recently, several groups purified BRCA2 and characterized it biochemically[157,158,359,360]. They confirmed that BRCA2 binds RAD51 through its BRC repeats, binding up to six RAD51 proteins at a time[157,158]. Prior studies using BRC fragments, and the *U. maydis* and *C. elegans* orthologs of BRCA2 (Brh2 and BRC-2, respectively) suggested a role for BRCA2 in DSB repair through RAD51[344,348,355,361-368]. Reports of full length BRCA2 purification showed that BRCA2 prefers binding ssDNA to dsDNA, with a slight preference for tailed substrates[157,359]. Electron microscopy data showed that BRCA2 specifically recognizes dsDNA with ssDNA overhangs, but not blunt dsDNA ends. BRCA2 was found to form rod-shaped complexes, which based on their dimension and mass were suggested to be dimers[359]. Also, there was no complex of BRCA2-RAD51-ssDNA observed, suggesting that BRCA2 leads RAD51 to ssDNA and does not become a stable part of filament[359].

Based on these reports and previous studies, BRCA2 is proposed to have four main mechanisms of promoting RAD51 function[360]. First, BRCA2 prevents RAD51 binding to dsDNA, which inhibits exchange[157,359]. Second, it stimulates binding of RAD51 to ssDNA or to dsDNA with ssDNA tails[157,359]. Third, it enables RAD51 to bind ssDNA in presence of RPA[157,158,359]. And fourth, BRCA2 inhibits RAD51-ssDNA dissociation by inhibiting ATP hydrolysis[157,158]. While other mediators, such as Brh2 in *U. maydis* and RecFOR in bacteria, have been shown to localize recombinase filaments to the ssDNA/dsDNA junction, this was not observed for BRCA2[368-370].

In one of the reports of purified full-length BRCA2, Jensen et al showed that BRCA2 binds RAD51, DMC1, and yRad51, but not RecA, RPA, SSB, or

RAD52. They showed that BRCA2 stimulates strand exchange by RAD51, at an optimal one to three RAD51:nucleotide ratio. In the exchange assay, when RAD51 was added to the tailed substrate and dsDNA at the same time, rather than being incubated with the ssDNA tail first, there were only background levels of exchange. However, the addition of BRCA2 promoted exchange in these conditions, confirming that BRCA2 directs RAD51 to ssDNA, or limits its binding to dsDNA, or both. This effect is even seen in the presence of RPA added at same time as the other proteins, though this reduces levels of exchange. At a concentration that saturates both ssDNA and dsDNA with RAD51, exchange is normally inhibited because of RAD51 binding the dsDNA, even when RAD51 is first incubated with ssDNA and then the dsDNA is added; BRCA2 also stimulated exchange in this case (in these assays RPA was not included). The authors further showed this ability of BRCA2 to direct RAD51 to dsDNA by EMSA analysis[157].

In exchange assays where the ssDNA representing the resected strand is incubated with RPA prior to the addition of RAD51 and BRCA2, BRCA2 promoted RAD51 exchange[157]. It mediated exchange when the ssDNA was 3' tailed and 5' tailed equally, but did not mediate exchange with untailed ssDNA quite as well. BRCA2 stimulated RAD51-exchange up to twenty fold, but it did not stimulate RecA exchange, suggesting its interaction with RAD51 is important for this function and is species specific. BRCA2 lacking polarity in mediation of exchange differs from Brh2 and RecFOR[368,371,372]. Replacing RPA with SSB did not affect BRCA2 stimulation; this observation, in conjunction with the data showing that BRCA2 does not interact with RPA, implies that BRCA2 does not need to interact with RPA to facilitate exchange[157].

Jensen et al also looked at the differences between BRCA2 and RAD52. While *U. maydis* and *C. elegans* BRCA2 orthologs can anneal ssDNA in presence of RPA[365,373] BRCA2 did not anneal RPA-coated ssDNA—in humans this function appears to be specific to RAD52. Although scRAD52 promotes scRAD51-exchange on scRPA-ssDNA, both hRAD52 and scRAD52 did not mediate exchange with hRPA and hRAD51, while BRCA2 did. This suggests this mediation activity is species-specific, and is primarily carried out by BRCA2 in humans[157]. In the next section, we discuss the role of RAD52 in humans, which despite its inability to promote RAD51 filament formation on RPA-ssDNA in these assays, still plays a role in HR.

**RAD52 phenotypes**

hRAD52 was initially identified as a homolog of RAD52 in *S. cerevisiae*, which was known to be required for HR repair of DSBs. The human RAD52 gene is 418 amino acids (mouse is 420), sharing 30% identity and 58% similarity amino acid sequence with scRAD52[374], primarily in the N-terminal region of the protein (human and mouse share 69% identity and 80% similarity) (Figure 3). The N-terminus is responsible for binding to DNA and for forming oligomers. Toward the C-terminus, where the human and yeast sequences diverge, RAD52 interacts with RPA and RAD51. Low amounts of RAD52 RNA were observed in adult mouse tissues, while a relatively high level of gene expression was observed in the testis and thymus, suggesting that the mammalian RAD52 protein, like its homolog from yeast, plays a role in recombination[374].

*S. cerevisiae* RAD52 is known to mediate the exchange of RPA for RAD51, promoting RAD51 filament formation on RPA-coated ssDNA and stimulating RAD51 strand invasion[155,156]. It is known to bind DNA[375], RAD51[376],

RPA[377,378], to target Rad51 to ssDNA[379], and to facilitate RPA displacement by RAD51[380]. Deletion of RAD52 leads to severe DSB repair and meiosis defects in yeast. *S. cerevisiae* RAD52 not only promotes Rad51 filament formation, but also functions in RAD51-independent ssDNA annealing for second-end capture, SDSA, and SSA, which is likely why yeast *rad52* mutants have more extreme phenotypes than *rad51* mutants[180].

Despite their similarities, RAD52 does not have a strong HR phenotype in vertebrates, as it does in yeast[93,381], which is likely due to the presence of BRCA2. In organisms that do have both a BRCA2 homolog and RAD52, like *U. maydis*, chicken, and mice, RAD52 disruption also leads to limited or no defects in HR or DNA repair; interestingly, *C. elegans* do not have RAD52 homolog, only containing BRCA2[159,160,382]. Nevertheless, there are some effects of RAD52 in cells and organisms, suggesting it does indeed play a role.

RAD52 knockouts in chicken and mice failed to show a strong phenotype[159,160]. RAD52 deletion mutants of the chicken B-cell line DT40 were not hypersensitive to DNA damage induced by IR, MMS, or cisplatin, and there was no effect on growth rate and cloning efficiency[160]. Furthermore, intrachromosomal recombination, measured by immunoglobulin gene conversion, and IRIF of RAD51, were unaffected in RAD52-deleted cells compared to WT cells[160]. Targeted integration frequencies, however, were consistently reduced in RAD52-deleted cells, showing a clear role for RAD52 in genetic recombination[160]. This phenotype was noted to be similar to that of the yeast *S. pombe*, where mutation of RAD51 and RAD54 leads to hypersensitivity to radiation and targeted integration deficiency, but deficiency in the RAD52 homolog rad22 has a less severe defect[383]. Similar to the chicken cells, mouse RAD52 mutants lack a strong HR phenotype[159]. RAD52-

deleted embryonic stem (ES) cells were not hypersensitive to DSB agents, and RAD52-null mice showed no abnormalities in viability, fertility, and the immune system, calling into question the importance of RAD52 in HR[159]. However, inactivation of RAD52 in mouse ES cells did cause a reduced frequency of HR measured by gene targeting[159]. Finally, I-SceI reporter constucts in RAD52-null mouse cells show no detectable HDR defect, although SSA is dependent on RAD52[93]. In sum, these data suggest that though RAD52 is not critical to DSB repair in chicken and mouse, it does still play some role in HR and is important in SSA.

Further supporting some role in the HR pathway despite RAD52's lack of severe phenotype, RAD52 still interacts with key players in HR. Several studies showed RAD52 interacts with RAD51 by IP, colocalization, and *in vitro* assays[298,376,381,384-386], and it interacts with RPA1 and RPA2 as described previously[292-294,298]. These interactions map to the C-terminal half of RAD52 (Figure 3), where it does not share homology with yeast Rad52, implying the interactions, and possibly the functional significance of these interactions, are species specific.

Studies of RAD52 overexpression have shown effects on HR readouts, again supporting a role for RAD52 in homology-directed DNA repair. Early studies showed that overexpressing RAD52 in monkey cells conferred resistance to IR and increased levels of HR[387], and that RAD52 mutants that lacked its interaction domain with RPA (amino acids 221-280 of RAD52) failed to enhance HR in monkey cells[298]. Mouse cells overexpressing a RAD52-GFP fusion show both increased survival and an increased number of RAD51 foci after MMS and IR[384], and RAD52 overexpression has also been found to

inhibit gene targeting and stimulate HR between separate transfected plasmids[388].

Double mutants of RAD52 with other HR factors have also pointed to a role for RAD52 in HR. In chicken DT40 cells, conditional mutants deficient in both RAD52 and XRCC3 are non-viable and have extensive chromosomal breaks, whereas rad52 and xrcc3 single mutants grew well[389]. Double mutants of RAD52 and RAD54 in mice have normal haemopoietic depression in bone marrow and micronuclei formation with MMC treatment, but a deficiency of RAD52 exacerbates the MMC survival of RAD54 mutant mice and also has a distinct effect on the survival of bone marrow cells after exposure to IR[390]. As will be discussed in more detail later, RAD52 is also synthetically lethal with BRCA2, BRCA1, PALB2, and the RAD51 paralogs, and acts independently of these proteins[162,391,392].

RAD52 is regulated by post-translational modifications in yeast and humans. Studies in yeast have shown that RAD52 is regulated by sumoylation, which is induced by DNA damage and involved in regulating RAD52 stability and activity[393-396]. Similarly, hRAD52 associates with the sumo-conjugating enzymes UBL1 and UBE2I in yeast two hybrid assays[397,398]. Further, in human cells, PTEN was shown to interact with RAD52 by IP and colocalization, and also colocalized with γH2AX, and depletion of PTEN reduced RAD52 sumoylation[399], supporting that RAD52 in humans is also regulated by sumoylation. RAD52 is also regulated by tyrosine phosphorylation; phosphorylation reduces RAD52 affinity for dsDNA and increases its ssDNA annealing rate, overcoming the inhibition of dsDNA on annealing[400]. This site is likely phosphorylated by c-abl[401] in response to IR, and is important for RAD52 foci formation in response to IR.

RAD52 foci are induced and immobilized to a greater extent by HU, which stalls replication forks, than by IR, suggesting its role is related to replication damage[386]. Interestingly, RAD52 has been shown to interact with MUS81 and to regulate its role in replication stress[402]. MUS81 generates DSBs during replication in checkpoint deficient cells, but the mechanism and its effects are not well understood. MUS81-induced DSBs, when CHK1 is inhibited, are independent of RAD51, and instead depend on RAD52. Depletion of RAD52 rescues chromosome instability after replication fork stalling in CHK1-deficient cells, and leads to the ability of stalled forks to restart and to more *de novo* origin firing in these conditions. Recovery from replication stress in RAD52-depleted cells requires MUS81, and loss of both these proteins results in increased RAD51 foci formation that is toxic. Thus, a RAD52/MUS81-dependency exists in checkpoint-deficient cells, and this suggests that RAD52 is associated with cleavage at stalled forks *in vivo* and *in vitro*[402].

RAD52 has also been suggested to interact with helicases in cells. WRN, a RECQ homolog, interacts physically with RAD52 *in vivo* at arrested replication forks and *in vitro*[403]. Biochemically RAD52 both inhibits and enhances WRN helicase activity, while WRN increases RAD52-mediated strand annealing[403]. RAD52 also interacts with human RECQ5 to promote SDSA; Paliwal et al show that the RECQ5 helicase, which can disrupt RAD51 filaments, promotes NCO during DSB repair through HR[168]. It counteracts the inhibition by RAD51 on RAD52 annealing *in vitro* and *in vivo*, and deficiency in RECQ5 leads to increased occupancy of RAD51 at DSBs[168]. RECQ5 deficiency causes elevated levels of SCEs in untreated cells when Holliday junction dissolution is impaired (by BLM deficiency), and in response to CPT

RECQ5-deficiency alone elevates SCEs, while depleting both BLM and RECQ5 increases SCEs even more[168]. These data suggest that RECQ5 has a role in CO suppression even in the presence of BLM if the load of DNA damage exceeds a certain threshold, and that RECQ5 and BLM act in two different pathways to suppress CO formation during HR. The authors suggest that RECQ5 acts in SDSA post-synaptically to prevent aberrant RAD51 filament formation on the extended invading strand, thus limiting crossovers. They also show a role for RAD52 in this pathway. Including an ATP-hydrolysis dead RAD51 mutant before RAD52 *in vitro* inhibits RAD52 annealing of RPA-ssDNA, and wild-type RAD51 also inhibits annealing but to a lesser extent. This inhibition was lost when RECQ5 was included, likely by disrupting RAD51 filaments since RECQ mutants (helicase deficient and RAD51-interacting mutant) did not alleviate inhibition; WRN and FBH1 also did not relieve inhibition[168].

Interestingly, alternative splice variants of hRAD52 have been identified that code for RAD52 proteins containing mostly the conserved N-terminal half[404-406]. Expression of two mouse RAD52 splice variants in CHO tissue culture cells elevates the frequency of recombination that uses a sister chromatid template[406]. A yeast homolog (Rad59) is also composed mostly of this domain, yeast do not contain splice variants. It has been suggested that RAD52 splice variants could function like yeast Rad59 in vertebrate cells[404-406].

In summary, the role of RAD52 in vertebrate cells and organisms is not well understood. Its function as an annealer has been shown to be important in the SSA pathway, and is also potentially important for SDSA and second end capture downstream in HR (Figure 3). Its function as a RAD51 mediator is likely only important in the absence of the BRCA pathway, as will be discussed

in more detail later on. Studies into the *in vitro* biochemical function of RAD52 help shed light on its role in cells; the next section focuses on these studies.

**RAD52 structure and biochemistry**

As described previously, RAD52 binds RPA and RAD51 through domains in the C-terminal half of the protein. At its N-terminus are the RAD52 DNA binding and self-association domains, where it contains 70% homology with the yeast protein, and these also catalyze homologous DNA pairing. From the N- to the C-terminus are the DNA-binding domain (residues 25–65), the RAD52 heptameric ring binding domain (125–185), the RPA32 binding domain (220–280), and Rad51-binding domain (290–340)[292](Figure 3). Some studies suggest while the N-terminal self-association domain mediates the assembly of monomers into rings, a C-terminal domain of the protein mediates higher order self-association of the rings[407,408]. RAD52-DNA filaments are proposed to contain either stacked rings or rings arranged side by side[409].

Looking at RAD52 rings by electron microscopy, it was shown that RAD52 forms rings with 10nm diameter and a hole at the center[410]. ssDNA-hRAD52 complexes have RAD52 distributed along the length of the DNA with a beads on a string appearance, and at higher RAD52 concentrations, “super rings” of 30nm are observed with the ssDNA collapsed on itself[410]. These rings were shown using transmission electron microscopy and scanning transmission electron microscopy to be heptameric with a large central channel[411]. RAD52 binds DNA non-cooperatively, and in contrast to how it binds ssDNA, dsDNA-RAD52 has some protein free regions[410]. RAD52 is proposed to bind four nucleotides per monomer of ring[412-414].

Crystal structures of N-terminal RAD52 truncations have been analyzed. These truncated forms of RAD52 form an undecameric ring with a

highly positively charged groove around the ring and extensive subunit contacts[412,413]. Though the truncations form an undecamer and full length RAD52 forms a heptamer, both rings are about the same size[407,411,412]. Each RAD52 monomer has a β-β-β-α fold[412], and a mutational analysis revealed that the amino acid residues located between the β-β-β-α fold and a hairpin loop are essential for ssDNA and dsDNA binding. Amino acids 79-156 make up a stem region of the β-β-β-α fold, while residues flanking both ends of the stem form a domed cap with a flat top[412]. Negative charges are found at the top of the domed cap, near the channel of the ring, while most of the bottom half of the ring is positively charged[412]. The region between the stem and the loop in the RAD52 monomer is most positively charged and aligned outside the ring[412]. The central channel of RAD52 does not contain any basic residues, suggesting RAD52 binds DNA along outside of the ring, and not within the channel[412]. A study of the crystal structure of the N-terminus identified a potential second DNA binding site in hRAD52[415]. This new binding sites includes residues from Lys102 to Arg173, which are exposed on the surface of the crystal structure of the N-terminal domain of RAD52. It is located at the rim of the stem region and edge of domed cap region, while the earlier established sites are clustered at the bottom of the groove between the stem and domed cap regions[415]. Singleton et al[413] propose that the large, positively charged groove that runs along the surface of the ring suggests a mechanism by which RAD52 presents the single strand for reannealing with complementary ssDNA, with ssDNA bound to RAD52 having bases facing outward, and RAD52 forming a ternary complex with ssDNA and dsDNA[413].

This model is supported by a report from Van Dyck et al, who show using tailed duplex DNA molecules that purified hRAD52 protein binds

resected DSBs and promotes associations between complementary DNA termini[416]. Heteroduplex intermediates of these recombination reactions visualized by electron microscopy reveal the specific binding of multiple rings of RAD52 to the resected termini and the formation of large protein complexes at heteroduplex joints formed by RAD52-mediated annealing[416]. This study suggests that RAD52 rings are active in SSA, and strengthens the argument that SSA is driven by RAD52-directed DNA–DNA contacts in which ssDNA lies exposed on the surface of the protein[416].

hRAD52 binds ssDNA and dsDNA, though it does not bind dsDNA as well as ssDNA[410,417,418]. In one study, hRAD52 was found to bind preferentially to the ends of duplex DNA with 300 nucleotides of 5' or 3' tails, and was suggested to have a higher affinity or greater stability on tailed dsDNA[419]; thus, RAD52 binds structures found at DSBs. Mutational analysis with DNA binding assays suggested that specific aromatic and basic side-chains, R55, Y65, F79, Y81, K152 and R156, are most important for mediating the interaction of hRad52 with DNA, which mostly agrees with the crystal structure[420]. A study looking at truncation mutants (amino acids 1–212, blocking higher order oligomers and 1–85, blocking 10nm ring formation) suggested that DNA binding depends on neither ring-shaped oligomers nor higher order oligomers, but that formation of oligomers consisting of multiple RAD52 rings is important for activities involving simultaneous interaction with more than one DNA molecule[408].

Parsons et al[414] showed that RAD52 binds ssDNA and tailed duplex DNA molecules via precise interactions with the terminal base. DNA in ssDNA-RAD52 complexes exhibit a four-nucleotide repeat hypersensitivity pattern when probed with hydroxyl radicals, which is due to the interaction of RAD52

with either a 5' or a 3' terminus of the ssDNA, and is sequence independent and phased precisely from the terminal nucleotide[414]. This hypersensitivity is observed for 36 nucleotides, consistent with the length of DNA that is protected by RAD52 in nuclease protection assays. The authors propose that RAD52 binds DNA breaks via specific interactions with the terminal base, leading to the formation of a precisely organized ssDNA-RAD52 complex in which the DNA lies on an exposed surface of the protein[414]. This protein-DNA arrangement may facilitate the DNA-DNA interactions necessary for RAD52-mediated annealing of complementary DNA strands[414].

As mentioned previously, RAD52 has been shown to be important for annealing *in vivo*, and studies also show that RAD52 is effective at annealing *in vitro*. Unlike BRCA2, RAD52 is able to overcome RPA inhibition to anneal ssDNA[157]. RAD52 annealing has been suggested not only to be important in SSA, but also may play a role in second end capture, SDSA, or BIR, which is supported by studies in yeast and with human protein[177,421].

Early studies demonstrated that RAD52 is efficient at annealing complementary DNAs *in vitro*[413,416,418]. It has been shown that RAD52 promoted annealing can be followed by branch migration, displacing another strand downstream[418]. As described earlier, purified hRAD52 binds tailed duplex DNA molecules and promotes associations between complementary DNA termini, with annealing likely driven by RAD52 rings directing DNA-DNA contacts with the ssDNA exposed on the surface of the protein[416]. Interestingly, tyrosine phosphorylation reduces RAD52 affinity for dsDNA and increases the ssDNA annealing rate, overcoming annealing inhibition by dsDNA[400] and WRN increases RAD52-mediated strand annealing[403]; thus, there is complex regulation of RAD52 annealing.

More detailed studies shed light on the mechanism of RAD52 annealing. Rothenberg et al[422] proposed that RAD52-annealing proceeds in successive steps involving rearrangements of the ssDNA–hRAD52 complex. After initial pairing, further search for extended homology occurs without dissociation of DNA and RAD52, which is driven by the interaction of two overlapping nucleoprotein complexes. The authors propose a model in which ssDNA release and dsDNA zippering are coordinated through successive rearrangement of overlapping nucleoprotein complexes[422]. Another study of annealiny by Grimme et al[295] studied RAD52-annealing using fluorescence resonance energy transfer (FRET). They found that RAD52 bound ssDNA or ssDNA-RPA in two modes: at low RAD52 concentration, the ssDNA wrapped around circumference of protein ring, while at high RAD52 concentration, the ssDNA was stretched between multiple RAD52 rings[295]. In annealing assays where RPA was included, RAD52 mutants with impaired RPA binding (RQK/AAA (261-263) and 1-212 truncation) competed with RPA for ssDNA binding and failed to counteract RPA duplex destabilization; the rate and extent of annealing was reduced in these mutants. In annealing assays without RPA, the rate and extent of annealing increased with RAD52 concentration up to 8nM, beyond that the rate of annealing was reduced but the extent remained the same; in this scenario the RAD52-RQK mutant behaved similarly to RAD52-WT, while the rate was reduced with the 1-212 truncation though the extent was similar to RAD52-WT. ssDNA annealing was fastest when the concentration of DNA molecules used was the one in which it is wrapped around individual RAD52 protein rings (low concentration), suggesting that optimal annealing occurs at conditions where both strands are bound by RAD52 and that homology search and annealing occur through two

RAD52-ssDNA complexes or two hRAD52-ssDNA-RPA complexes rather than a RAD52-ssDNA(-RPA) complex and free ssDNA[295].

Studies of human RAD52 have suggested that it can also promote second-end capture and SDSA. In a reconstituted system, RAD54 can cause dissociation of joint molecules through its ATP-dependent branch migration activity[423]. In a reaction analogous to SDSA, RAD52 anneals the extended invaded strand after it dissociates from the D-loop with a second complementary tail, though it is dispensable for this activity in the absence of RPA[423]. RAD52 can also promote formation of a "double D-loop," annealing the displaced strand of a D-loop to another complementary ssDNA, which would be analogous to second end capture/dHJ formation of DSBR[423]. RAD54 was capable of dissociating double D-loops as well[423]. Additionally, DNA repair synthesis catalyzed by human DNA polymerase η (which has been suggested to play a role in HR repair) acting upon priming strand of a D-loop leads to capture and annealing of the second end of a resect DSB, and this reaction is mediated by RAD52[176]. This second-end capture was not seen with other polymerases, or RAD51 instead of RAD52. The RAD52-dependent reaction is stimulated by RPA, and following repair synthesis and second-end capture, *de novo* synthesis from the captured second end occured[176]. Taken together, these studies suggest that RAD52 may function in cells to anneal during SDSA and second end capture.

