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RPA and RAD51: Fork reversal, fork protection, and genome stability

Kamakoti P. Bhat and **David Cortez***

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

Abstract

Replication Protein A (RPA) and RAD51 are DNA binding proteins that help maintain genome stability during DNA replication. These proteins regulate nucleases, helicases, DNA translocases, and signaling proteins to control replication, repair, recombination and the DNA damage response. Their different DNA binding mechanisms, enzymatic activities, and binding partners provide unique functionalities that cooperate to ensure that the right activities are deployed at the right time to overcome replication challenges. Here we discuss the latest discoveries of the mechanisms by which these proteins work to preserve genome stability with a focus on their actions in fork reversal and fork protection.

Introduction

Accurately and completely copying the genome each cell division cycle is essential to prevent disease. This is a daunting challenge considering the trillions of cell divisions in a human lifetime during which billions of bases need to be replicated. Replication stress caused by DNA lesions and conflicts with transcription create additional challenges that must by overcome each cell division cycle[1].

The DNA in mammalian cells is replicated by the replisome, which synthesizes DNA in discontinuous stretches on the lagging strand and long continuous sections on the leading strand. This mode of DNA synthesis results in small regions of single strand DNA (ssDNA) on the lagging strand template. When DNA replication is challenged by stress, replication forks “stall”. In many cases, this stalling generates larger stretches of ssDNA on the leading strand because of polymerase and helicase uncoupling[2]. In addition, ssDNA can be generated by exonucleases that enlarge ssDNA gaps[3] or resect reversed or broken forks (see more below).

Management of ssDNA by DNA binding proteins is essential during replication. These proteins protect the DNA from nucleases, recruit replication stress response proteins like checkpoint kinases and regulate enzymes that control replication fork stability and restart. In this review we will focus on the functions of the major eukaryotic ssDNA binding protein RPA and the single- and double-strand DNA binding protein RAD51 in the regulation of replication fork reversal and fork protection.

*Corresponding author: David Cortez, Ph.D., Vanderbilt University, 613 Light Hall, 2215 Garland Avenue, Nashville, TN 37232 USA, Phone: 615-322-8547, david.cortez@vanderbilt.edu.

First responder: RPA

As a highly abundant (~4 million molecules/cell[4]), high affinity ssDNA binding protein, RPA is expected to be the first responder to ssDNA whether it is formed during normal DNA replication or in response to replication stress. Below we discuss how its biochemical characteristics facilitate its activities in a variety of DNA metabolic processes with a focus on how it regulates replication fork reversal.

Biochemical characteristics of RPA

RPA is a heterotrimer of three subunits RPA70, RPA32 and RPA14 (Figure 1A). The three RPA subunits have a total of six OB (oligonucleotide/oligosaccharide binding) fold domains[5]. Four of these (70A, 70B, 70C, 32D) act as ssDNA binding domains (Figure 1A). 32D, 70C, and RPA14 help assemble the RPA trimer while 70N acts as a protein interaction domain. In addition to these OB-folds, RPA32 has a winged-helix C terminal domain (RPA32C) that is involved in mediating protein interactions.

RPA binds ssDNA with a K_d as low as 10^{-10} M for optimal DNA ligands of approximately 30 nucleotides[6]. However, this tight affinity masks the dynamic nature of the binding that is made possible by its modular domain architecture. Individual domains can microscopically associate and dissociate from the DNA[7]. Two of the four DNA binding domains 70A and 70B act as a single unit to bind 8-10nts of ssDNA[8]. The other two DNA binding domains, 70C and 32D, are part of the trimer core and engage the ssDNA with lower affinities[9]. At least 20nts of DNA are required for all of the binding domains to be occupied, but the full DNA footprint is ~28-30nts[6]. The linker between the 70B and 70C domains also makes DNA contacts and likely participates in overcoming ssDNA secondary structure as RPA transitions between binding modes[10]. RPA can also diffuse along ssDNA, leading to melting of hairpins, other secondary structures and even short regions of duplex DNA[11-13]. Thus, RPA-ssDNA interactions should be viewed as highly dynamic with individual domains dissociating and re-associating as RPA adopts different conformations, destabilizes duplex regions and reveals small ssDNA stretches that can act as binding sites for other replication and repair proteins.

Importantly, the domain architecture also means that RPA binding to ssDNA is polar, with 70A-B-C and RPA32D domains binding DNA 5′-3′. The polarity of RPA binding facilitates the proper assembly of protein complexes in some repair contexts[14], and as we will discuss later, dictates activities of fork reversal enzymes[15].