While it is clear biochemically that RAD52 functions as an annealier, the role of RAD52 as a mediator of RAD51 filament formation and strand exchange in humans and other vertebrates is controversial. The prevailing view seems to be that DNA strand exchange by hRAD51 is not affected by RAD52 when RPA is present[157]. RAD52's relative unimportance as a mediator

is consistent with no or mild effects of RAD52 deficiency[159,160]. Nevertheless, as there is some recombination phenotype *in vivo*, RAD52 has also been shown to have some activity in biochemical reactions of exchange and strand invasion.

hRAD51 promotes homologous pairing and DNA strand exchange *in vitro* on its own in certain conditions[424-426]. A one to three, RAD51 to nucleotide ratio is optimal for exchange[425]. hRAD51 initiates joint molecule formation preferentially at the 5' end of the complementary strand of the linear duplex, and heteroduplex DNA is subsequently formed by the 5' to 3' transfer of this strand to the ssDNA[425]. Thus, the heteroduplex forms with a 3' to 5' polarity (polarity of transfer is defined relative to ssDNA on which nucleoprotein filament assembled), which is analogous to scRad51 but opposite to RecA[425]. RAD51 reactions are stimulated by RPA at low RPA concentrations when RPA is added before RAD51[425]. However, hRAD51 requires a mediator to overcome inhibition by RPA added before RAD51 at higher concentrations, and to prevent RAD51 binding to dsDNA, which inhibits the reaction. At sub-optimal RAD51 concentrations (1:6 RAD51:nucleotide) few joint molecules are formed by RAD51 in the absence of RPA, but preincubation with low concentrations of RPA prior to RAD51 addition stimulates efficiency of joint molecule formation, while high RPA concentrations inhibit exchange[427]. However, at a one to three RAD51 to nucleotide concentration, there is no stimulation by low concentrations of RPA[427]. There is also a stimulatory effect at suboptimal RAD51 concentrations with bacterial SSB, and there is RAD51 stimulation at low RPA concentration with optimal RAD51 concentration at low temperatures (0 and 22 degrees); these data suggest that RPA stimulates RAD51 exchange by removing secondary structure[427]. Another study by

McIlwraith et al[428] also shows that RAD51 stimulates D-loop formation, though less efficiently than RecA, and requires magnesium and ATP to do so. They also found that RPA (added after RAD51) does not stimulate the reaction when RAD51 is added in excess, but RPA did stimulate D-loop formation when using sub-optimal Rad51 concentrations. At higher RPA concentrations, RPA inhibited the reaction[428]. Thus, there is a complex relationship between RPA and RAD51 in strand exchange and D-loop formation.

Some studies have found that RAD52 can stimulate strand exchange or D-loop formation on its own when incubated with ssDNA before the addition of dsDNA, and at concentrations of RAD52 that saturate the ssDNA, and this activity was localized to the N-terminus of RAD52[385,409,412,415,429]. Other studies have disputed this observation[428]. The effect of RAD52 is possibly executed by its annealing activity, but it has not been shown to overcome RPA inhibition. The *in vivo* relevance of these observations is not clear.

Stimulation of RAD51 activity by RAD52 using human proteins has been observed when RPA is not included, or when RAD51 is added at suboptimal concentrations[157,417]. At substoichiometric RAD51 concentrations (1:6 RAD51:nucleotide), RAD52 stimulates joint molecule formation by RAD51; this effect is greater when RAD52 is incubated with the ssDNA before the addition RAD51 than when the proteins are added simultaneously or RAD51 is added first. Preincubation of RAD51 and RAD52 before adding ssDNA is worse than adding RAD51 alone, suggesting that in this case the RAD52 may sequester the RAD51 from DNA[417].

Using stoichiometric amounts of RAD51, RAD52 is inhibitory to hRAD51 joint molecule formation (2.1uM RAD52:10uM RAD51, no inhibition observed at 0.6uM RAD52)[427]. Adding RAD52, then RPA at low

concentrations (0.3-0.6 uM RPA), then RAD51 (10uM), then labeled duplex, removed this inhibition[427]. However, at higher RPA concentrations (1.2uM) adding RPA inhibits the reaction even more than RAD52, and RAD52 does not overcome this inhibition[427]. Adding excess heterologous dsDNA also removes the RAD52 inhibition, which was proposed to be due to the prevention of RAD51 binding to the dsDNA. Including excess heterologous DNA also allowed RAD51 and RAD52 preincubated together to form joint molecules[417,427]. RPA and RAD52 do not stimulate RAD51 D-loops when RAD51 is added in excess, but they do stimulate D-loop formation when using suboptimal RAD51 concentrations[428]. The N-terminal fragment of RAD52 (1-209), using suboptimal conditions, also stimulates RAD51 in presence of RPA and ammonium sulfate[413]. Thus, RAD52 cannot overcome RPA inhibition of RAD51 exchange, but it does stimulate RAD51 activity at suboptimal conditions.

One study found that there is higher homologous pairing activity in presence of both hRAD51 and hRAD52 than either alone using suboptimal RAD51 concentrations[385]. A mutant of RAD51 that does not interact with RAD52 reduced homologous pairing in these conditions, but has normal activity compared to WT-RAD51 in the absence of RAD52. This suggests that the interaction between these two proteins is important for RAD52 stimulation when it is present[385].

It has been suggested that RAD52 may promote RAD51 binding to ssDNA[430,431]. Interestingly, RAD52 binds RAD51-ssDNA filaments by EMSA analysis and by gold antibodies in electron microscopy[410]. At saturating concentrations of RAD51, it was able to displace RAD52 from ssDNA, but dsDNA-RAD52 was more resistant to RAD51 nucleoprotein filament

formation[410], suggesting that RAD52 could potentially direct RAD51 to ssDNA in some conditions.

Stimulation of RAD51 exchange and D-loop formation by RAD52 has been observed in some cases, but RAD52 has not been observed to remove facilitate RAD51 filament formation on RPA-ssDNA, an important step *in vivo*. Furthermore, mutants of RAD52 do not have strong HR phenotypes. As a result, RAD52-RAD51 mediator function in humans and other vertebrates is not believed to be essential to HR, but its residual activity is likely still biologically relevant, particularly in the context of BRCA deficiency, as will be described in the following section.

**RAD52-BRCA synthetic lethality**

hRAD52 can anneal *in vivo*[93] and *in vitro*[157] but RAD52 deficiency does not cause phenotype expected of an protein important for HR repair, like hypersensitivity to DNA damage or depletion in HR, and RAD52 is inefficient at promoting RAD51-exchange *in vitro*. *S. cerevisiae* Rad52 mutants have more severe defects than Rad51 mutants, believed to be due to its dual role as a mediator and an annealer. It appears that the functions of scRad52 have split between BRCA2, which can mediate and not anneal, and hRAD52, which can anneal and not mediate effectively[161]. The lack of strong phenotype in vertebrate RAD52 mutants suggests that annealing is not important to homology-directed DSB repair, or that there is some redundancy or way to compensate for the loss of RAD52.

Despite RAD52's apparent lack of mediator activity in humans, recent evidence suggests that hRAD52 provides an alternative mediator pathway to BRCA2 (Figure 4)[161,162]. In BRCA2-deficient human cancer cell lines, depletion of RAD52 reduces cell survival and proliferation, thus there is a synthetic lethal

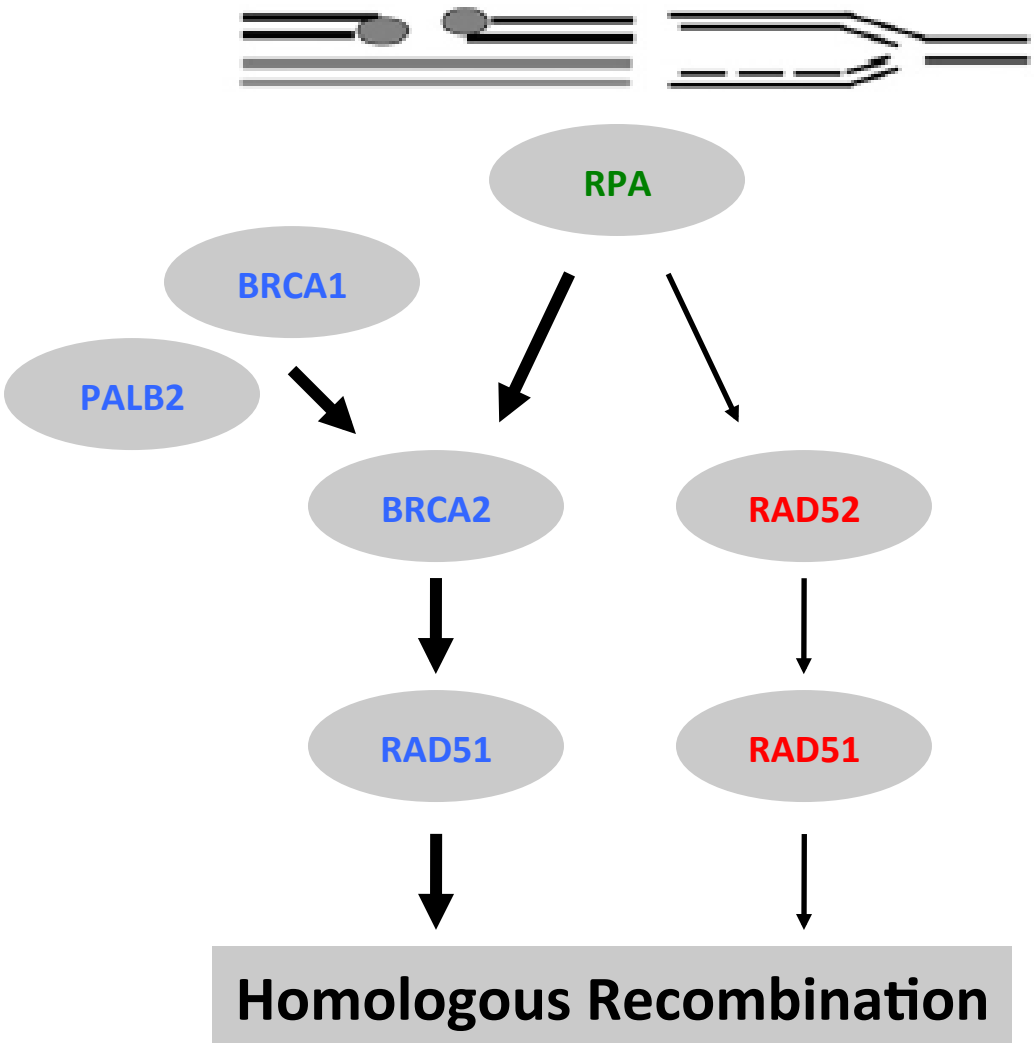



**Figure 4: Synthetic lethality: Two mediator pathways in humans.** BRCA2, BRCA1, and PALB2 form the primary RAD51 mediator pathway in human cells. In cells that lack the BRCA pathway, RAD52 can act as a backup mediator, promoting RAD51 function and HR.

relationship between the two proteins[162]. In cells lacking both BRCA2 and RAD52 there is increased chromosomal instability, evidenced by telomere end associations and radials, pointing to HR deficiency as the cause of the synthetic lethality. RAD52 functions independently of BRCA2, since its localization to damage and with RPA2 and ssDNA is no different between BRCA2-complemented and BRCA2-deficient cells, and RAD52 interacts with RAD51 independently of BRCA2[162]. Additionally, RAD52 was necessary for RAD51-mediated HR in BRCA2-deficient cells[162]. Depleting RAD52 in EUFA423 cells, which are BRCA2-deficient, reduces RAD51 foci formation, and expressing RAD52 in Capan-1 cells, which are deficient in BRCA2 and RAD52, increases levels of RAD52 foci. DR-GFP HR is also dependent on RAD52 in BRCA2-deficient cells. These results suggest that RAD52 provides a backup HR pathway in human cells: when BRCA2 is present, RAD52 has little effect on HR and viability, whereas in BRCA2-deficient cells, RAD52 is important for viability and for HR[162]. This suggests that residual RAD52 mediator activity is sufficient for survival in cells lacking BRCA2

Lok et al showed that not only is RAD52 synthetically lethal with BRCA2, but with BRCA1 and PALB2[392]. Double knockdown of RAD52 and either BRCA1 or PALB2 reduces plating efficiency in MCF7 and U2OS cells[392]. There is a further reduction in RAD51 foci with BRCA1-RAD52 double knockdown compared to BRCA1 knockdown, and DR-GFP HR is further reduced in BRCA1- and PALB2-depleted cells also depleted of RAD52. Similar to its independence from BRCA2 status, RAD52 foci formation is normal in the absence of BRCA1[392]. Further supporting the independence of the RAD52 pathway, Van Veelen et al found that in mammalian cells, though RAD52 is not required for foci formation of RAD51 and RAD54, RAD52 foci

formation is not influenced by a mutation in the RAD51 paralog-mutated (XRCC2, XRCC3, RAD51C) and BRCA2-mutated cell lines tested[391]. Evidence from Wray et al also supports the independence of the RAD52 pathway, as that RAD51 colocalizes with a BRCA2-interacting protein (BCCIP) early after IR and with RAD52 at later timepoints, with little colocalization of BCCIP and RAD52[386]. These results suggest that RAD52 promotes RAD51 recruitment and HR in the absence of and independent of the BRCA pathway. Thus, evidence points to RAD52 acting in an alternative, or backup, mediator pathway in humans, independent of BRCA pathway.

It is possible that RAD52 performs its mediator functions in conjunction with other proteins, and there are factors that would allow it to perform this role if included in reactions *in vitro*. Candidates for partners of RAD52 include the RAD51 paralogs, however recent evidence suggests that they are epistatic to the BRCA pathway rather than the RAD52 pathway. RAD52 is synthetically lethal in chicken DT40 with XRCC3, a RAD51 paralog[389], BRCA2 is epistatic to the RAD51 paralogs in response to DNA damage as measured by cellular survival after MMC[432], and RAD51 paralog complexes are epistatic to BRCA2 and synthetically lethal with RAD52 in human cells[433], suggesting the paralogs function with the BRCA pathway and not with RAD52. One study did find that RAD52 (as well as BRCA1, SFR1, SWS1, and XRCC3) is epistatic to BRCA2 in DT40 cells[434], as measured by cell growth and CPT and cisplatin sensitivity. There was no effect on RAD51 after IR when RAD52 was depleted on top of BRCA2 depletion, however, this did increase sensitivity to Olaparib, a PARP inhibitor[434]. The majority of evidence, however, supports an independent role for RAD52. So far the factors needed for RAD52 mediation in cells and in biochemical assays have not been determined.

In cancer patients carrying *BRCA* mutations only, tumor cells are BRCA-deficient (usually heterozygous BRCA$^{mut/-}$), while normal cells are proficient in HR, containing one functional and one nonfunctional copy of *BRCA1* or *BRCA2*. Thus, targeting a protein, such as RAD52, that is synthetically lethal with BRCA2 would result in death specifically of tumor cells. The synthetic lethal relationship between Poly (ADP Ribose) Polymerase (PARP) and the BRCA proteins provides a proof of principle example that synthetic lethality with BRCA proteins may be utilized for cancer treatment. PARP depletion has been shown to be lethal to cells lacking BRCA1 or BRCA2[435,436], and inhibition of PARP is being exploited to selectively kill tumors in patients with *BRCA* mutations[437-440].

The PARP family consists of 17 proteins, each containing a conserved catalytic domain which is responsible for poly (ADP-ribose) polymerization[441], which generates long chains of poly (ADP) ribose on target proteins, a process known as PARylation. PARP proteins are important in genomic stability, DNA repair, cell cycle progression, and apoptosis[441]. PARP1 and PARP2 specifically have been shown to be involved in single strand break (SSB) repair (SSBR), and also facilitate HR and fork restart by promoting MRE11, RPA, and RAD51 recruitment to collapsed replication forks[441-447]. Mouse knockouts of PARP1 or PARP2 are viable and fertile and do not develop early onset tumors[448]. However, PARP1 knockout mice and normal cells treated with PARP inhibitors do exhibit defective SSBR, increased HR and increased SCE, suggesting that HR may be vital to repairing lesions in PARP deficient cells[435,449-453]

The current model for the synthetic lethality relationship between PARP and HR hinges on PARP's role in the repair of SSBs[454,455], though recent

evidence suggests that the interaction is more complicated. PARP inhibition leads to the accumulation of SSBs, which stall replication forks and lead to the formation of DSBs[435,436,456]. These DSBs are normally repaired by the HR pathway, but cannot be repaired effectively in BRCA-deficient cells[456]. Thus, PARP inhibition in HR-deficient cells results in synthetic lethality, as the resulting DNA damage induces apoptosis or other forms of cell death. This model focuses on PARP's role in SSBR, but its function in HR-mediated restart of stalled replication forks[447] as well as in alt-NHEJ[457] may contribute to the sensitivity of BRCA-deficient cells to PARP inhibitors[458]. Another mechanism has been proposed in which PARP inhibitors trap PARP on a SSB intermediate[459], which may then be converted to a more toxic lesion during replication[458]. PARP proteins are also involved in DNA methylation, transcription, chromatin modification, and cell death pathways, and these functions may contribute to the sensitization of BRCA-deficient cells as well[441]. Sensitivity to PARP inhibitors has also been demonstrated in cells deficient in other HR genes, supporting the hypothesis that BRCA-mutant cells are sensitive to PARP inhibitors specifically because of HR deficiency[460]. Currently, many PARP inhibitors are in early phase clinical trials[439,461,462].

PARP therefore provides an established example of the clinical implications of synthetic lethality with the BRCA pathway. Targeting RAD52 may therefore similarly be effective in treating tumors in patients with BRCA mutations or other HR defects.

**Other mediators**

Beyond BRCA2 and RAD52, other proteins play a role in mediating and supporting RAD51 function. These include the RAD51 paralogs, PALB2 (as described), SWS1, SWSAP1, RAD54, RAD54B, SWI5, and SFR[463]. There are

five RAD51 paralogs in humans: RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3, which are likely tumor suppressor proteins and are analogous to the yeast Rad55 and Rad57 proteins. They have 20-30% amino acid sequence similarity to RAD51 and each other, mostly at the Walker A and B motifs, which are responsible for ATP-binding. RAD51 paralogs form two major complexes: BCDX2 (RAD51B, RAD51C, RAD51D, XRCC2), and CX3 (RAD51C, XRCC3) [464-466]. RAD51 interacts with the RAD51 paralogs[466-468], but their functions in HR are not well understood. Cells deficient in the paralogs in mammals have reduced RAD51 foci formation[469-472], increased chromosomal abnormalities, increased sensitivity to damaging agents, reduced HR-mediated gene targeting and DSB repair, and reduced SCEs[470,473-476]. Disruption of these genes in mice leads to early embryonic lethality with accumulation of unrepaired DNA damage[477-479]. Though these proteins have been implicated as mediators of the RAD51 recombinase, their precise roles remain unclear, as part of their phenotype may be due to other roles in the cell such as control of centrosome integrity and function in checkpoint signaling[480].

The RAD51 paralog complexes have been shown to promote exchange activity *in vitro*. The CX3 complex catalyzes the formation of D-loops on its own *in vitro*[481]. Directly supporting a role as RAD51 mediators, the BC subcomplex partially alleviates the competition by RPA with RAD51 for substrate binding, thereby stimulating RAD51-filament formation and strand exchange, though this enhancement was more evident in the formation of joint molecules than the final exchanged product[482,483]. Beyond roles in mediation, paralogs have also have been suggested to have functions downstream in HR,

such as regulating gene conversion tract length[484,485] and playing a role in Holliday junction resolution[486,487].

The different RAD51 paralog complexes have been shown to function differently in HR. The BCDX2 complex acts downstream of BRCA2 recruitment and upstream of RAD51, while the CX3 complex functions downstream of RAD51 recruitment, but still affects HR[433]; both are epistatic to BRCA2 and synthetically lethal with RAD52[433]. Double depletion of the paralogs with RAD52 reduces plating efficiency (51D and X3), RAD51 foci (51D), and DR-GFP HR. Depletion of all complex members reduces DR-GFP HR, but depleting both complexes does not further deplete HR, so they likely function in the same pathway[433]. Other studies have also found that BRCA2 is epistatic to the paralogs in response to DNA damage by cellular survival after MMC[432].

SWS1, an ortholog of the yeast Shu complex, and SWSAP1, which interacts with SWS1 and has a predicted RecA-core, have also been proposed to act as recombination mediators[488,489]. SWS1 also interacts with RAD51D, while SWSAP1 interacts with RAD51 and most of the paralogs and its depletion causes defects in HR. The hSWS1-SWSAP1 complex binds ssDNA and exhibits DNA-stimulated ATPase activity. These proteins are not well characterized, but their interactions have lead to the suggestion that they may be mediators as well.

The RAD54 protein, a Swi/Snf family protein that translocates on DNA, is also important in HR through numerous mechanisms**.** Humans have two RAD54 homologs: RAD54 and RAD54B. These proteins have been implicated as mediators through stimulation of RAD51 filament formation, homology search, and strand exchange, and disruption of RAD51 binding to dsDNA, and

also function in chromatin remodeling, branch migration of Holliday junctions, and resolution of Holliday junctions through endonuclease interactions[165]. Human RAD54 has been shown to associate with RAD51, promote its recruitment, and stimulate its DNA pairing[490-492].

RAD51AP1 enhances joint molecule formation through Rad51, but its exact role is unclear. It interacts with PALB2, and PALB2 stimulates joint molecule formation[351,352], though it is believed to promote an interaction with the dsDNA target and stabilizing the D-loop rather than promoting RAD51 filaments[352,463]. Interestingly, PALB2 has also been shown to interact with the RAD51C paralog[467].

Finally, the SWI5-SFR1 complex, related to the *S. pombe* Swi5-Sfr1 and *S. cerevisiae* Sae3-Mei5 complexes, may act as a mediator. These proteins function only in meiosis in budding yeast. In mouse, Swi5 and Sfr1 are nuclear proteins; deletion mutants in ES cells are sensitive to DNA damaging agents, have increased chromosomal aberrations, and reduced PARP-inhibitor-induced SCE, supporting a role for the complex in homology based DSB repair[493]. Expression of a BRC repeat has been shown to inhibit RAD51 focus formation and to decrease HR in mammalian cells, likely by sequestering RAD51 in cells[340,346,349,494]; in Sfr1 and Swi5 deleted cells, though their loss does not decrease HR frequency on their own, expression of the BRC repeat reduced HR in Sfr1 and Swi5 deleted cells even more than in WT cells, again implicating these proteins in a role supporting RAD51[493]. In humans cells, SWI5-SFR1 depletion reduces RAD51 foci and also enhances sensitivity to IR[495]; *in vitro* the complex interacts with RAD51 and stimulates RAD51-strand exchange by stabilizing the filament[496] paradoxically by

enhancing ATP hydrolysis by RAD51 and facilitating the release of ADP from the presynaptic filament to keep it in its active ATP bound form[497].

These effects are interesting due to the role of the Swi5-Sfr1 complex in *S. pombe*, which may be relevant to the mediator proteins in human cells. While *S. cerevisiae* Rad52 protein promotes Rad51-dependent DNA-strand exchange on RPA-coated DNA by itself, *S. pombe* Rad52 (Rad22) cannot. Though Rad22 is important to overcome the inhibitory effect of RPA, it requires the Swi5–Sfr1 complex in a downstream step to stabilize the Rad51 filament, so it can progress to strand exchange[480,498,499]. Although this is possibly due differences between *S. pombe* and *S. cerevisiae* RAD51, it could be because of differences in Rad52 mediator activity between budding and fission yeast. Similar to RAD52 knock-outs, mouse cells in which the *Swi5* or *Sfr1* are knocked out are viable and display no defect in HR. Thus, it is plausible that RAD52 in humans and other mammals requires the SWI5-SFR1 complex to perform its mediator function[480,498,499].

**Thesis aims**

RAD52 provides a backup pathway to BRCA2 in human cells as a RAD51-mediator. However, the factors important to this pathway have yet to be uncovered. There may be factors that allow RAD52 to perform its mediator role more efficiently *in vitro*, and RPA is a good candidate. RPA is an important interacting partner of RAD52, and studies of its hyperphosphorylation leave much to be uncovered. In this thesis, we look at the effect of RPA hyperphosphorylation on the RAD52-mediator pathway both *in vivo* and in *in vitro* assays.

# CHAPTER TWO

# RPA HYPERPHOSPHORYLATION PROMOTES RAD52 MEDIATOR FUNCTION IN HUMAN CELLS THROUGH IMPROVED RPA-RAD52 ASSOCIATION

## INTRODUCTION

Double strand breaks (DSBs) are the most deleterious type of DNA damage and arise during endogenous processes, such as DNA replication, or after exposure or DNA damaging agents, such as ionizing radiation or topoisomerase poisons. DSBs are resolved by two main pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ).

In HR, DSBs are resected to generate single-stranded DNA (ssDNA) overhangs, which are bound by the heterotrimeric ssDNA binding complex, RPA[1-3,72]. RPA is exchanged for RAD51, which then performs homology search and strand invasion. *S. cerevisiae* RAD52 mediates the exchange of RPA for RAD51, promoting RAD51 filament formation on RPA-coated ssDNA and stimulating RAD51 strand invasion[155,156]. BRCA2 performs this mediator function in humans[157,158]. Moreover, RAD52 mouse knockouts show little phenotype or sensitivity to DSB-inducing agents, hRAD52 is inefficient at displacing RPA and stimulating strand exchange *in vitro*, and hRAD52 is not essential for RAD51 function or HR[157,159,160]. Thus the role of human RAD52 and its ability to function as a RAD51 mediator in HR is unclear.

Nevertheless, recent evidence suggests that hRAD52 provides an alternative mediator pathway to BRCA2[161,162]. In BRCA2-deficient human cancer cell lines, depletion of RAD52 reduces cell survival and proliferation, such that there is a synthetic lethal relationship between the two proteins[162]. RAD52 is necessary for RAD51-mediated HR in BRCA2-deficient cells[162], and

RAD52 functions independently of BRCA2, since its localization to damage is not affected by the presence of the BRCA2 protein, and it interacts with RAD51 independently of BRCA2[162]. These results suggest that RAD52 provides a backup HR pathway in human cells: when BRCA2 is present, RAD52 has little effect on HR and viability, whereas in BRCA2-deficient cells, RAD52 is important for viability and for HR[162]. What factors are required for this backup pathway are currently unknown.

hRAD52 has been shown to interact with the RPA2 subunit of the RPA complex, and this interaction facilitates HR in mammalian cells[298]. RPA2 is hyperphosphorylated in response to DNA damage, which is important for RAD51 recruitment and HR[249,253]. Dephosphorylation of RPA2 by PP4 and PP2A phosphatases is also needed for HR[112,230]. How RPA phosphorylation affects RAD52 function is not well understood. *In vitro* evidence suggests that more RAD52 associates in RPA-RAD52-ssDNA complex when RPA is phosphorylated, and pRPA promotes RAD52 contacts with ssDNA[216].

This study aims to address if and how RPA phosphorylation affects RAD52 function. We are interested in this relationship as a potential mechanism to target RAD52 function, since targeting RAD52 may lead to tumor specific synthetic lethality in patients. The main questions we address are whether RPA phosphorylation affects RAD52-mediated HR directly, and whether phosphorylation affects RPA's interaction with RAD52. Using human cell lines expressing RPA-phosphorylation mutants we investigate RAD52-dependent HR using endonuclease-induced recombination assays and RAD51 foci formation assays. We also look at the how the interaction of RPA and RAD52 is affected by RPA hyperphosphorylation by IP and colocalization.

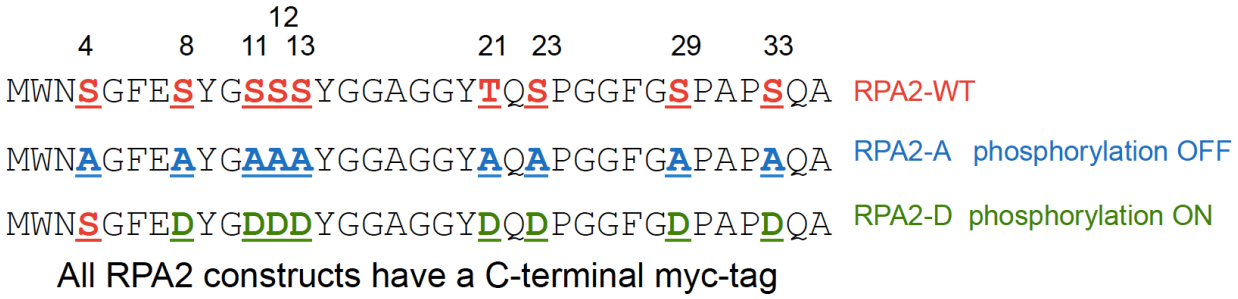
## RESULTS

### RAD52-mediated HR is promoted by RPA hyperphosphorylation

In order to study the effect of RPA hyperphosphorylation on RAD52-mediated HR, we used MCF7 breast carcinoma cells stably expressing myc-tagged RPA2 phosphorylation mutants, which are similar to systems used in other studies of RPA phosphorylation (Figure 5). These cells express either wild-type RPA2 (RPA2-WT), RPA2 with all of the phosphorylation sites mutated to alanine (RPA2-A), or with most of the sites mutated to aspartate (RPA2-D), mimicking non-phosphorylatable and constitutively phosphorylated RPA2, respectively. Alanine is structurally similar to serine, lacking the hydroxyl group that is phosphorylated, and therefore acts as a phospho-dead analog. Aspartate has a carboxyl group that can mimic the negative charge of a phosphate group, though aspartate substitutions do not always perfectly mimic a phosphorylated protein. The exogenous RPA2-WT and RPA2-mutants each have a C-terminal myc tag. We depleted endogenous RPA2 using siRNA to study the effects of each exogenous RPA specifically. As in previous studies, these RPA mutants still interact with RPA1 and RPA3 by immunoprecipitation and colocalization (Figure 5B). Although some studies saw an effect on the cell cycle by expressing RPA phosphorylation mutants, in these cell lines cell cycle distributions were normal after endogenous RPA2 depletion, suggesting that RPA phosphorylation mutants do not affect the normal cell cycle. Expression of the RPA2-D mutant was very low in all clones tested, and unpublished data from our lab suggests that RPA phosphorylation plays a role in regulating RPA protein stability or turnover. Thus, it is possible that effects seen in the RPA2-D line are due to low expression of RPA and not due to mutation of the phosphorylation sites.

**Figure 5: Methods—RPA phosphorylation mutants a**. RPA phosphorylation mutants. Either RPA2-WT, RPA2-A (non-phosphorylatable) or RPA2-D (phospho-mimic) were stably expressed in MCF7 cells. **b**. IP pull down with myc antibody, showing RPA2-WT, -A, and –D, exogenous proteins interact with RPA1 and RPA2.

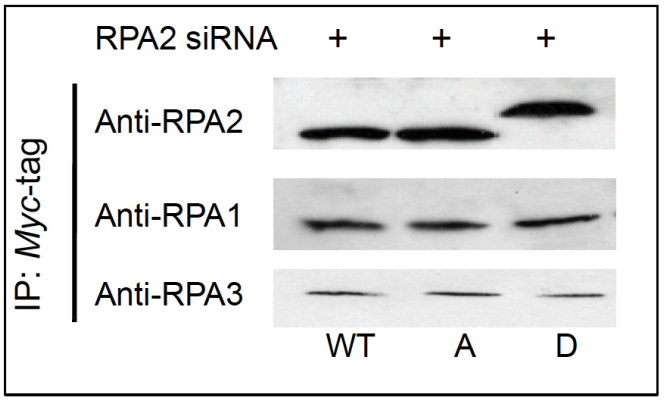
**A**




All RPA2 constructs have a C-terminal myc-tag

**B**

RPA2 mutants form a complex with RPA1 and RPA3

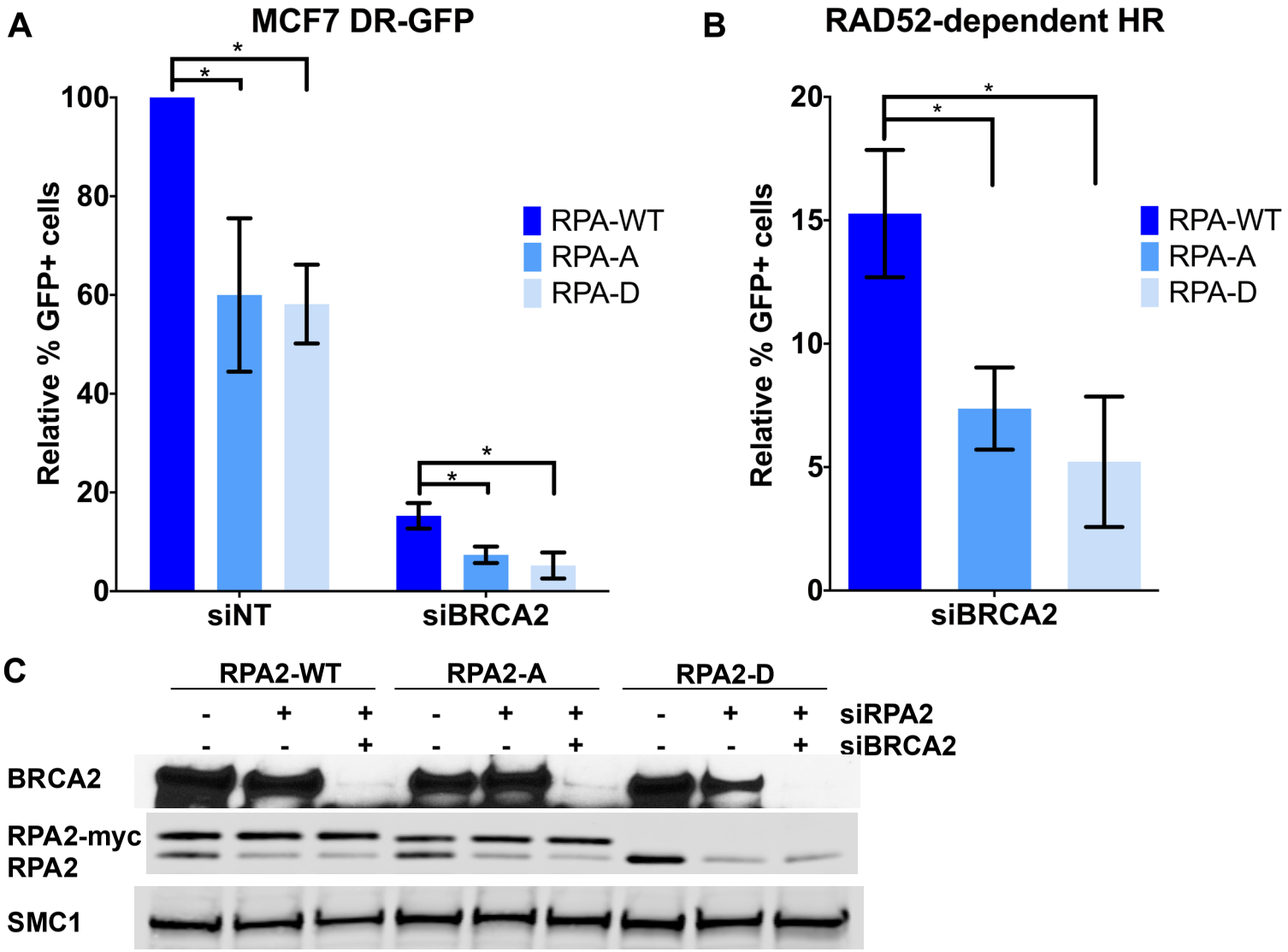


Each of these cell lines also expresses the DR-GFP construct to measure levels of HR (Figure 5). In the DR-GFP assay, which was developed in the Jasin lab, a site-specific DSB is induced in DR-GFP cells by transfecting the I-SceI endonuclease. The I-SceI site is located in a truncated GFP gene (SceGFP), and generates early stop codons so there is no functional GFP expressed. Downstream of the SceGFP gene is an iGFP gene that is also truncated. When an I-SceI break in SceGFP is repaired by HR with the iGFP gene on the sister chromatid and gene conversion occurs, a functional GFP gene results and is expressed[475]. The percentage of cells expressing GFP can then be determined using flow cytometry analysis.

Our first set of experiments suggests that RPA phosphorylation facilitates RAD52-dependent HR. Consistent with earlier reports, cells expressing RPA2 phosphorylation mutants have reduced HR as measured by the DR-GFP assay (Figure 6A), confirming that RPA phosphorylation and dephosphorylation are important for HR. In order to study RAD52-dependent HR, we depleted BRCA2 with siRNA (Figure 6C). Evidence suggests that the remaining DR-GFP HR and RAD51 foci in these cells is dependent on RAD52[162]. As expected, depletion of BRCA2 significantly reduces levels of HR (Figure 6B). In these conditions, we also see reduced HR in the RPA phosphorylation mutant cell lines compared to RPA2-WT (Figure 6B). This suggests that RPA phosphorylation and dephosphorylation are not only important in the BRCA pathway of HR, but also for the RAD52-dependent pathway.

In order to confirm the effect of RPA phosphorylation on RAD52-dependent HR, we next looked at RAD51 foci formation. The RAD51 recombinase is recruited to foci in normal cells and in response to DNA

**Figure 6. RPA phosphorylation is important for RAD52-dependent HR. a**. MCF7 DR-GFP cells expressing RPA2-WT or phospho-mutant RPA2 (RPA2-A and RPA2-D) were depleted of endogenous RPA2 by siRNA and also transfected with siNT or siBRCA2. 48 hours later, cells were transfected with the I-SceI endonuclease. 72 hours after that, $10^5$ cells per condition were tested by flow cytometry for expression of GFP. Experiments were normalized to RPA-WT siNT treated cells. Error bars represent SEM. n=5 (*: $p < .05$ by t-test). **b.** siBRCA2 data, or RAD52-dependent HR events, plotted on different scale. **c.** Western blots confirming depletion of BRCA2 and endogenous RPA2


A
MCF7 DR-GFP
Relative % GFP+ cells
100
80
60
40
20
0
siNT
siBRCA2
RPA-WT
RPA-A
RPA-D
*
*
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*
B
RAD52-dependent HR
Relative % GFP+ cells
20
15
10
5
0
siBRCA2
RPA-WT
RPA-A
RPA-D
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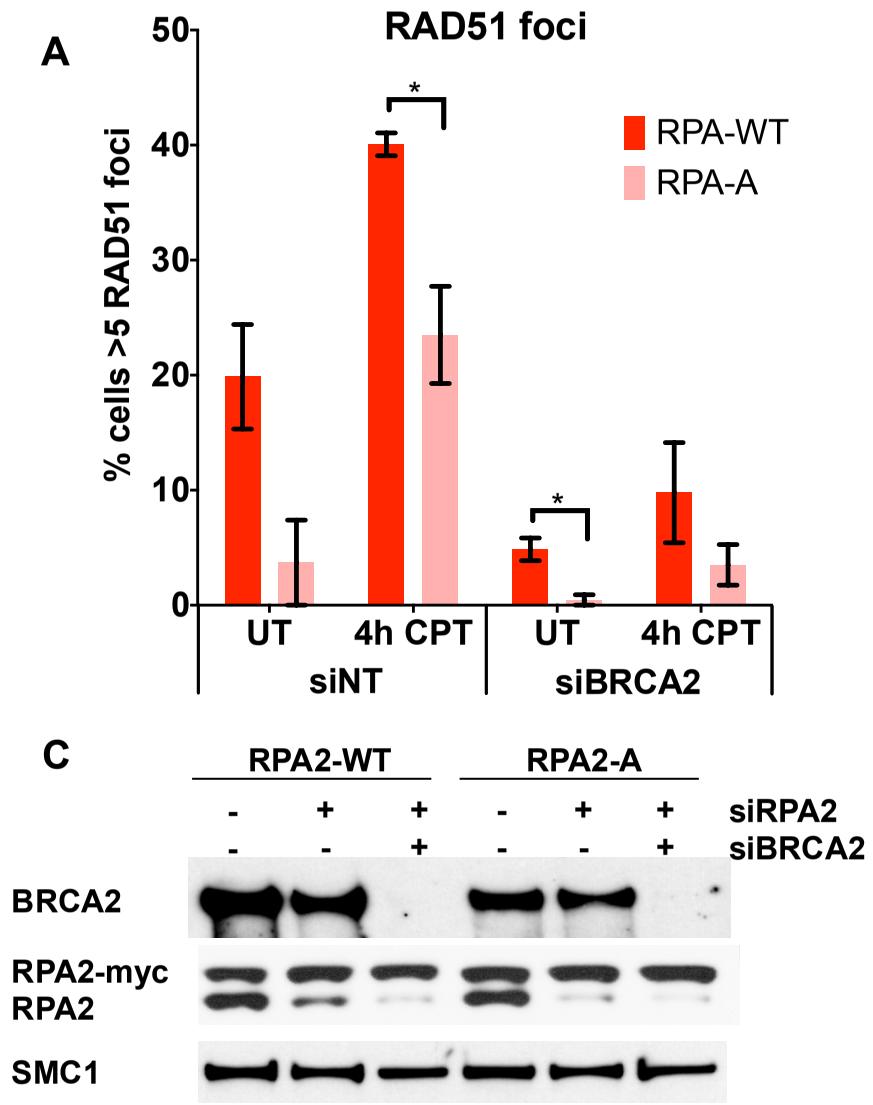
RPA2-WT
RPA2-A
RPA2-D
siRPA2
siBRCA2
BRCA2
RPA2-myc
RPA2
SMC1

damage that causes DSBs. Levels of RAD51 foci are reduced in cells that have impaired HR. In order to generate DSBs we treated cells for 4 hours with 4 μM camptothecin (CPT). Camptothecin is a topoisomerase I inhibitor, which stabilizes the topoisomerase bound to DNA and prevents religation, thus generating a break in DNA, that is converted to a DSB when encountered by the replication fork. We looked at RAD51 foci by immunofluorescence, and the percentage of cells with greater than five RAD51 foci were counted as positive. In cells treated with non-targeting siRNA with BRCA2-dependent RAD51 recruitment intact, we again see a dependence of RAD51 recruitment on RPA phosphorylation: RPA2-A cells have reduced RAD51 foci in both untreated cells and in cells treated with CPT compared to RPA2-WT cells, in agreement with previous reports that RPA phosphorylation is important for RAD51 recruitment and HR (Figure 7A and 7B). In cells depleted of BRCA2, where RAD51 foci formation is dependent on RAD52, we again see reduced RAD51 foci formation in the RPA2-A cells compared to RPA2-WT cells. This suggests that RPA phosphorylation is necessary for RAD52-dependent recruitment of RAD51 and subsequent HR.

Since it appears that RAD52-dependent HR is promoted by RPA phosphorylation and dephosphorylation, we looked at the effect of RPA phosphorylation mutants on RAD52 foci recruitment, as RAD52-dependent HR may be defective due to a failure to recruit RAD52. RAD52 is difficult to detect by immunofluorescence, so RAD52 tagged with GFP was expressed in MCF7 cells expressing RPA phosphorylation mutants or RPA2-WT, and after 6 hours with 4 μM CPT (or no treatment in control cells), the cells were fixed and imaged on the confocal microscope. The number of cells with greater than five RAD52-GFP foci per condition were counted and normalized to RPA2-WT

**Figure 7. RPA phosphorylation is important for RAD52-dependent RAD51 foci.** MCF7 cells expressing RPA2-WT or phospho-mutant RPA2 (RPA2-A) were depleted of endogenous RPA2 by electroporation of siRPA. 48 hours later cells were depleted of BRCA2 by Lipofectamine RNAiMax transfection of siBRCA2. 24 hours after that, cells were plated on glass slides. The next day, cells were treated with CPT for 4 hours, then fixed with formaldehyde and permeabilized with Triton X-100. Cells were stained with RAD51 primary antibody, fluorescent secondary antibody, and then imaged on the confocal microscope. **a**. 300 cells were counted per condition in each experiment, and cells with >5 RAD51 foci were counted as positive for RAD51. Error bars represent SEM. n=3 (*: $p < .05$ by t-test). **b**. Representative images for data in **a**. **c**. Western blots confirming depletion of RPA2 and BRCA2.


A
RAD51 foci
% cells >5 RAD51 foci
50
40
30
20
10
0
*
*
RPA-WT
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UT
4h CPT
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siBRCA2
BRCA2
RPA2-myc
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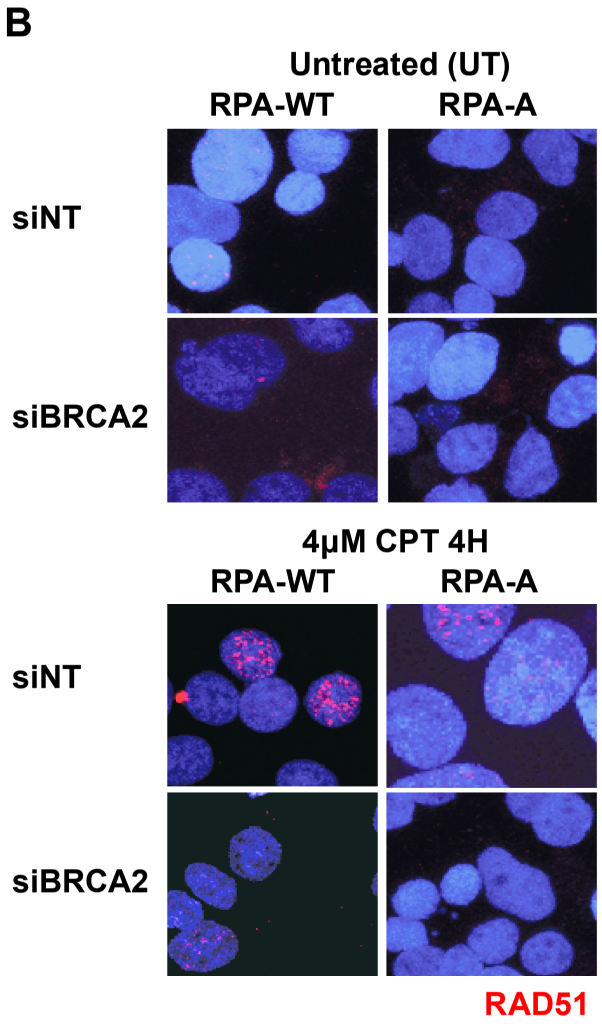

B
Untreated (UT)
RPA-WT
RPA-A
siNT
siBRCA2
4μM CPT 4H
RPA-WT
RPA-A
siNT
siBRCA2
RAD51

untreated controls. After treatment with CPT, there was no difference in the percentage of cells with RAD52-GFP foci between RPA2-WT, RPA2-A, and RPA2-D cells (Figure 8). The levels of RPA foci in each cell line were also not significantly different. This suggests that after damage, RPA phosphorylation and dephosphorylation do not affect the recruitment of RAD52. RAD52 is either able to interact sufficiently with the RPA mutants for its recruitment, or RAD52 is recruited independently of RPA. Interestingly, in RPA2-D cells, RAD52-GFP foci levels are elevated in the absence of damage compared to RPA2-WT and RPA2-A cells, which have similar levels of RAD52-GFP foci. RPA2-D untreated cells have RAD52-GFP foci levels similar to the levels of foci in CPT-treated cells of both RPA2-WT and RPA2-mutant lines, and there is no increase in the number of cells with RAD52-GFP foci after damage treatment in RPA2-D cells. This suggests some deficiency in RPA2-D cells, perhaps that RPA2-D signals to RAD52 that there is damage in untreated cells, leading to RAD52 recruitment.

To investigate why RAD52 is more efficient at HR and RAD51-recruitment in RPA2-WT compared to RPA2-phosphomutant cells, we looked at colocalization of RAD52-GFP with RAD51. More RAD52-GFP colocalizes with RAD51 in the RPA2-WT line compared to the RPA2-A line with and without BRCA2-depletion after DNA damage (Figure 9A and 9B). Furthermore, the percentage of cells with RAD52-GFP foci in which those foci are colocalized with RAD51 is reduced in RPA2-A cells compared to RPA2-WT cells (Figure 9D), and the percent of cells with RAD51 foci in which those foci are colocalized with RAD52-GFP is also reduced in RPA2-A cells, though not significantly (Figure 9E). Interestingly, we did not observe any RAD52-RAD51 colocalization in unperturbed cells, suggesting that the interaction between

**Figure 8. RAD52 recruitment after damage is unaffected by RPA phosphorylation.** MCF7 cells expressing RPA2-WT or phospho-mutant RPA2 (RPA2-A and RPA2-D) were depleted of endogenous RPA2 by electroporation of siRPA and RAD52-GFP was expressed, also by electroporation. 48 hours later cells were plated on glass slides; 24 hours after that they were fixed with formaldehyde and permeabilized 24 hours later. Cells were imaged on the confocal microscope. **a**. 300 cells were counted per condition in each experiment, and cells with >5 RAD52-GFP foci were counted as positive for RAD52. Error bars represent SEM. n=5 (*: $p < .05$ by t-test). **b**. Representative images for data in A.

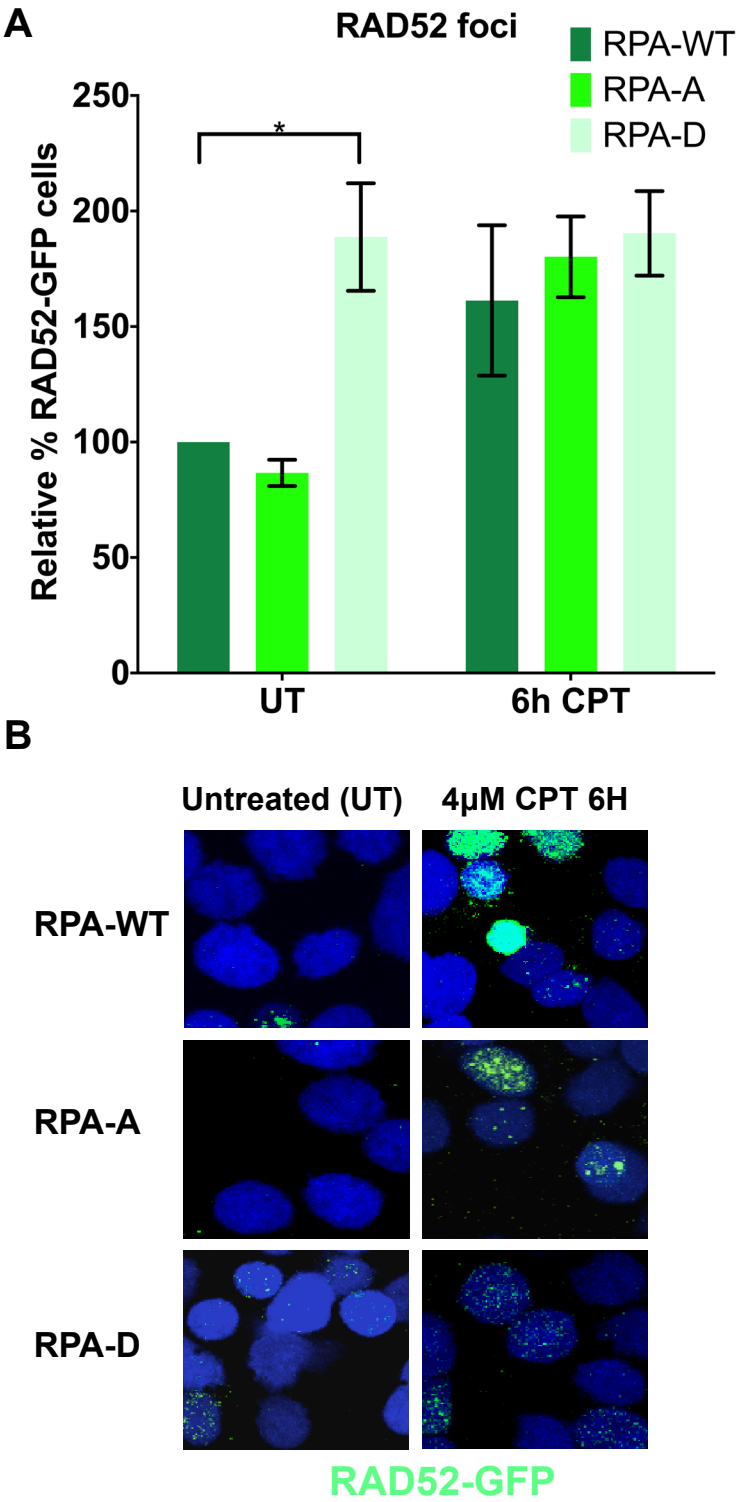
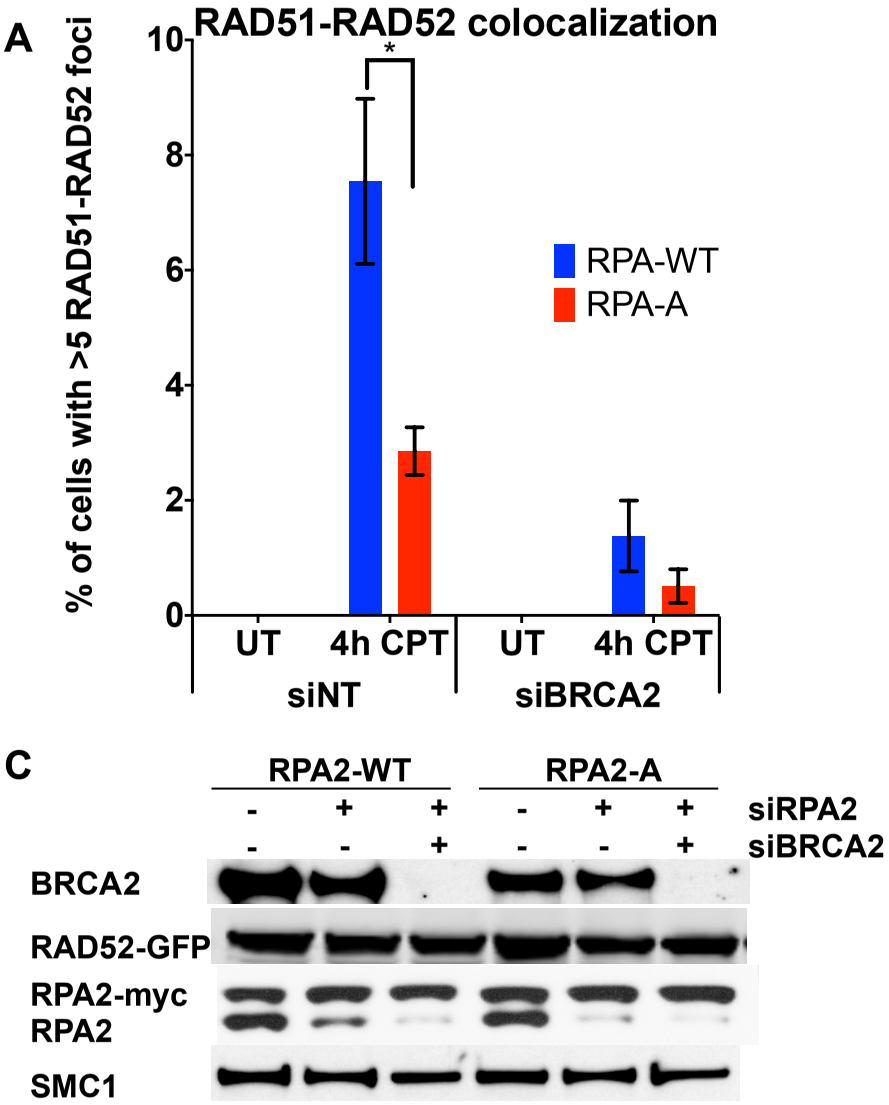
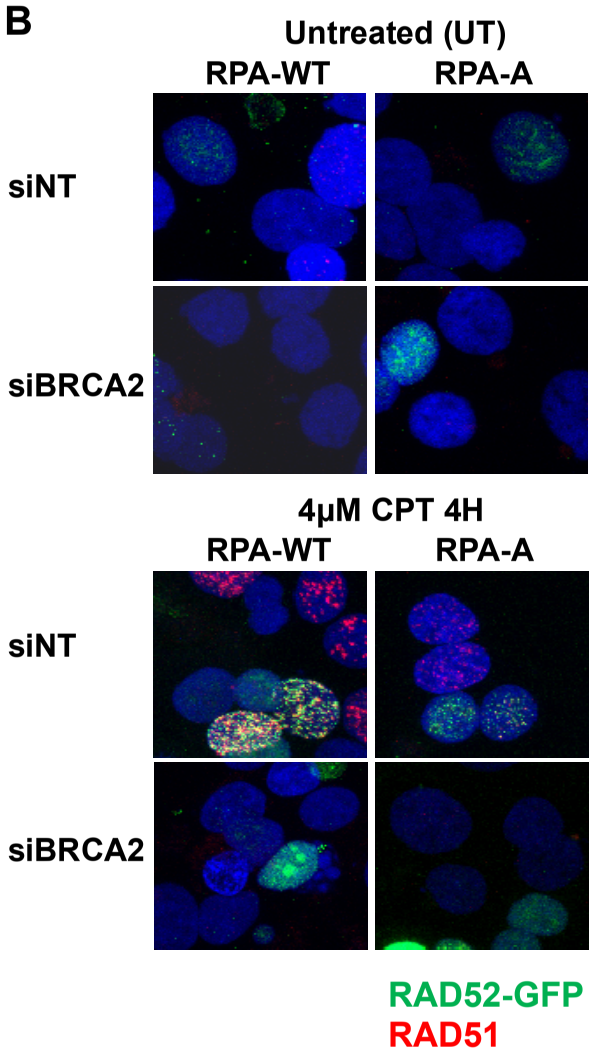

A
RAD52 foci
RPA-WT
RPA-A
RPA-D
Relative % RAD52-GFP cells
250
200
150
100
50
0
*
UT
6h CPT
B
Untreated (UT)
4μM CPT 6H
RPA-WT
RPA-A
RPA-D
RAD52-GFP

**Figure 9. RPA phosphorylation promotes RAD51-RAD52 colocalization.** MCF7 cells expressing RPA2-WT or phospho-mutant RPA2 (RPA2-A) were depleted of endogenous RPA2 by electroporation of siRPA2 and RAD52-GFP was expressed. 48 hours later cells were depleted of BRCA2 by Lipofectamine RNAiMax transfection of siBRCA2. 24 hours after that, cells were plated on glass slides. The next day, cells were treated with CPT for 4 hours, then fixed with formaldehyde and permeabilized with Triton X-100. Cells were stained with RAD51 primary antibody, fluorescent secondary antibody, and then imaged on the confocal microscope. **a.** 300 cells were counted per condition in each experiment, and cells with >5 RAD51 foci colocalized with RAD52-GFP were counted as positive for colocalization. Error bars represent SEM. n=3 (*: $p < .05$ by t-test). **b.** Representative images for data in **a**. **c**. Western blot confirming BRCA2 and endogenous RPA knockdown, and RAD52-GFP expression. **d.** The percent of cells with RAD52-GFP foci in which >5 of those foci are colocalized with RAD51. **e.** The percent of cells with RAD51 foci in which >5 of those foci are colocalized with RAD52-GFP.


A
RAD51-RAD52 colocalization
% of cells with >5 RAD51-RAD52 foci
10
8
6
4
2
0
*
RPA-WT
RPA-A
UT
4h CPT
UT
4h CPT
siNT
siBRCA2
C
RPA2-WT
RPA2-A
-
+
+
-
+
+
siRPA2
-
-
+
-
-
+
siBRCA2
BRCA2
RAD52-GFP
RPA2-myc
RPA2
SMC1


B
Untreated (UT)
RPA-WT
RPA-A
siNT
siBRCA2
4μM CPT 4H
RPA-WT
RPA-A
siNT
siBRCA2
RAD52-GFP
RAD51

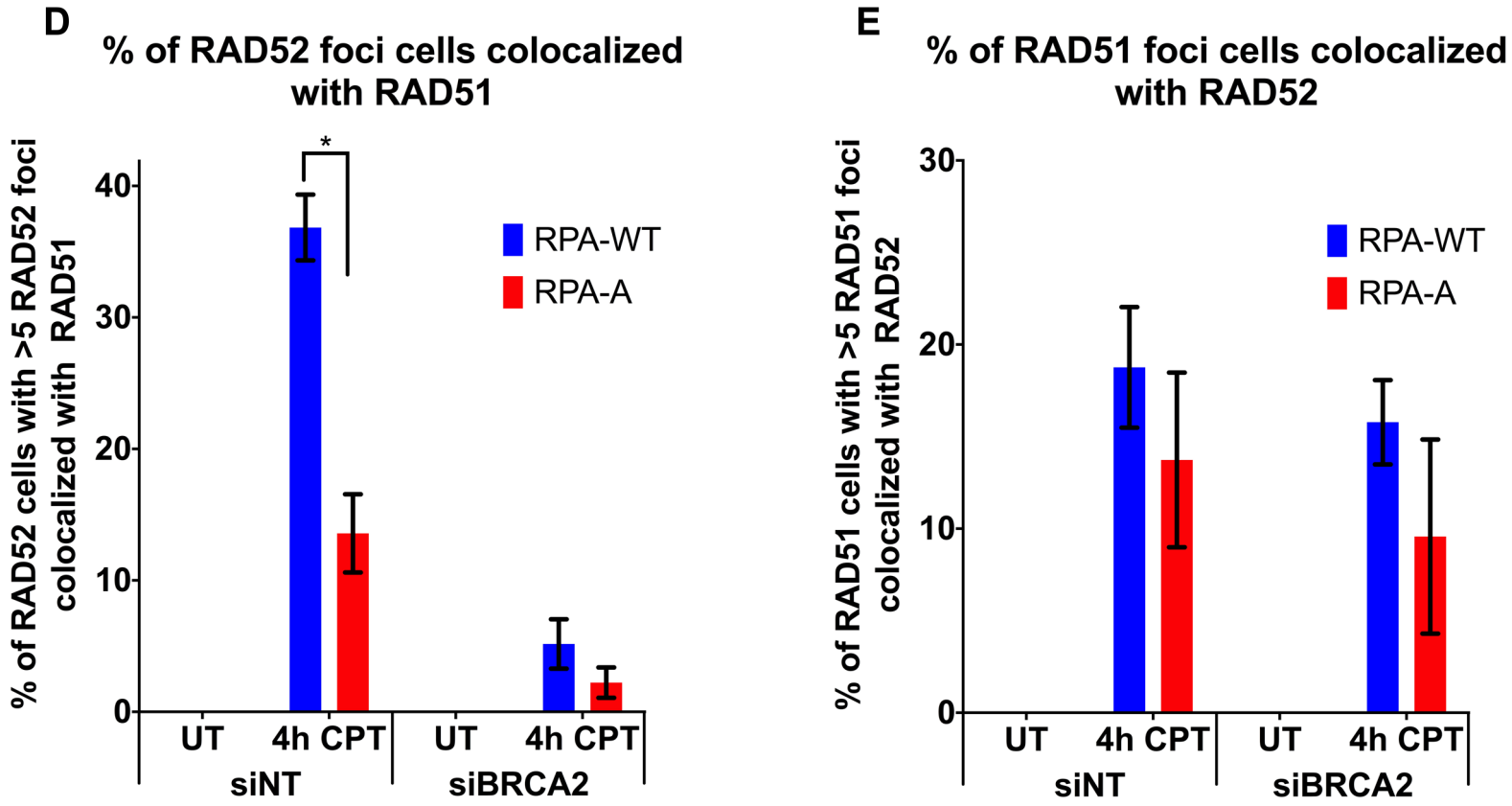



**Figure 9 (continued)**

RAD51 and RAD52 is DNA damage dependent in both RPA2-WT and RPA2-A cells. Thus, although RAD52 forms foci in RPA-phosphorylation mutants, it fails to recruit RAD51 normally, resulting in lower levels of HR.

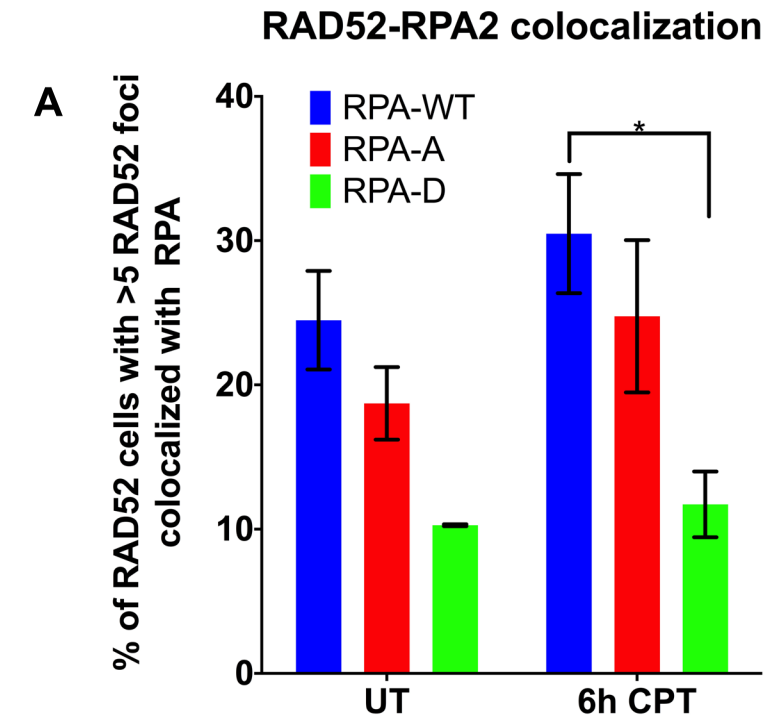
**RAD52-RPA interaction is promoted by RPA hyperphosphorylation**

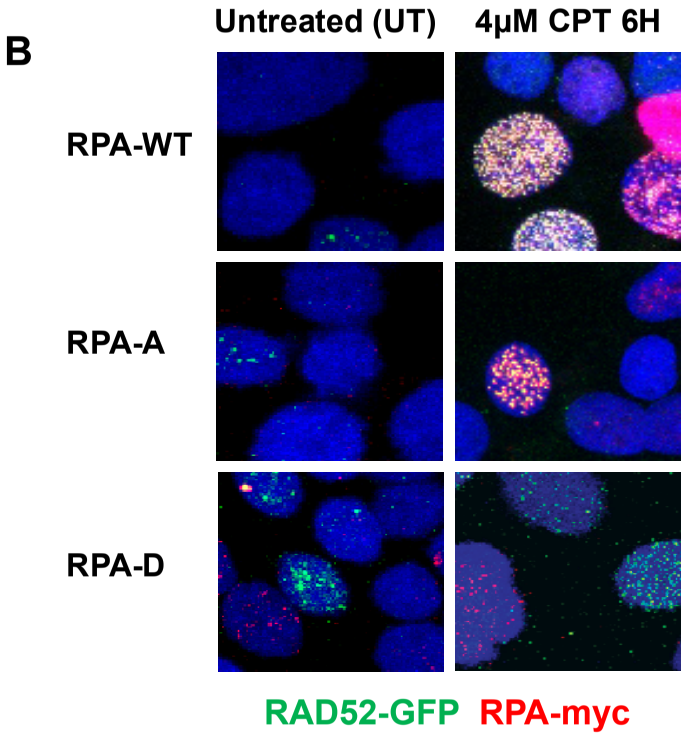
Our results indicate that RPA hyperphosphorylation promotes RAD52 function in HR. RPA and RAD52 interact, and this interaction has been suggested to be important for RAD52 function. There is some evidence that RPA hyperphosphorylation promotes the interaction of RAD52 and RPA[216,255], which could possibly explain the differences we see in RPA2-WT and RPA-phosphomutant cells in RAD52-dependent HR and RAD51 foci. Thus, we looked at the effect of RPA phosphorylation on the interaction of RAD52 and RPA.

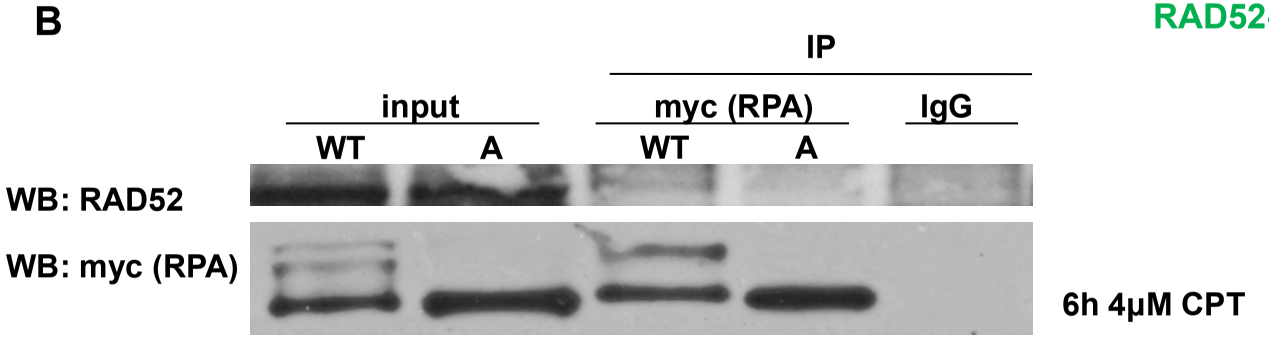
Using immunofluorescence of the RPA2-myc tagged proteins and RAD52-GFP foci, we looked at the co-localization of RAD52-GFP and RPA2 (with a myc antibody) (Figure 10). We calculated the percentage of cells that had RAD52-GFP foci in which at least five of those foci were colocalized with RPA. The RPA2-A and RPA2-D mutants did not colocalize with RAD52-GFP as well as the RPA2-WT protein did in untreated cells after treatment with CPT, although this result was only significant for the RPA2-D cells. Interestingly, despite the elevated levels of RAD52 foci in RPA2-D untreated cells, relatively few of those foci colocalize with RPA, suggesting that RPA dephosphorylation is important for effective interactions between RPA and RAD52.

RPA2-phosphorylation mutants have defective colocalization with RAD52, suggesting that proper regulation of RPA phosphorylation is important for their interaction. Therefore, we looked at the association of RPA and RAD52 by immunoprecipitation (IP). Consistent with a disruption in the

**Figure 10. RPA phosphorylation promotes RPA-RAD52 association. a.** MCF7 cells expressing RPA2-WT or phospho-mutant RPA2 (RPA2-A or RPA2-D) were depleted of endogenous RPA2 by electroporation of siRPA2 and RAD52-GFP was expressed. 48 hours later cells were plated on glass slides. The next day, cells were treated with CPT for 6 hours, then fixed with formaldehyde and permeabilized with Triton X-100. Cells were stained with myc primary antibody, fluorescent secondary antibody, and then imaged on the confocal microscope. 300 cells were counted per condition in each experiment, and cells with >5 RAD52-GFP foci colocalized with RAD52-GFP were counted as positive for colocalization. Plotted are the percent of cells with RAD52-GFP in which >5 of those foci are colocalized with RPA2-myc. Error bars represent SEM. n=5 (*: $p < .05$ by t-test). **b.** Representative images for data in **a**. **c**. Co-immunoprecipitation pull down with myc (RPA2) antibody.


RAD52-RPA2 colocalization
A
% of RAD52 cells with >5 RAD52 foci colocalized with RPA
40
30
20
10
0
RPA-WT
RPA-A
RPA-D
*
UT
6h CPT


B
Untreated (UT)
4μM CPT 6H
RPA-WT
RPA-A
RPA-D
RAD52-GFP
RPA-myc


B
IP
input
myc (RPA)
IgG
WT
A
WT
A
WB: RAD52
WB: myc (RPA)
6h 4μM CPT

association between RPA and RAD52 in phosphorylation-defective cells, more RAD52 immunoprecipitates with RPA2-WT than RPA2-A after CPT treatment, using an antibody against the RPA-myc tag to IP. Thus, although RAD52 can be recruited to foci regardless of RPA phosphorylation status, RPA phosphorylation improves RAD52 association with RPA. RAD52 association is different between RPA2-WT and the mutants, with RAD52 interacting with RPA more efficiently in RPA2-WT cells. RPA's association with RAD52 is therefore dependent on phosphorylation and dephosphorylation. In cells in which RPA phosphorylation is defective, RPA fails to associate properly with RAD52, which does not affect RAD52 recruitment but does result in the failure of RAD52 to recruit RAD51 and thereby promote HR.

## MATERIALS AND METHODS

*Note*: MCF7 cell lines expressing RPA2-myc proteins were generated by Rohini Roy, who also determined cell cycle distributions and confirmed immunoprecipitation of the RPA2-myc proteins with RPA1 and RPA3 in these cells. Alison Carley performed all other experiments in this thesis.

### MCF7 cell lines

RPA-myc cell lines were generated by electroporating linearized plasmids into MCF7 cells, a breast adenocarcinoma cell line, containing the DR-GFP reporter construct. Cells were grown in blasticidin and resistant colonies were expanded and tested for expression of RPA mutant cell lines. Cells were maintained in DMEM with 100U/mL penicillin, 100 µg/mL streptomycin, 10% BGS, 20 mM Hepes, and 15 µg/ml blasticidin. RPA2-WT (-A, -D) plasmids were generated from the pEF6 by insertion into the XbaI and BstBI sites of the pEF6/Myc-HisA vector (Invitrogen). Expression of the His6

tag from pEF6/Myc-HisA was prevented by mutating the ATG codon at position 1863 to a TGA codon.

**DR-GFP assay:** As described by Pierce et al. The DR-GFP system was developed in the Jasin lab and has been used extensively since then. Briefly, gene conversion events in cells containing this substrate result in expression of GFP protein, which can then be assayed by flow cytometry. DR–GFP contains two mutated *GFP* genes oriented as direct repeats and separated by a drug selection marker. SceGFP is mutated to contain a recognition site for the endonuclease I-SceI, the substitution of the recognition site also generates in-frame stop codons. The second mutated GFP is downstream of SceGFP, termed iGFP, a 5′ and 3′-truncated *GFP* gene. When the I-SceI nuclease is expressed, it results in a DSB in the SceGFP site. Upon a gene conversion event between Sce-GFP and iGFP on the same chromatid or sister chromatid, a functional GFP gene results and is expressed[475].

**Transfections**

- *DR-GFP*: 1 million cells were transfected with 2µg each siRPA and siBRCA2 (or siNT control) using the Amaxa electroporation system. 48 hours later, the 2µg pCMV-ISceI-3xNLS plasmid along with 0.5 µg each siRNA were transfected using the Lipofectamine 2000 reagent (Life Technologies). 72 hours later, the cells were harvested and percentages of GFP-positive cells per 100,000 cells were determined by flow cytometry (FACSCalibur; Becton, Dickinson).
- *RPA-myc -RAD52-GFP foci:* 1.5 million cells were transfected with 4.5 µg RAD52-GFP plasmid and 2 µg siRPA (or siNT control). Cells were plated on 8 well glass slides 48 hours later, then treated with 4µM camptothecin for 6 hours the next day fixed 72 hours later. Foci were imaged as

described below in immunofluorescence. (RAD52-GFP plasmid: RAD52 was C-terminally tagged with GFP by cloning *Rad52* cDNA into the pEGFP-C1 vector; Kitao H and Yuan ZM. JBC 2002.)

- *RAD51-RAD52 foci:* 1.5 million cells were transfected with 4.5 µg RAD52-GFP plasmid and 0.75µg siRPA (or siNT control). 48 hours later, 0.75 µg siBRCA2 was transfected using Lipofectamine RNAiMax from Life Technologies. Cells were plated on glass slides 24 hours later, then treated with 4µM camptothecin the next day and fixed.
- siRNAs were obtained from Dharmacon as follows:
    - siRPA: 2 sequences mixed in equal amounts:

      *sense sequence:*
        - AAC UGG AUC UAA CUG GGU ACC UU
        - GCU UCU AGG AAG UAG GUU UCA UU
    - siBRCA2: ON-TARGETplus SMARTpool from Dharmacon (L-003462-00)

      *target sequences:*
        - GAA ACG GAC UUG CUA UUU A
        - GGU AUC AGA UGC UUC AUU A
        - GAA GAA UGC AGG UUU AAU A
        - UAA GGA ACG UCA AGA GAU A
    - siNT: ON-TARGETplus SMARTpool from Dharmacon (D-001810-10)

      *target sequences:*
        - UGG UUU ACA UGU CGA CUA A
        - UGG UUU ACA UGU UGU GUG A

- UGG UUU ACA UGU UUU CUG A
- UGG UUU ACA UGU UUU CCU A

**Western blots**

- *Preparation of protein lysates*: Cells were trypsinized and pelleted by centrifugation. They were lysed using RIPA buffer containing 1X protease inhibitor cocktail (HaltTM Protease Inhibitor Cocktail, ThermoScientific) and incubated on ice for 5 minutes. Lysates were then sonicated and incubated on ice for 30 minutes. Lysates were then centrifuged at 16.1 RCF for 20 minutes, and the supernatant was collected. Protein concentrations were determined by using Bio-Rad Protein Assay Dye Reagent Concentrate and comparing against a BSA protein standard curve, measured on a Tecan Infinite M200 reader.
- *Western blot*: 20-100µg of protein lysates were loaded onto pre-cast gels from Life Technologies (NuPAGE® Novex® 10% Bis-Tris SDS page gels were run in NuPAGE® MOPS SDS running buffer for all proteins except BRCA2; 3-8% Tris-Acetate gels run in NuPAGE® Tris-Acetate running buffer for BRCA2 and SMC-1) in Novex Mini-Cell or Midi apparatus at 110 V for 2 hours. Gels were then transferred onto Nitrocellulose membranes using transfer buffer (1.4% glycine, 0.3% tris-base, 20% methanol) at 40V overnight in a Mini-PROTEAN® Tetra System (Bio-Rad). Membranes were stained with Ponceau S solution to verify even transfer, and then blocked in 5% milk in TBS-T(tris-buffered saline with 0.1% tween) or Odyssey TBS blocking solution for 1 hour. Membranes were incubated in primary antibodies diluted in 5% milk TBS-T (2.5% for BRCA2) or Odyssey TBS blocking solution with 1% tween overnight. Antibody was removed and the membrane was washed 3 times for 10 minutes in TBS-T. Membranes were

then incubated in secondary antibodies for 1 hour, and then again washed 3 times with TBS-T. Membranes incubated in HRP secondary were then incubated in Western Lightning ® Plus-ECL (Perkin Elmer) for 5 minutes, and then exposed to autoradiography film. Membranes incubated in IRD secondary were imaged on the Odyssey CLx System (Li-Cor).

- *Antibodies:*
    - RPA2 (cell signaling RPA2 (4E4) Rat mAb #2208) 1:1000
    - pRPA (abcam Anti-RPA32/RPA2 (phospho S4 + S8) antibody (ab87277)) 1:400
    - myc (cell signaling myc rb polyclonal #2272) 1:400
    - myc (Myc-Tag (9B11) Mouse mAb #2276) 1:1000
    - RAD52 (rb polyclonal RAD52 Antibody H-300, sc-8350) 1:500
    - RAD52 (ms monoclonal Rad52 Antibody F-7, sc-365341) 1:500
    - BRCA2 (BRCA2 Mouse mAb, OP-95, EMD Millipore) 1:300
    - SMC-1 (rabbit polyclonal, Bethyl Laboratories) 1:5000
    - Actin (mouse monoclonal, EMD Millipore MAB1501)
    - Secondary: Pierce goat anti-mouse and goat anti-rabbit HRP conjugated; Li-or IRDye® 800CW Goat anti-Mouse, IRDye® 800CW Goat anti-Rabbit, IRDye® 800CW Goat anti-Rat IgG

**Immunoprecipitation**

MCF7 cells were incubated in 4μM CPT for 6 hours. Nuclear lysates were collected using the Universal Magnetic Co-IP kit (Active Motif). Protein A/G SpinTrapTM Buffer Kit (GE Healthcare) was used to immunoprecipitate proteins as follows: Protein A Magnetic Sepharose Beads (GE Healthcare) were equilibrated by resuspension in 500 μl binding buffer, which was then removed. Beads were incubated with 10 μg primary antibody (myc mouse, cell

signaling #2276) for 4 hours. They were washed in 500 µl binding buffer, then in crosslink solution A. The beads were crosslinked to the primary antibody twice in 500 µl crosslink solution A with 50mM DMP for 30 minutes, with a wash in crosslink solution A in between. After crosslinking, the beads were washed in 500 µl crosslink solution A. They were blocked for 15 minutes in crosslink solution B, and unbound antibody was then eluted for 10 minutes at 50 degrees in elution buffer. Antibody-crosslinked beads were washed twice in 500 µl wash buffer, and then nuclear lysates were added to the beads and incubated for 3 hours. Unbound lysates were removed, and the antibody- and protein-bound beads were then washed twice with wash buffer. Bound protein was eluted in twice for 10 minutes in elution buffer. The eluate was concentrated using Amicon® Ultra 0.5 mL Centrifugal Filters (Ultracel® 10K, Millipore), and run on western blots as described above. Binding and wash buffer (50mM Tris, 150 mM NaCL pH 7.5); Elution Buffer (0.1 M glycine-HCl , pH 2.9); Crosslink Solution A (200 mM triethanolamine pH 8.9); Crosslink Solution B (100 mM ethanolamine, pH 8.9).

**Confocal Microscopy and Immunofluorescence**

Cells were simultaneously fixed and permeabilized in 0.5% triton and 0.5% formaldehyde diluted in PBS for 15 minutes. They were then blocked at 4 degrees overnight in 5% BGS in PBS. The following morning, they were incubated in primary antibody for 3 hours at room temperature. Cells were washed 3 times for 5 minutes in 0.5% triton in PBS, and then incubated in secondary antibody for 1 hour. Cells were washed again 3 times for 5 minutes in 0.5% triton in PBS. Mounting Medium with DAPI (Vectashield) was added, and then coverslips. Cells were imaged on a Zeiss confocal microscope.

- *Antibodies:*
  - myc (Myc-Tag (9B11) Mouse mAb #2276) 1:2000
  - RAD51 (rabbit monoclonal, abcam ab133534) 1:2500
  - Secondary: goat anti mouse Alexa Fluor® 568 (Life technologies), Goat anti-Rabbit, Alexa Fluor® 555 conjugate

# CHAPTER THREE

# INVESTIGATING THE EFFECT OF RPA HYPERPHOSPHORYLATION ON ITS *IN VITRO* FUNCTIONS

## INTRODUCTION

While our studies in Chapter Two show that RPA hyperphosphorylation is important for RAD52 mediator function in cells, the effect of RPA phosphorylation on RAD52 mediator function *in vitro* has not been determined previously. Thus, to complement these studies, we looked at *in vitro* assays of recombination. Human RAD52 has been shown to be inefficient at acting as a mediator of RAD51 filament formation and strand invasion on RPA-coated ssDNA, but it is possible that if phosphorylated RPA were included it would allow RAD52 to function as a mediator *in vitro*. Several lines of evidence suggest this may be true: the interaction of RPA and RAD52 is improved with RPA phosphorylation as shown in chapter two and published work[255], hypRPA promotes RAD52 contacts with ssDNA in RPA-RAD52-ssDNA complexes[216], the interaction between RPA and RAD52 is functionally significant as RAD52 mutants that do not interact with RPA fail enhance HR in monkey cells while RAD52-WT does[298], and the RAD52-RPA interaction facilitates binding of RAD52 to RPA-ssDNA, and this interaction is necessary for RAD52 to counteract RPA's helix destabilizing activity[295]. Biochemically, RAD52 has been shown have annealing activity and RAD51-mediation activity in the absence of RPA or in suboptimal conditions for RAD51, but it has not been shown to remove RPA from ssDNA to allow RAD51 filament formation[157]. Phosphorylated RPA is present in cells at DNA damage but has not been studied in *in vitro* strand exchange assays to date. The effect of RPA phosphorylation on RAD51 exchange itself also has not previously been

determined, though other studies have found that hyperphosphorylation promotes RPA's interaction with RAD51: RAD51 preferentially co-immunoprecipitates with an RPA2-D mutant[230] and RAD51 *in vitro* pulled down hypRPA with little non-phosphorylated RPA2[255,268]. Thus, it is possible that RPA phosphorylation may affect RAD51 exchange on its own.

The relationship between RPA phosphorylation and RPA DNA binding is not clear, as there have been mixed reports[207,223,240,244]. In one study, no difference in hyperphosphorylated and non-phosphorylated RPA binding to pyrimidine-rich ssDNA sequences was observed, but hyperphosphorylated RPA (hypRPA) had decreased binding to purine-rich ssDNA and dsDNA[244]. Simarlarly, IR-induced hypRPA exhibited decreased binding to ssDNA compared to non-phosphorylated RPA in crude mouse cell extracts[223], while in contrast, using a mixed sequence 25 nucleotide oligomer, a relative increase in ssDNA binding by hypRPA over RPA was observed[216]. Other studies have seen no difference in ssDNA binding[240,259,265]. Thus, while it is possible that phosphorylation of RPA may regulate its DNA binding, how it does so is still unclear. Studies investigating phosphorylated RPA binding to dsDNA have been more consistent, finding a reduction in pRPA dsDNA binding[206,240], such that phosphorylation has been proposed to reduce RPA duplex destabilization, while a higher affinity of RPA for damaged dsDNA has been observed[244], leading to the proposal that hypRPA may signal DNA damage to other repair proteins.

We have evaluated the effect of RPA phosphorylation on various biochemical activities. Since published data are inconsistent, we first looked at RPA and hypRPA binding to ssDNA. We then investigated the effect of RPA phosphorylation on RAD51 strand exchange. Since our experiments show that

RPA phosphorylation promotes RAD52 mediator function in cells, we looked at RAD52 mediator activity using ssDNA coated with unphosphorylated versus phosphorylated RPA2, as using phosphorylated RPA may allow RAD52 to promote RAD51 strand exchange. Finally, we looked at the effect of RPA phosphorylation on RAD52 annealing activity, as the promotion of RAD52 activity and RPA interaction by RPA phosphorylation may not be limited to its mediator function.

## RESULTS

### RPA ssDNA-binding is not significantly affected by phosphorylation

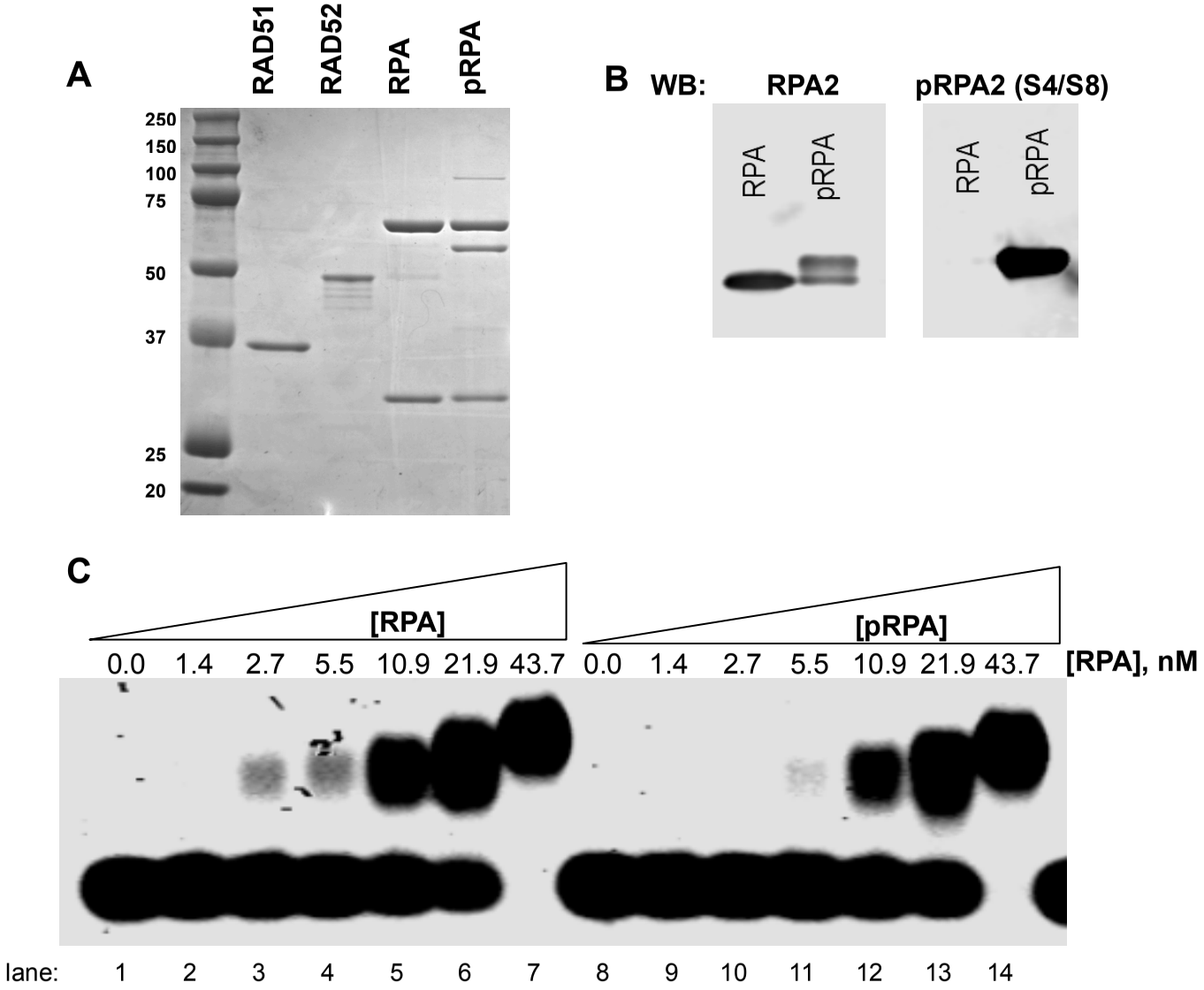
To study the effects of RPA phosphorylation, we used purified RPA and RPA that had been purified, hyperphosphorylated using HeLa cell extracts, and then re-purified, in the Borgstahl lab. We used purified RAD51 and RAD52 from the Jensen lab. We verified their purity (Figure 11A) and phosphorylation status of RPA (Figure 11B) using antibodies specific to the S4/S8 epitope on RPA.

We compared DNA binding of RPA and pRPA with 5nM of 80 nucleotide ssDNA oligomer. There was no significant consistent difference observed in DNA binding between the two proteins—they saturated the DNA at the same concentration, though in some cases either RPA or pRPA bound ssDNA at a slightly lower concentration, this effect was not seen consistently and there was not a significant difference (Figure 11C and 11D).

### RAD51 strand exchange is not affected by RPA phosphorylation *in vitro*

Strand exchange and annealing experiments were modeled on those in Jensen et al[157], which characterized the biochemical function of full length BRCA2, using one-third the concentrations of nucleotides and protein in our assays. We first verified the strand exchange activity of RAD51. RAD51 was

**Figure 11. RPA phosphorylation does not affect DNA binding. a.** Coomassie stain of purified RPA, pRPA, RAD51 and RAD52. **b.** Western blots of purified RPA, pRPA. **c.** ssDNA binding EMSA. RPA and pRPA were incubated with an 80-nucleotide ssDNA oligo with an infrared label for five minutes, cross-linked with glutaraldehyde, run on an agarose gel, and imaged on a Li-Cor Odyssey machine. **d.** Quantification of c. Bands were measured using Image Studio, points represent the percent of ssDNA signal bound by protein as a fraction of the total of the signal in each lane. Error bars represent SEM, n=3.


A
RAD51
RAD52
RPA
pRPA
250
150
100
75
50
37
25
20
B
WB:
RPA2
pRPA2 (S4/S8)
RPA
pRPA
RPA
pRPA
C
[RPA]
[pRPA]
0.0 1.4 2.7 5.5 10.9 21.9 43.7
0.0 1.4 2.7 5.5 10.9 21.9 43.7
[RPA], nM
lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

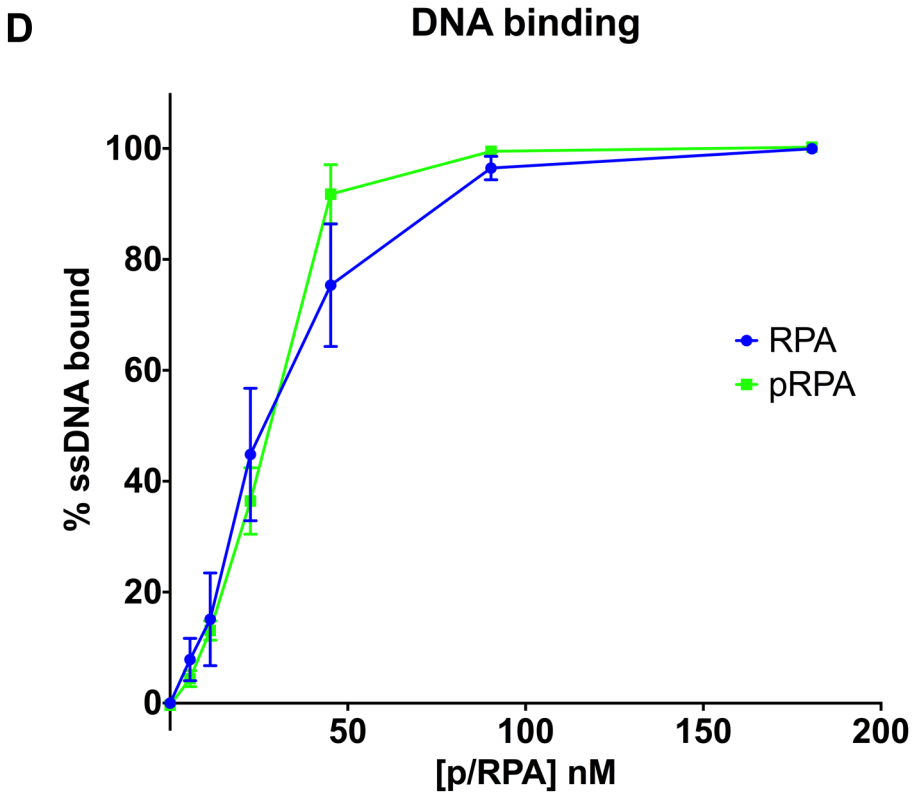
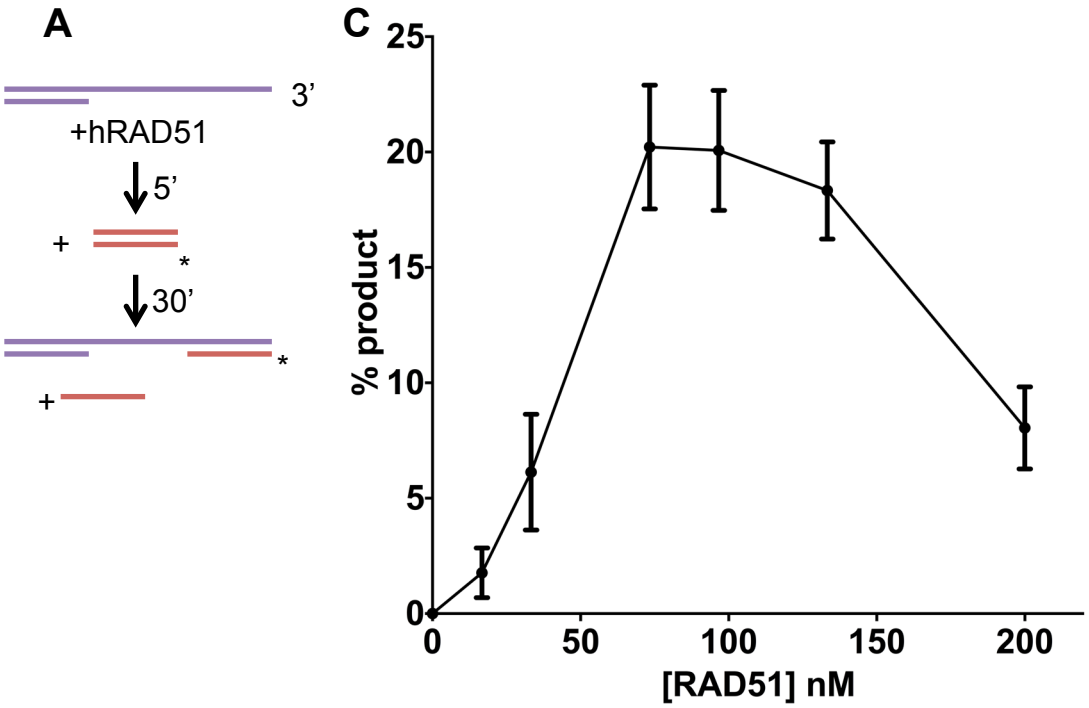



**Figure 11 (continued)**

incubated with DNA containing a 3' overhang (a 167 nucleotide oligo base-paired at the 5' end with a 40 nucleotide oligo). After 5 minutes, dsDNA was added, one of the strands of which is homologous to the end of the 3' overhang and is radiolabeled with $^{32}P$. After 30 minutes, the reaction was stopped and the samples were run on a polyacrylamide gel, imaged on a phosphorimager, and analyzed. The shifted band is the strand exchange product, and the amount of this product as a percentage of the signal in the lane as a whole was calculated. In agreement with published results, RAD51 activity was greatest around 73.3 nM, a 1:3 RAD51:nculeotide ratio, and activity was reduced at higher concentrations (Figure 12). RAD51 generally produced between 15 and 30 percent strand exchange products at this concentration.

We then looked at the effect of RPA phosphorylation on RAD51 exchange *in vitro.* Published studies show that incubation of ssDNA or DNA overhangs with RPA before the addition of RAD51 inhibits RAD51 exchange, due to the inability of RAD51 to form filaments by displacing RPA. For these experiments, we incubated RPA (or hypRPA) with the 3' overhang substrate for five minutes before adding RAD51 (Figure 13A). Adding increasing amounts of RPA up to 33nM reduced exchange activity, which is analogous to the concentration where inhibition was seen in the report by Jensen et al. There was no significant difference in the amount of inhibition by RPA versus hypRPA on RAD51 exchange inhibition (Figure 13). Thus, RPA phosphorylation does not prevent it from inhibiting RAD51-filament formation.

**Figure 12. RAD51 strand exchange. a.** Strand exchange protocol for b. and c. **b.** Autoradiogram of reaction. 3' overhang ssDNA was incubated with RAD51 for 5 minutes, labeled complementary dsDNA was then added for 30 minutes. The reaction was stopped, run on a polyacrylamide gel, and imaged on a phosphorimager. **c**. Quantification of b. Bands were quantified, and the signal of the exchange product as a percent of the total signal in each lane was determined and plotted. Error bars represent S.D., n=3.


A
3'
+hRAD51
5'
+
*
30'
*
+
C
% product
25
20
15
10
5
0
0
50
100
150
200
[RAD51] nM

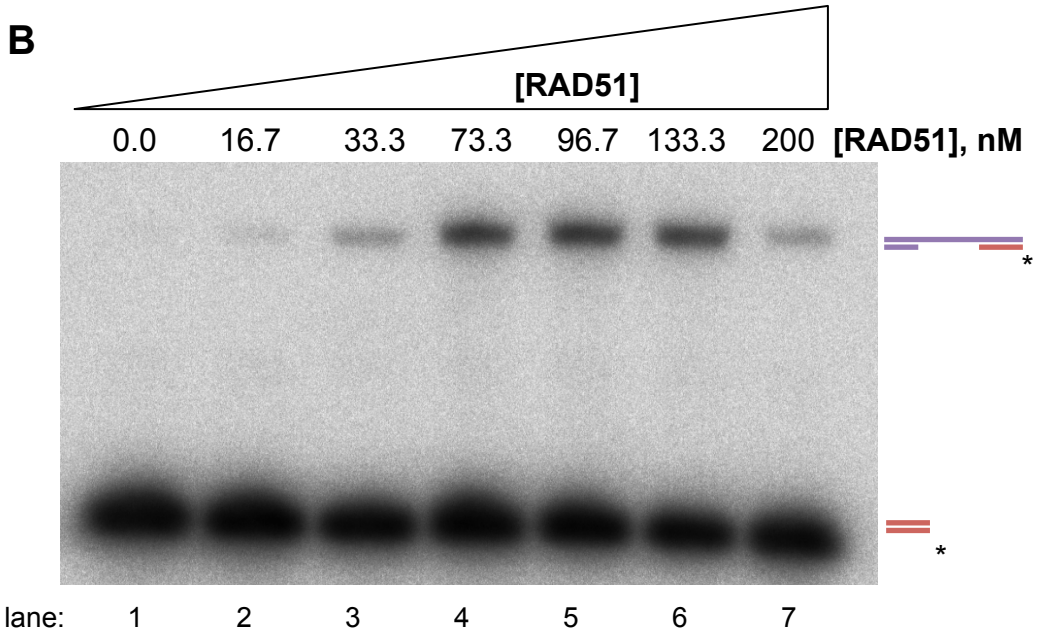
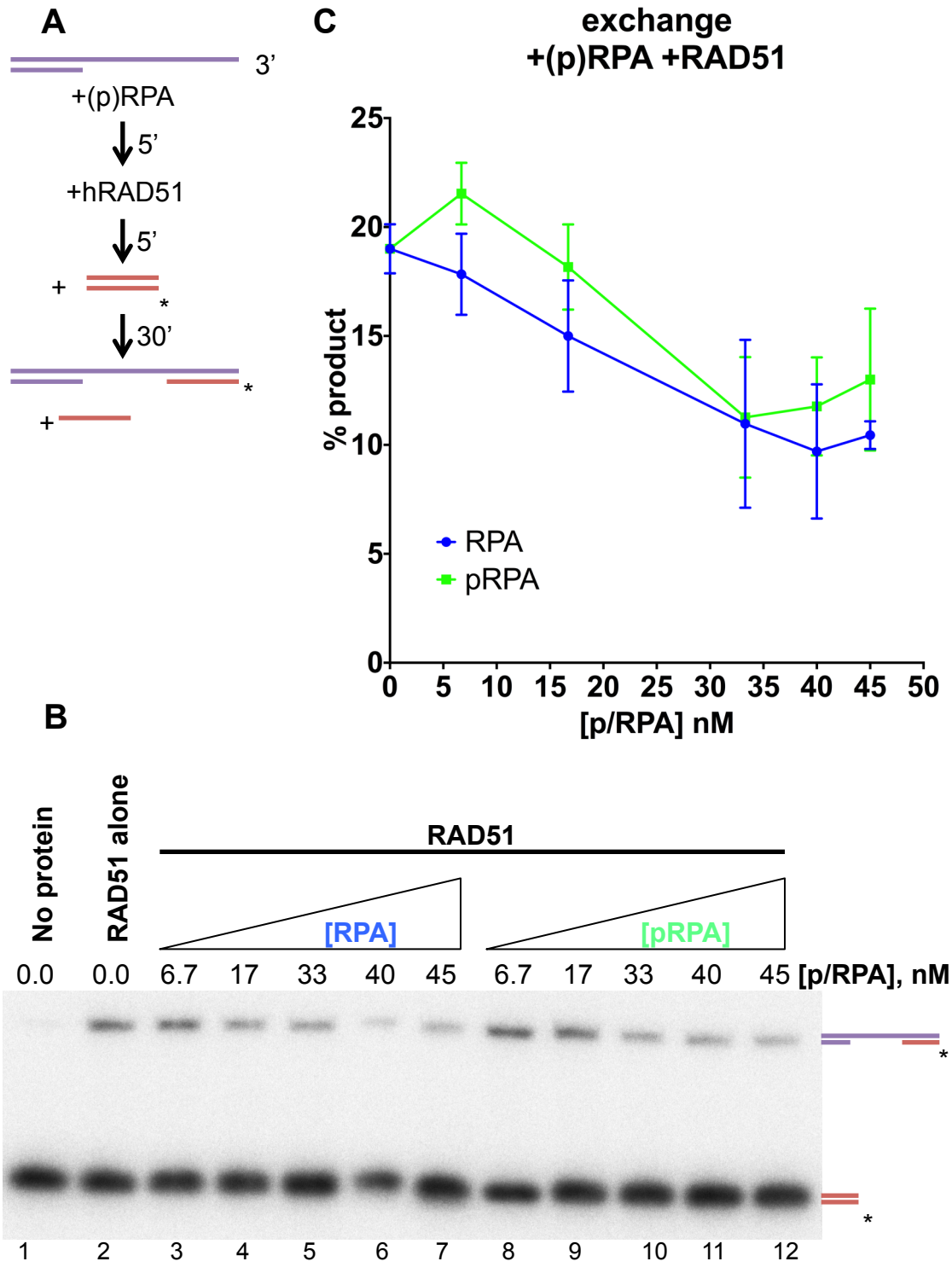

B
[RAD51]
0.0
16.7
33.3
73.3
96.7
133.3
200
[RAD51], nM
*
*
lane:
1
2
3
4
5
6
7

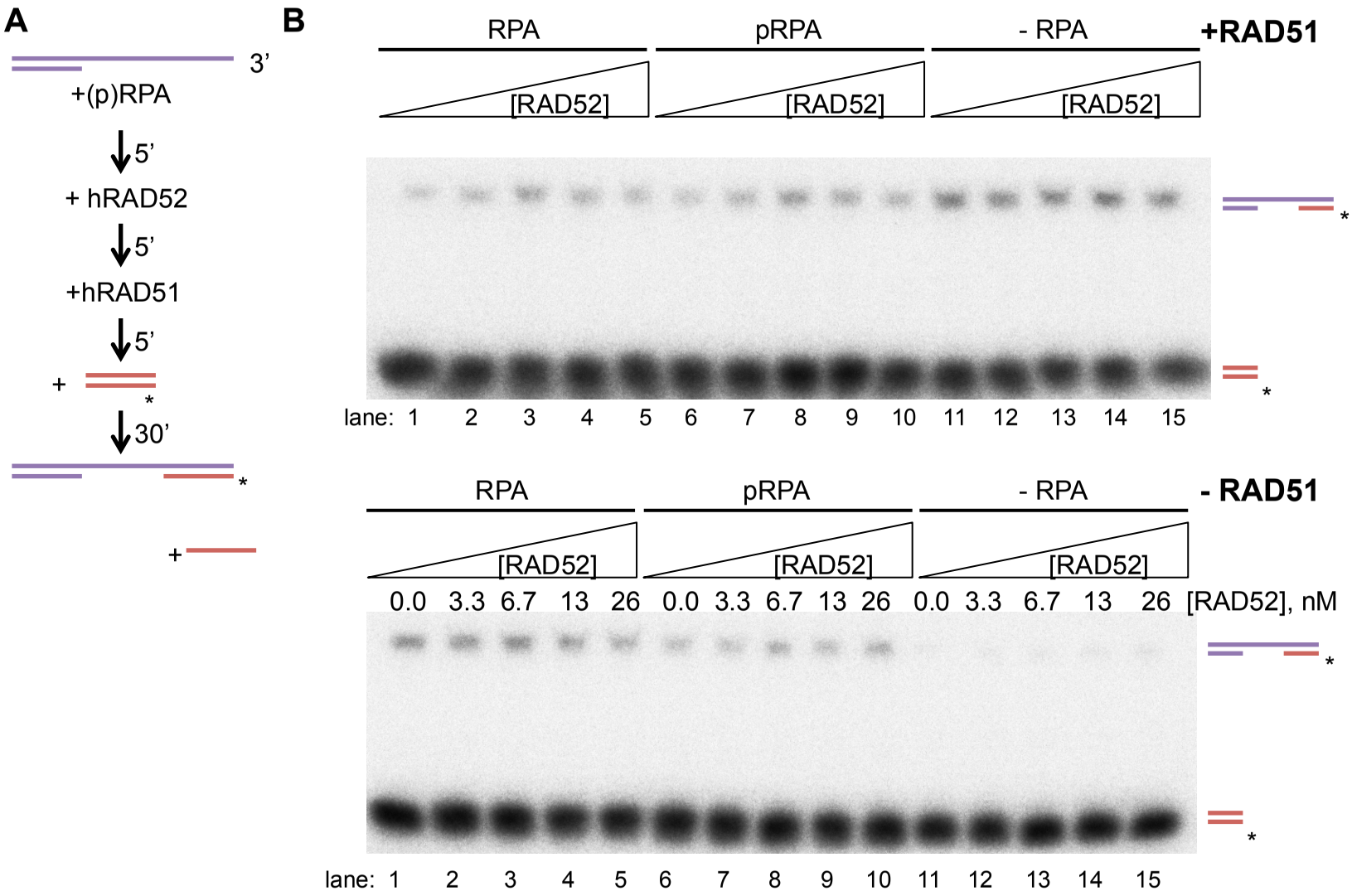
**Figure 13. RPA phosphorylation doesn't affect RAD51 strand exchange a.** Strand exchange protocol for **b**. and **c**. **b.** Autoradiogram of reaction with RPA and pRPA. 3' overhang ssDNA was incubated with RPA or pRPA for 5 minutes, RAD51 was added for 5 minutes, then labeled complementary dsDNA was then added for 30 minutes. The reaction was stopped, run on a polyacrylamide gel, and imaged on a phosphorimager. **c**. Quantification of b. Bands were quantified, and the signal of the exchange product as a percent of the total signal in each lane was determined and plotted. Error bars represent S.D., n=3.


A
3'
+(p)RPA
5'
+hRAD51
5'
+
*
30'
*
+
C
exchange
+(p)RPA +RAD51
% product
25
20
15
10
5
0
RPA
pRPA
0 5 10 15 20 25 30 35 40 45 50
[p/RPA] nM
B
No protein
RAD51 alone
RAD51
[RPA]
[pRPA]
0.0 0.0 6.7 17 33 40 45 6.7 17 33 40 45 [p/RPA], nM
*
*
1 2 3 4 5 6 7 8 9 10 11 12

**RAD52-mediated strand exchange is not promoted by RPA phosphorylation *in vitro***

Finally, we looked at the effect of RPA phosphorylation on RAD52's ability to stimulate RAD51 filament formation and exchange on RPA-covered ssDNA. For these experiments, we incubated the overhang DNA with RPA or phosphorylated RPA for five minutes, then added RAD52 and incubated for five minutes, then added RAD51 and incubated for five minutes, and finally added the labeled dsDNA (Figure 14A). RAD52 did not promote RAD51 exchange in the presence of RPA or phosphorylated RPA, and it did not promote RAD51 exchange in the absence of RPA (Figure 14B top and Figure 14C). The presence of some exchange activity with RPA and hypRPA alone is curious (Figure 14B, bottom lane 1 and 6 compared to 11), as RPA and hypRPA should inhibit exchange. It is possible this is duplex destabilization activity, however in Figure 13 we see that these concentrations do not promote more activity and actually inhibit RAD51 exchange compared to lower concentrations—adding more RPA in these conditions (up to 90nM) lead to the loss of inhibition (not shown), likely due to duplex destabilization activity. Nevertheless, we can still compare the effect of RAD52 on RPA inhibition, and we see no difference between RPA and hypRPA in their inhibition of RAD52 mediator function—there is no promotion of RAD51 exchange in either case. We also do not see strand exchange by RAD52 protein on its own as some previous publications observed (Figure 14B bottom, lanes 11-15). We conclude that there may be a factor in the cell not present in these reactions that allows hypRPA to promote RAD52 function, and that another factor or other conditions may exist that allows RAD52 to promote RAD51 exchange on RPA-ssDNA.

**Figure 14. RPA phosphorylation doesn't affect RAD52-mediated strand exchange a.** Strand exchange protocol for **b**. and **c**. **b.** Autoradiogram of reaction with and without RAD51. 3' overhang ssDNA was incubated with RPA, pRPA, or RPA buffer for 5 minutes, RAD52 or RAD52 buffer was added for 5 minutes, RAD51 or RAD51 buffer was added for 5 minutes, then labeled complementary dsDNA was then added for 30 minutes. The reaction was stopped, run on a polyacrylamide gel, and imaged on a phosphorimager. **c**. Quantification of b. +RAD51. **d.** Quantification of b. -RAD51. For c. and d., bands were quantified, and the signal of the exchange product as a percent of the total signal in each lane was determined and plotted. Error bars represent S.D., n=3.


A
3'
+(p)RPA
5'
+ hRAD52
5'
+hRAD51
5'
+
*
30'
*
+
B
RPA
pRPA
- RPA
+RAD51
[RAD52]
[RAD52]
[RAD52]
*
*
lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
RPA
pRPA
- RPA
- RAD51
[RAD52]
[RAD52]
[RAD52]
0.0 3.3 6.7 13 26 0.0 3.3 6.7 13 26 0.0 3.3 6.7 13 26 [RAD52], nM
*
*
lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

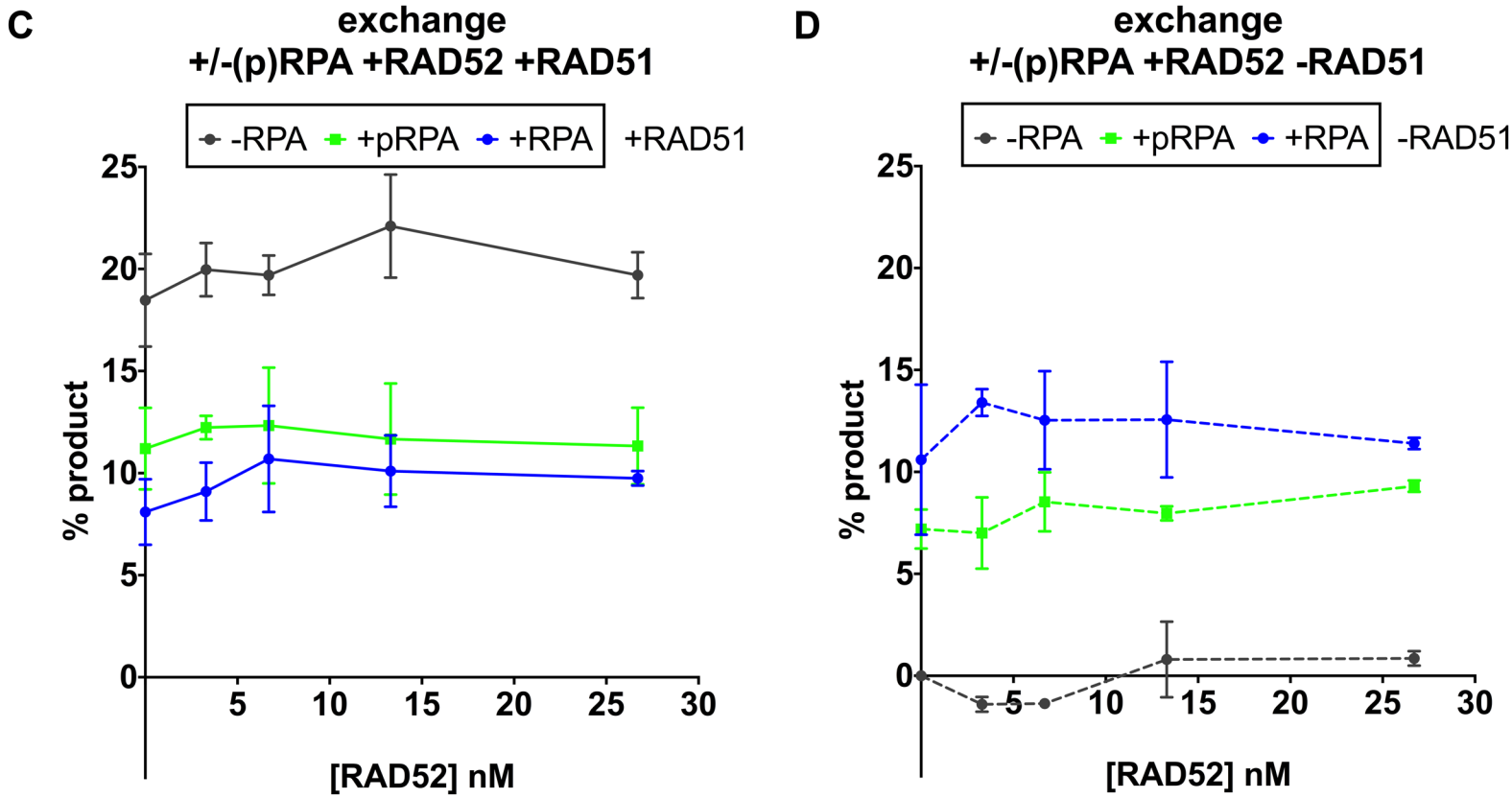



**Figure 14 (continued)**

**RAD52-mediated annealing is not promoted by RPA phosphorylation *in vitro***

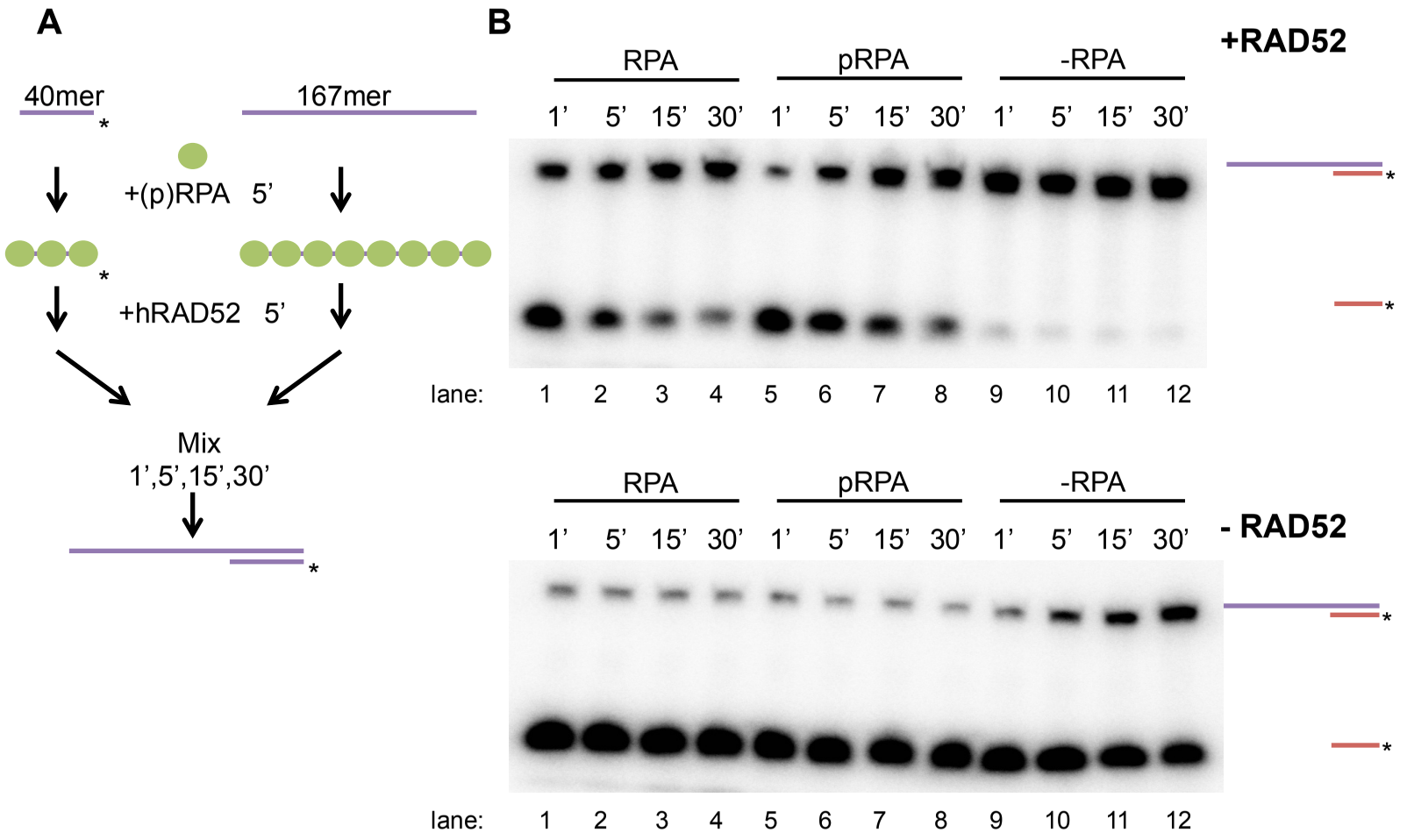
It is also possible that RPA phosphorylation affects RAD52 annealing activity. This is supported by evidence that RPA phosphorylation promotes RAD52-ssDNA contacts and the RPA-RAD52 interaction. To study this we used an *in vitro* strand-annealing assay with purified RAD52, RPA and hypRPA. We incubated complementary ssDNAs (one radiolabeled) separately with RPA or hypRPA for five minutes, then added RAD52 for five minutes, and then mixed the two solutions to allow annealing and stopped the reaction at 1, 5, 15, and 30 minutes (Figure 15A). RPA and hypRPA on their own reduced annealing compared to the control with no protein (Figure 15B, bottom). When RAD52 was incubated without RPA or hypRPA, in promoted almost 100 percent exchange (Figure 15B top, lanes 9-12). Adding RPA or hypRPA before RAD52 inhibited RAD52 annealing, and there was no significant difference between them (Figure 15B top, lanes 1-4 vs. 5-8). Thus, RPA phosphorylation does not promote RAD52 mediator function or annealing *in vitro* under the conditions tested.

## MATERIALS AND METHODS

***Purification of proteins***:

RPA and phosphorylated RPA were purified in Gloria Borgstahl's lab as described previously (Deng et al, Biochemistry, 2009)[216]. To generate phosphorylated RPA, RPA was purified as previously described and then mixed with HeLa extracts supplemented with an ATP regenerating system, ssDNA, and phosphatase inhibitors. The phosphorylated RPA was then purified again in the same was as previously.

**Figure 15. RPA phosphorylation doesn't affect RAD52-mediated strand annealing a.** Strand annealing protocol for **b**. and **c**. **b.** Autoradiogram of reaction. Complementary ssDNAs in separate tubes were incubated with RPA, pRPA, or buffer for 5 minutes, RAD52 was added for 5 minutes, and then the ssDNAs were mixed. Aliquotes were added to stop buffer at 1, 5, 15, and 30 minutes, run on a polyacrylamide gel, and imaged on a phosphorimager. **c**. Quantification of b. Bands were quantified, and the signal of the annealed product as a percent of the total signal in each lane was determined and plotted. Error bars represent S.D., n=3.


A
40mer
167mer
*
+(p)RPA 5'
*
+hRAD52 5'
Mix
1',5',15',30'
*
B
+RAD52
RPA
pRPA
-RPA
1' 5' 15' 30' 1' 5' 15' 30' 1' 5' 15' 30'
lane: 1 2 3 4 5 6 7 8 9 10 11 12
- RAD52
RPA
pRPA
-RPA
1' 5' 15' 30' 1' 5' 15' 30' 1' 5' 15' 30'
lane: 1 2 3 4 5 6 7 8 9 10 11 12

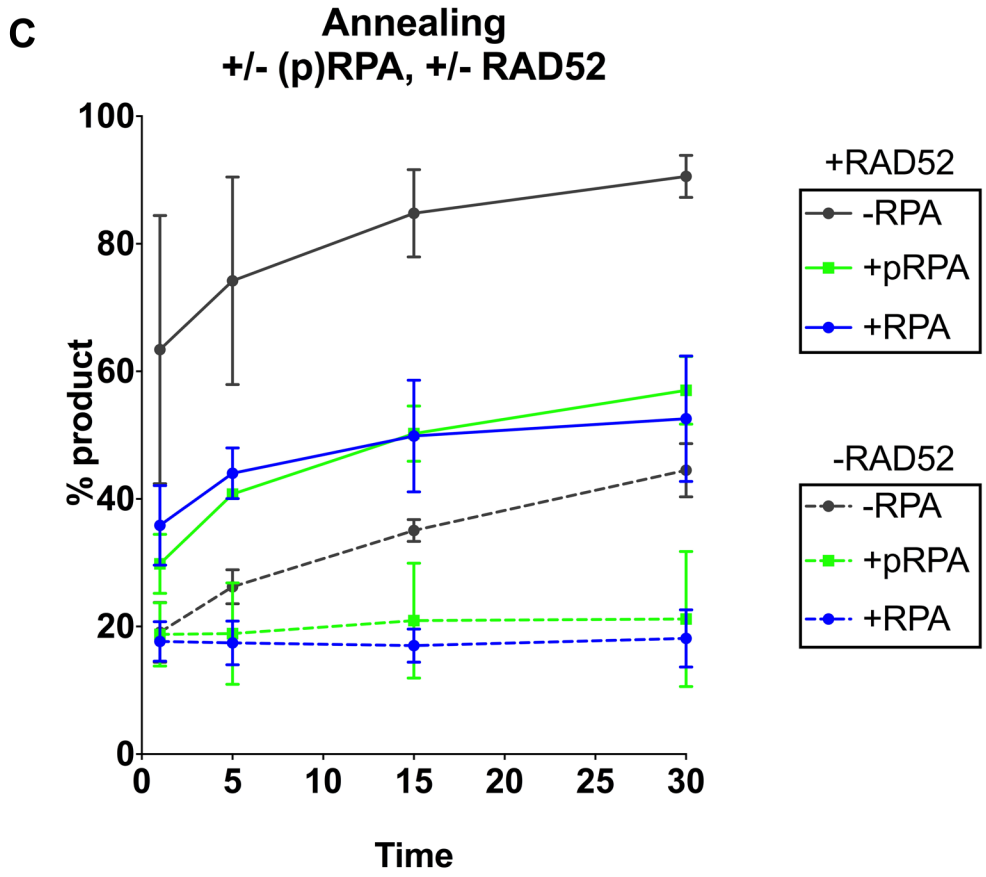



**Figure 15 (continued)**

RAD51 and RAD52 were purified in Ryan Jensen's lab as described previously (Jensen et al, Nature, 2009)[157].

***DNA binding***

RPA and pRPA were incubated at indicated concentrations in 35mM HEPES pH 7.5, 1mM DTT, and 5nM 80mer (5' IRD700 labeled oligo with sequence: TT TGT TAA AAT TCG CGT TAA ATT TTT GTT AAA TCA GCT CAT TTT TTA ACC AAT AGG CCG AAA TCG GCA AAA TCC CTT ATA, ordered from IDT HPLC purified) at 37 degrees for 5 min. Reactions were then cross-linked for 10 minutes at room temperature with 0.2% gluteraldehyde, and stopped with 100mM Tris pH 8.0. Reactions were loaded on a 1% agarose gel for 2 hours, and imaged on a Li-Cor Odyssey CLx.

***DNA strand exchange and single-stranded DNA annealing assays***

Based on experiments in Jensen et al*., Nature*, 2010, and performed as follows:

Oligonucleotide substrates were obtained from Sigma or IDT and were purified by polyacrylamide gel electrophoresis (PAGE). The following oligonucleotides were used:

*RJ-167-mer*

(5',CTGCTTTATCAAGATAATTTTTCGACTCATCAGAAATATCCGTTTCCTAT ATTTATTCCTATTATGTTTTATTCATTTACTTATTCTTTATGTTCATTTTTTAT ATCCTTTACTTTATTTTCTCTGTTTATTCATTTACTTATTTTGTATTATCCTT ATCTTATTTA-3′);

*RJ-PHIX-42-1*

(5′,CGGATATTTCTGATGAGTCGAAAAATTATCTTGATAAAGCAG-3′);

*RJ-Oligo1* (5′,TAATACAAAATAAGTAAATGAATAAACAGAGAAAATAAAG-3′);

*RJ-Oligo2* (5′,CTTTATTTTCTCTGTTTATTCATTTACTTATTTTGTATTA-3′).

To generate the 3′ tailed DNA substrate, RJ-167-mer annealed at a 1:1 molar ratio to RJ-PHIX-42-1. The dsDNA was generated by radio-labelling RJ-Oligo1 with $^{32}$P at the 5′-end and annealing it to RJ-Oligo2. The ssDNA substrate was RJ-167-mer radio-labelled with $^{32}$P at the 5′-end.

***DNA strand exchange assays***

The assay buffer contained 25 mM Tris acetate (pH 7.5), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 µg/µl BSA, 2 mM ATP and 1 mM DTT. All pre-incubations and reactions were at 37 °C. The DNA substrates and proteins were at the following concentrations unless otherwise indicated in the figure legend: p/RPA (33 nM); RAD51 (73 nM); 3′ tail (1.3 nM molecules); and dsDNA (1.3 nM molecules). Where proteins were omitted, storage buffer was substituted. The reaction was terminated with Proteinase K in 0.5% SDS for 10 min. The reactions were loaded on a precast 6% polyacrylamide gel in TBE buffer (Life Technologies) and electrophoresis was at 30 V for 120 min. The gel was then dried and exposed to PhosphorImager screen overnight. The screens were scanned on a Fujifilm FLA-7000 phosphorimager and bands quantified using Quantity One. The percentage of exchange product was calculated as the radio-labelled product divided by the total radio-labelled input DNA in each lane.

***Single-stranded DNA annealing assays****:*

Cold 167-mer at 4 nM (molecules) and 5′ radio-labelled oligo 1 at 2 nM (molecules) were each incubated separately in 10 µl reactions containing 25 mM Tris acetate (pH 7.5), 1 mM $MgCl_2$, and 1 mM DTT for 5 min with RPA(50 nM), pRPA(50 nM) or storage buffer. The 40-mer is complementary to the 167-mer at the 3′ end. All incubations were at 37 °C. The oligonucleotides were then incubated with either RAD52 (100 nM) or protein storage buffer for

5 min. The two separate reactions were then mixed and incubated for 1, 5, 15 or 30 min to allow for annealing. At the indicated time points aliquots were removed and added to stop buffer (4 mg/ml proteinase K, 1% SDS and 0.2 µM unlabelled Oligo2 (complementary to Oligo1) for 15 min. Loading dye was then added to the samples and they were run on precast 6% polyacrylamide gels in TBE buffer (Life Technologies) for 2 h at 30 V. The gels were dried onto Whatman paper and exposed to a PhosphorImager screen overnight. The screens were scanned on a Fujifilm FLA-7000 phosphorimager and bands quantified using Quantity One. The percentage of annealed product was calculated as the radio-labelled product divided by the total radio-labelled input DNA in each lane.

*Protein buffers*

RPA and pRPA: 30mM HEPES, 0.5% inositol, 0.5mM EDTA, 1mM DTT, 250mM KCl. Final pH is 7.8.

RAD52: 50 mM Tris-Cl (pH=7.5), 200 mM KCl, 15% Glycerol, 10 mM Beta-mercaptoethanol.

RAD51: 20 mM HEPES (pH=7.5), 150 mM NaCl, 0.1 mM EDTA, 10% Glycerol, 2 mM B-me.

# CHAPTER FOUR

# DISCUSSION

## RAD52's function as an alternative mediator

Studies of RAD52 in vertebrates show no or a limited role for RAD52 in HR, as there is little or no phenotype associated with RAD52 loss[93,159,160], and BRCA2 is the primary mediator of RAD51 filament formation in humans and other vertebrates[157,158]. Despite RAD52's apparent lack of mediator activity in humans, recent evidence suggests that hRAD52 provides an alternative mediator pathway to BRCA2[161,162]. In cells lacking both BRCA2 and RAD52 there is increased chromosomal instability, pointing to HR deficiency, and RAD52 is necessary for RAD51-mediated HR in BRCA2-deficient cells[162]. RAD52 is also synthetically lethal with BRCA1 and PALB2[392], and HR and RAD51 foci are dependent on RAD52 in BRCA1 and PALB2 depleted cells[392]. The RAD51 paralogs also likely function with BRCA2 and not with RAD52, since RAD52 is synthetically lethal in chicken DT40 cells with XRCC3[389], BRCA2 is epistatic to the paralogs in response to DNA damage as measured by cellular survival after MMC[432], and both paralog complexes are epistatic to BRCA2 and synthetically lethal with RAD52 in human cells[433]. Several lines of evidence suggest this RAD52-backup pathway functions independently of the BRCA pathway: RAD52 localization to damage and colocalization with RPA2 and ssDNA is not affected by BRCA2-status[162], RAD52 interacts with RAD51 independently of BRCA2[162], RAD52 foci formation is normal in the absence of BRCA1[392] and the Rad51 paralogs (XRCC2, XRCC3, RAD51C)[391], RAD52 does not interact with BRCA2[157], there is little colocalization of BCCIP (a BRCA2-interacting protein) and RAD52 while both localize with RAD51 separately[386], and synthetic lethality has been observed with BRCA1, BRCA2,

and PALB2, and RAD51 paralogs, resulting in reduction in HR and viability after DNA damage[162,392,433].

RPA hyperphosphorylation has been shown to play a role in the repair of replication associated DSBs through HR. Several studies have shown that RPA phosphorylation defects lead to sensitivity to DNA damaging agents[112,230,249] and persistent DNA breaks[112,204,233,249]. Phosphorylation mutants can associate with damage sites[253], supporting a model where RPA can localize to breaks and sites of stress without phosphorylation but RPA function at damage sites and recruitment of downstream factors is impaired when the regulation of hyperphosphorylation is defective. RPA phosphorylation has also been shown to play a role in HR, as RAD51 foci formation[230,249] and HR by the DR-GFP assay[230,249,268] are reduced in phosphorylation mutants, and aberrant SCEs are increased in phosphorylation mutants[231]. RPA phosphorylation also affects its interactions with HR and DNA damage response proteins including MRN[245,267], p53[210,268], RAD51[230,255,268], PALB2[236], and ATR[255]. Thus, RPA phosphorylation is important for the BRCA DNA repair pathway. However, the effect of RPA phosphorylation on the RAD52 mediator pathway was previously not well understood.

There is published evidence that RPA phosphorylation is also important for the RAD52 pathway. RPA phosphorylation promotes its interaction with RAD52[255], hypRPA co-localizes with RAD52 in foci[255], and RAD52 focus formation is reduced in an RPA2-A mutant[268]. The RPA-ssDNA-RAD52 complex is more stable when it includes hypRPA compared to unphosphorylated RPA, and more RAD52 cross-links to ssDNA when the RPA in the complex is hyperphosphorylated[216]. Our data in cells supports this

evidence that RPA phosphorylation promotes its interaction with RAD52 and RAD52 function as an alternative mediator.

**RAD52 mediator function in human cells is promoted by RPA phosphorylation**

In this study we showed that in BRCA2-deficient cells, RAD52-dependent HR as measured by RAD51 foci and the DR-GFP assay are dependent on functional phosphorylation and dephosphorylation of RPA. This supports the importance of RPA and its phosphorylation in both the BRCA and RAD52 pathways of HR. While it is possible that these results are due to the effects of residual levels of BRCA2 protein, the relationship between RPA and RAD52, as well as our results and previous publications showing that phosphorylation regulates their interaction, suggests that RPA phosphorylation is important to RAD52 function.

Furthermore, our results show that RPA phosphorylation promotes the association of RAD51 and RAD52 by colocalization, as there is more colocalization of RAD51 and RAD52 in RPA2-WT cells than in RPA2-A mutant cells. We show that the association of RAD51 and RAD52 is DNA-damage dependent, further supporting a role for RAD52 in facilitating RAD51 function in DNA repair. While we might expect that the percent of cells in which RAD52 colocalizes with RAD51 should increase when BRCA2 is depleted, this was not observed, suggesting that in normal cells RAD52 colocalizes with RAD51, a fraction of this is due to RAD52 mediator function, and the absence of BRCA2 does not enhance its mediator activity. In both BRCA2-proficient and BRCA2-depleted cells, the percentage of cells with RAD52 foci that colocalize with RAD51 is reduced in RPA2-A cells compared to RPA2-WT, suggesting that RAD52 requires functional phosphorylation of RPA to recruit RAD51.

Although this result could also simply be due to the presence fewer RAD51 foci in RPA2-A cells, considered in conjuction with the result that there are fewer RAD52-dependent (in BRCA2-depleted cells) RAD51 foci and less RAD52-dependent HR in RPA2-phosphomutant cells, it is clearer that RPA phosphorylation and dephosphorylation promote RAD52-dependent RAD51 recruitment. Furthermore, although the result was not significant, a smaller percent of RAD51 foci colocalize with RAD52 in RPA-phosphomutant cells compared to RPA2-WT cells in both control and BRCA2-depleted cells. We do not see an increase in the percentage of cells with RAD51 foci that colocalize with RAD52 when BRCA2 is depleted, such that RAD52 recruitment of RAD51 is not affected by BRCA2 loss, supporting its independence from the BRCA pathway. Also, it is possible that the levels of RAD51 foci are higher than they should be in these experiments, as overexpression of RAD52-GFP, which was also transfected in these experiments, has previously been shown to promote RAD51 foci formation and HR[298,384,387,388]. Finally, preliminary evidence not shown here indicate that RAD52-depletion does not cause a further reduction of DR-GFP recombination in RPA2-phosphomutant cells compared to RPA2-WT cells, supporting our model in which RAD52 and RPA phosphorylation function in the same pathway. As a whole, these data show that RPA phosphorylation is important to RAD52 mediator function.

Depleting RAD52 in BRCA2-depleted RPA phosphorylation mutant cells was not technically feasible using our system, however this would show directly that RPA phosphorylation mutants are important to RAD52 mediator function. We would expect RPA phosphorylation mutants to cause even more toxicity and HR deficiency in BRCA2 and RAD52 depleted cells compared to RPA2-WT cells. Other endpoints that we could use to show that RPA

phosphorylation is important to RAD52 function are the resolution of γH2AX foci and cell survival.

**RPA phosphorylation and RAD52-RPA interaction in human cells**

Unlike some previous publications, we saw no effect of RPA phosphorylation on levels of RPA foci[204,241,252,253,262]. We did see a reduction in the association of RAD52 with phosphorylation mutants by IP and colocalization, in agreement with published literature[216,255]. This suggests that the reduction in RAD52-dependent HR in phosphorylation mutants is due to impaired interactions between RAD52 and RPA. Paradoxically, though RPA phosphorylation promotes RAD52-dependent HR and improved its interaction with RAD52 in our studies and others, RAD52 foci form normally in RPA phosphorylation mutant cells after damage. It is possible that although RAD52 is recruited to foci independently of RPA phosphorylation, either through its interactions with RPA or through its own DNA binding activity, that the association between RAD52 and RPA is not efficient with phosphorylation mutants, or that the lack of cycling between phosphorylation and dephosphorylation prevents a hand off of tighter ssDNA contacts from RPA to RAD52, thereby causing defective HR. It is also possible that RPA phosphorylation mutants fail to recruit other factors important for RAD52 to function, for example, RAD51. Interestingly, while after damage RAD52 forms foci normally in RPA phosphorylation mutant cells, in RPA2-D untreated cells there is an increase in RAD52 foci formation to the level of CPT-treated cells. This could mean that phospho-mimic RPA2 signals to RAD52 that there appears to be DNA damage in untreated cells, or there could be buildup of DNA damage even in untreated RPA2-D mutant cells due to the mimic of constitutive RPA2 phosphorylation that results in RAD52-recruitment. On the

whole, our data suggest that it is the faulty association between RAD52 and RPA that leads to the failure of RAD52 to recruit RAD51 and therby promote HR in BRCA2-depleted cells.

It would be interesting to see if there is a difference in the interaction of RPA and RAD52 in the absence of BRCA2. The independence of the RAD52 pathway, and the lack of effect of BRCA2 on RAD52 function, suggests that there would be no difference in this association between BRCA-proficient and deficient cells. Additionally, a reverse IP pulling down with a RAD52 antibody would confirm the interaction deficiency of the RPA phospho-mutant. There may be differences at different timepoints and with different DNA damaging agents. Experiments using phospho-specific antibodies would also confirm these results by IP and colocalization. Preliminary evidence not shown here suggests that endogenous phosphorylated RPA does not preferentially colocalize with RAD52, however this may be due to the specific timepoint studied or phospho-site used. We used a pS4/8 antibody for these experiments; it is possible that other phosphorylation sites mediate the RAD52-RPA interaction. Using phosphorylation mutants with mutations at individual damage sites, or using a series of different phospho-specific antibodies, to determine which phosphorylation sites are important, could resolve this issue.

**RPA phosphorylation and RAD52 biochemical mediation**

We show that RAD51 is not able to replace RPA and form filaments on ssDNA on its own, as previously shown, and also that including phosphorylated RPA has no effect on this RPA inhibition. We also found that RPA phosphorylation does not promote RAD52 mediator function *in vitro* to allow RAD52 to mediate RAD51 filaments on RPA coated ssDNA like BRCA2

or yeast RAD52, under the conditions used. This suggests that there either there are other biochemical conditions necessary for hRAD52 mediator function, or there is a factor present in cells not included in the reaction that allows RAD52 to function as a mediator in the absence of BRCA2. This finding may also be attributable to a limitation of the assay: in cells, phosphorylation and dephosphorylation of RPA is needed for HR and this is under complex regulation, and phosphorylation at different sites may have different functions. Thus, the failure of phosphorylated RPA to promote RAD52-mediator function in our *in vitro* assays may be because phosphorylation of RPA needs to be cycled on and off to promote RAD52 function, or because we are lacking the specific phosphorylation pattern that promotes RAD52 function in cells.

It is possible that the reactions could be optimized further to show a difference between RPA and pRPA inhibition and its effect on RAD52 mediation. Other publications showed greater RAD51 activity on its own (up to 50%), and our background activity was high in the presence of RPA, possibly due to helix destabilization activity of RPA. Other conditions that may allow RAD52 to promote RAD51 mediation *in vitro* include adding RPA after RAD51 and RAD52 or at lower concentrations, including RAD51 at suboptimal concentrations, or including ammonium sulfate or spermidine, which have been shown to improve RAD51 activity[157,413,417,427,428]. Nevertheless, we see no difference in RAD52 mediator activity between RPA and phosphorylated RPA.

It may be that even though including phosphorylated RPA does not allow RAD52 to remove RPA from ssDNA, that phosphorylation of RPA in cells promotes RAD52 function through some other mechanism, such as recruiting another factor. There may be other biochemical conditions that

would show this effect, and these results could be negative because we are not seeing enough activity of RAD51 on its own and have too much background of RPA on its own. Perhaps, as in cells there is clearly a need for phosphorylation and dephosphorylation, cycling phosphorylated RPA and nonphosphorylated RPA *in vitro* would promote the reaction.

**RPA phosphorylation and RAD52 annealing**

We also show that RPA phosphorylation does not promote RAD52 annealing activity in vitro. It is possible that similar to RAD52 mediator function, there is some factor or condition in cells that allows RPA phosphorylation to promote RAD52 annealing, however there was no difference in the conditions tested. It would be interesting to see the effect of RPA phosphorylation on SSA *in vivo*. The impaired interaction between RPA and RAD52 in RPA phosphorylation mutants suggests that SSA in cells would be reduced in these phosphorylation mutants.

**Other potential partners of RAD52 in backup mediation**

RAD52 appears to be important for back-up mediation of RAD51 in human cells, however it has not been shown to be effective *in vitro*, suggesting a few possibilities. Some other function of RAD52, such as ssDNA annealing, and not RAD52-dependent promotion of RAD51 function, may be responsible for the synthetic lethal relationship. This is unlikely, since data including the dependence of RAD51 foci and HR on RAD52 in the absence of the BRCA pathway, suggests that RAD52 is acting as a mediator in the absence of BRCA2. Alternatively, RAD52's inefficient yet present mediation of RAD51 that has been shown *in vitro* may be sufficient *in vivo* for the synthetic lethality. Finally, there may be some other factor in cells that has not been tested in *in vitro* assays that allows RAD52 to mediate RAD51 displacement of RPA.

There are some candidates for RAD52 functional partners beyond RPA and RPA phosphorylation. Perhaps *in vitro* assays containing other known vertebrate SSBs, like a-RPA, or hSSB1 and hSSB2, will promote RAD52 function. Evidence that these proteins are important specifically in DNA damage repair and not in DNA replication supports this hypothesis. RPA4 replaces RPA2 in some RPA complexes in cells to form a-RPA, contains an N-terminal putative phosphorylation domain[199,299,300,302-304], preferentially binds damaged DNA[301], supports DNA repair through recombination, localizes to damage, interacts with RAD51 and RAD52, and supports RAD51 strand exchange *in vitro*[300-302]. This suggests that it is important at DSB sites, and therefore could play a role in promoting RAD52 function. hSSB1 and hSSB2[305-321] also function in the DNA damage response: hSSB1 forms foci in response to DSBs but not replication foci[313], hSSB1/2 and their SOSS partners have been shown to play a role in HR repair and ATM checkpoint signaling, and cells defective in these proteins have hypersensitivity to damaging agents, chromosomal instability, reduced HR and RAD51 recruitment, and reduced ATM-dependent phosphorylation[306,308,311,313]. hSSB1 and hSSB2 are therefore potential partners of RAD52. RPA4 and hSSBs are not well characterized, but it would be interesting to study their interactions with RAD52 in cells and their effect on RAD52 mediator function *in vitro*.

Beyond BRCA2 and RAD52, there are other proteins that play a role in mediating and supporting RAD51 function. These include the RAD51 paralogs, PALB2, SWS1, SWSAP1, RAD54, RAD54B, SWI5, and SFR1[463]. The RAD51 paralogs and PALB2 have been suggested to be epistatic to BRCA2 and not RAD52, but these other proteins are potential RAD52 mediator partners.

SWS1, an ortholog of the yeast Shu complex, and SWSAP1, which interacts with SWS1 and has a predicted RecA-core, have been proposed to act as recombination mediators[488,489]. SWS1 also interacts with RAD51D, while SWSAP1 interacts with RAD51 and most of the RAD51 paralogs and its depletion causes defects in HR. These proteins may function epistatically with RAD52, although evidence that RAD52 is not epistatic to the RAD51 paralogs and that SWS1 and SWSAP1 function with the paralogs suggests otherwise. Similarly, double mutants of RAD52 and RAD54 in mice have exacerbated MMC survival reduced survival of bone marrow cells after IR[390], suggesting these proteins are not epistatic.

Finally, the SWI5-SFR1 complex, which is related to the *S. pombe* Swi5-Sfr1 and *S. cerevisiae* Sae3-Mei5 complexes, may act as a mediator and function with RAD52. These proteins function only in meiosis in budding yeast. In mouse, Swi5 and Sfr1 are nuclear proteins; deletion mutants in ES cells are sensitive to DNA damaging agents, have increased chromosomal aberrations, and reduced PARP-inhibitor-induced SCE supporting a role for the complex in homology based DSB repair[493]. Expression of a BRC repeat has been shown to inhibit RAD51 focus formation and to decrease HR in mammalian cells, likely by sequestering RAD51 in cells[340,346,349,494]; in Sfr1 and Swi5 deleted cells, though their loss did not decrease HR frequency on their own, expression of the BRC repeat reduced HR even more than in WT, again implicating these proteins in a role supporting RAD51[493]. In humans cells, SWI5-SFR1 depletion reduced RAD51 foci and also enhanced sensitivity to IR[495]; *in vitro* the complex interacts with RAD51 and stimulates RAD51-strand exchange by stabilizing the filament[496] paradoxically by enhancing ATP

hydrolysis by RAD51 and facilitating the release of ADP from the presynaptic filament to keep it in its active ATP bound form[497].

These effects are interesting due to the function of the complex in *S. pombe*, which may be relevant to the mediator proteins in human cells. While *S. cerevisiae* Rad52 protein promotes Rad51-dependent DNA-strand exchange on RPA-coated DNA by itself, *S. pombe* Rad52 (Rad22) cannot. Though Rad22 is important to overcome the inhibitory effect of RPA, it requires the Swi5–Sfr1 complex in a downstream step to stabilize the Rad51 filament, so it can progress to strand exchange[480,498,499]. Although it is possible this effect is due to differences between *S. pombe* and *S. cerevisiae* RAD51, it could be because of differences in Rad52 mediator activity between budding and fission yeast. Similar to RAD52 knockouts, mouse cells in which the *Swi5* or *Sfr1* are knocked out are viable and display no defect in HR. Thus, it is plausible that RAD52 in humans and other mammals requires the SWI5-SFR1 complex to perform its mediator function.

**RAD52 roles in BRCA-proficient cells**

While RAD52 appears to play a backup mediator role in the absence of the BRCA pathway, its role in cells with a functional BRCA pathway is unclear. Despite RAD52's lack of severe HR phenotype, there are some recombination phenotypes of RAD52, suggesting that RAD52 does play a role even in BRCA-competent cells. Targeted integration frequencies are reduced in RAD52-null chicken cells[160], and inactivation of RAD52 in mouse ES cells causes a reduced frequency of HR[160], showing a clear role for RAD52 in genetic recombination. RAD52-null mutant mouse cells have no detectable HDR defect measured with I-SceI reporter constructs[93], but depletion of RAD52 has been shown reduce levels of HR repair of nuclease breaks in

some cases, although the effect is not as dramatic as would be expected for an essential HR protein[162]. Overexpressing RAD52 in monkey cells confers resistance to IR and increases levels of HR[387], while in mouse cells overexpression increases survival and RAD51 foci after MMS and IR[384] and inhibits gene targeting while stimulating HR between separate transfected plasmids[388]. Thus, mutations in RAD52 do affect HR. Furthermore hRAD52 interacts with RAD51 and RPA[292-294,298,376,381,384-386], though these interactions map to regions in which it does not share homology with yeast Rad52, suggesting species-specific functions of the interactions. RAD52's synthetic lethality with factors in DNA repair also point to a role for RAD52. RAD52 foci are induced and immobilized to a greater extent by hydroxyurea, which stalls replication forks, than by ionizing radiation, suggesting its role is related to replication damage[386].

RAD52 annealing, which is effective *in vitro* and i*n vivo* while BRCA2 annealing is not, may play a role in BRCA-proficient cells. The SSA pathway is clearly dependent on RAD52[93], and RAD52 has been proposed to be important for second-end capture, and for SDSA downstream in HR[295,413,416,418,422]. Yeast RAD52 mutations are more toxic than RAD51 mutations, which is believed to be due to scRAD52's dual function as both a mediator and an annealer. In contrast, loss of hRAD52 is not lethal to cells and BRCA2 is incapable of annealing RPA-ssDNA, suggesting that these annealing pathways are not necessary for functional DSB repair in cells, or that there is some redundancy in pathways or other proteins in cells that can compensate for the loss of RAD52. What factors function with RAD52 to promote its annealing and back-up mediation function in cells is unclear, as

most double knockouts tested suggest that RAD52 is synthetically lethal with the BRCA pathway including RAD51 paralogs.

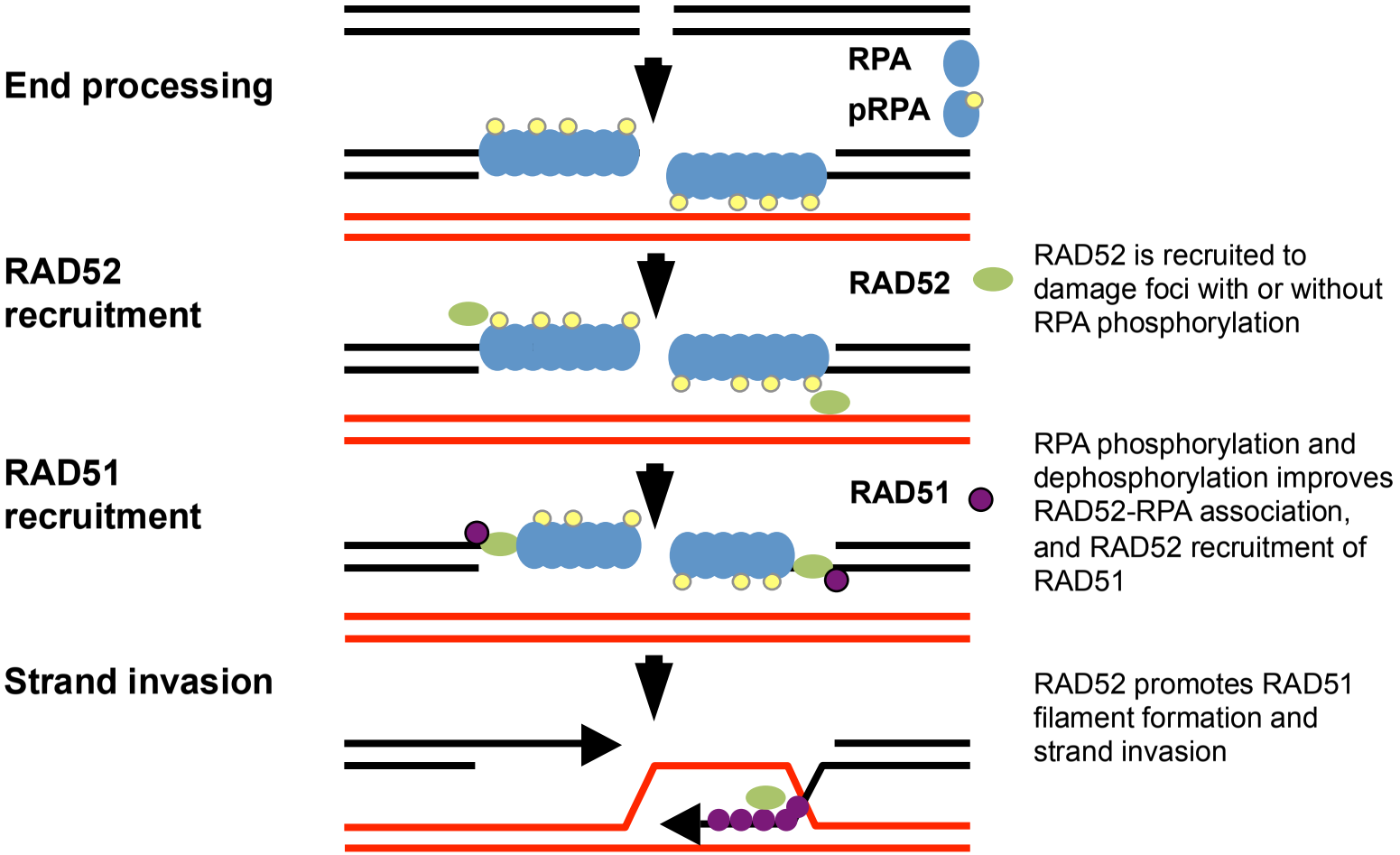
Insight into RAD52s function in BRCA-proficient cells comes from its interactions with other proteins. For example, MUS81-induced DSBs generated by CHK1 inhibition are independent of RAD51, and depend on RAD52. Recovery from replication stress in RAD52-depleted cells requires MUS81, and loss of both these proteins results in increased RAD51 foci formation that is toxic and massive cell death that can be suppressed by RAD51 depletion. Thus, a RAD52/MUS81-dependency exists in checkpoint-deficient cells, and this suggests that RAD52 is associated with cleavage at stalled forks *in vivo* and *in vitro*[402]. RAD52 has also been shown to interact with helicases in cells. Baynton et al[403] find that WRN, a RECQ homolog, interacts with RAD52 *in vivo* at arrested replication forks and *in vitro*. Biochemically RAD52 both inhibits and enhances WRN helicase activity, while WRN increases RAD52-mediated strand annealing[403]. RAD52 also interacts with human RECQ5 to promote SDSA[168]. RECQ5 counteracts the inhibition by RAD51 on RAD52 annealing in *vitro* and *in vivo*, and deficiency in RECQ5 leads to increased occupancy of RAD51 at DSBs. This suggests that RECQ5 acts in SDSA post-synaptically to prevent aberrant RAD51 filament formation on the extended invading strand, thus limiting crossovers[168]. RAD52 also interacts with PTEN, regulating RAD52 sumoylation[399].

In a reaction analogous to SDSA, RAD52 can anneal the extended invaded strand with a second complementary tail after it dissociates from the D-loop (dissociation is mediated by RAD54 in these experiments), though RAD52 was dispensable for this activity in the absence of RPA[423]. RAD52 can also promote the formation of a “double D-loop,” annealing the displaced

strand of a D-loop to another complementary ssDNA, which would be analogous to second end capture/dHJ formation of DSBR[423]. DNA repair synthesis catalyzed by human DNA polymerase η (which has been suggested to play a role in HR repair) acting upon priming strand of a D-loop leads to capture and annealing of the second end of a resected DSB, and this reaction is mediated by RAD52[176]. Thus, RAD52 may play an annealing role in second-end capture and SDSA in cells, though the importance of this role in vivo is unclear.

Interestingly, recent unpublished evidence from our lab suggests HelQ regulates RAD52 function in DSB repair by HR and SSA. HelQ depletion in U2OS cells reduces HR and SSA and leads to reduced RAD52-GFP foci formation after CPT. Depleting HelQ and RAD52 revealed no additive effect on either HR or SSA suggesting they are epistatic. HelQ acts downstream of RAD51 filament formation to facilitate RAD52 activity in second end capture—no change was observed in foci formation of γH2AX, BRCA1, RPA2, and RAD51 after CPT treatment in HelQ-depleted cells, suggesting a role for HelQ downstream of resection and RAD51 filament formation. HelQ interacts with RPA2, pRPA2, and RAD52 after 2h CPT by IP. Loss of HelQ results in a greater frequency of long tract gene conversion, similar to RAD52 depletion, which is due to the inability of non-invading strand of the double-strand break to support appropriate termination of the short tract gene conversion by annealing, suggesting a role for HelQ in second-end capture. Finally, HelQ suppresses RAD52 mediator activity in HR, as depleting HelQ in BRCA2-depleted cells resulted in an increase in HR by DR-GFP and RAD51 foci formation, and this recovery of RAD51 and HR can be attributed to the activity of the RAD52-mediated repair pathway, as the simultaneous depletion of HelQ,

**Figure 16: Model for RAD52-mediated HR.** In BRCA-deficient cells, RAD52 mediates HR after a DSB. DSBs are resected to generate 3' ssDNA overhangs, which are bound by RPA and pRPA. RAD52 is recruited to these damage sites independent of RPA phosphorylation, but proper regulation of RPA phosphorylation and dephosphorylation improves the RPA-RAD52 association. This improved association allows RAD52 to successfully recruit RAD51, and to promote RAD51 filament formation and strand invasion.




RAD52 is recruited to damage foci with or without RPA phosphorylation

RPA phosphorylation and dephosphorylation improves RAD52-RPA association, and RAD52 recruitment of RAD51

RAD52 promotes RAD51 filament formation and strand invasion

BRCA2, and RAD52 blocked RAD51 foci formation. Taken together, these data suggest that HelQ appears to facilitate RAD52 dependent annealing activity while blocking RAD51 mediator activity in HR.

**CANCER THERAPIES**

In cancer patients carrying *BRCA* mutations only, tumor cells are BRCA deficient (usually heterozygous $BRCA^{mut/-}$), while normal cells are proficient in HR, containing one functional and one nonfunctional copy of *BRCA1* or *BRCA2*. Thus, targeting a protein such as RAD52, which is synthetically lethal with BRCA2 would result in death specifically of tumor cells. The synthetic lethal relationship between PARP and the BRCA proteins provides a proof of principle example that synthetic lethality with BRCA proteins may be utilized for cancer treatment. Our works demonstrates that RPA phosphorylation is important not only for the BRCA pathway but the RAD52 pathway. Thus, this interaction is a potential therapeutic target.

**MODEL FOR RAD52-DEPENDENT HR**

Our data supports a model of RAD52-dependent HR that is promoted by hyperphosphorylation of RPA (Figure 16). After a DSB or fork collapse, the ends are resected to generate 3' ssDNA overhangs that are bound by RPA, which is then hyperphosphorylated. RAD52 is recruited to the damage site by RPA regardless of RPA phosphorylation status. However, RPA phosphorylation enhances its interactions with RAD52, allowing it to recruit RAD51 and promote filament formation. RAD51 then catalyzes homology search and strand invasion. Our *in vitro* evidence suggests that cycling of RPA phosphorylation or other factors besides RPA hyperphosphorylation are needed, to allow RAD52 to promote RAD51 nucleoprotein filaments to form on RPA-ssDNA.

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