RPA interacts with a large number of replication and repair proteins. These interactions are often mutually exclusive since many occupy the same 70N and 32C binding surfaces on RPA (Figure 1A). For example, the 70N domain binds to the ATRIP subunit of the ATR replication stress kinase to recruit it to regions of ssDNA[16,17]. The same 70N surface binds to RAD9, MRE11, p53, PRIMPOL, and ETAA1[18-21]. The 32C domain interacts with several proteins including ETAA1, SMARCAL/HARP, UNG2, XPA, and RAD52[21-24]. The 70N and 32C domains are connected to the DNA binding domains via flexible, unstructured tethers. Thus, these interactions happen independently of DNA binding. The affinities of the interactions are low, usually in the micromolar range, indicating that they are likely to be

transient. The picture that emerges is that both soluble and DNA-bound RPA molecules are constantly exchanging binding partners. Larger assemblies of proteins may form on the DNA in some circumstances to stabilize specific complexes.

The dynamic nature of both DNA and protein interactions allows RPA to fulfill multiple functions at replication forks. First, by binding the lagging strand template as the helicase extrudes it, RPA removes secondary structures[25]. Second, it promotes priming activity on the lagging strand[26]. Third, when replication forks stall, it acts as a platform to assemble checkpoint signaling proteins[27]. Finally, it recruits and regulates the function of numerous fork repair proteins. These activities are critical to prevent fork collapse[28].

RPA as a regulator of fork remodeling

Replication fork remodeling is the process of inter-converting the replication fork from a three-way junction to four-way junction and back (Figure 2A). Conversion from a three-way to four-way junction is called fork reversal or fork regression, while the opposite reaction is fork restoration. During fork reversal, rewinding of the parental DNA duplex and the protrusion of the nascent DNA strands forms a reversed replication fork or “chicken-foot”.

There are at least four ways fork reversal can promote genetic stability (Figure 2B)[29,30]. First, fork reversal could stabilize the stalled fork until a converging fork from a nearby origin passively replicates the region. Second, the formation of the reversed fork places any DNA lesion on the template strand that caused polymerase stalling back into the context of duplex DNA. This would then allow repair of the damage by excision repair mechanisms. Third, annealing of the nascent strands could provide an undamaged template to synthesize past the lesion in a template switching mechanism. Finally, the reversed fork can be cleaved by endonucleases such as MUS81 or other SLX4-dependent nucleases to promote recombination-mediated repair[31].

In contrast to an active replication fork, a subset of stalled forks would be expected to have RPA on the leading template strand due to the stalling of Polε. During the process of fork reversal, RPA must be removed from the parental ssDNA as the template strands re-form a DNA duplex[32]. The SNF2 family DNA translocase SMARCAL1/HARP can catalyze both reversal and restoration reactions on RPA-bound substrates[33]. SMARCAL1 interacts directly with the 32C domain of RPA, and this interaction is required for its recruitment to stalled replication forks[22,23]. Moreover, the interaction regulates SMARCAL1 activity. Specifically, RPA stimulates SMARCAL1 mediated fork reversal activity when it is bound to the leading template strand and inhibits its function when bound to the lagging strand[32]. The polar binding of RPA to the ssDNA facilitates this regulatory difference (Figure 2A)[15]. Thus, SMARCAL1 has exactly the kind of activity required to reverse replication forks that stall because of a leading strand template problem.

SMARCAL1 works in repetitive bursts in which small amounts of reversal are followed by pausing events[32]. Pausing provides opportunities for regulation, direction switching, or hand-off to another enzyme. For example, after SMARCAL1 engages the fork DNA, it is phosphorylated by ATR[22,34-36]. This phosphorylation inhibits SMARCAL1 activity and is important to prevent “excessive” fork reversal which can lead to fork breakage[36]. Second,

pausing allows the fork to test whether the obstacle to replication has been resolved. The T4 UvsW protein, highly similar in structure to SMARCAL1[37], also exhibits repetitive fork reversal activity and an ability to switch branch migration direction to catalyze fork restoration[38]. Combined, these activities promote lesion bypass by template switching without replisome dissociation[39]. Thus, fork reversal may often be a transient intermediate that rapidly resolves back to an elongating fork unless the replication stress is persistent.

SMARCAL1 is only one of several proteins that can reverse replication forks. Other SNF2 family members including ZRANB3 and HLTF also perform this function *in vitro* and in cells[32,40-42]. Each has unique functional domains and regulation as reviewed elsewhere[43]. For example, in contrast to SMARCAL1, RPA bound to a leading strand gap that mimics a stalled fork acts as a block to ZRANB3 fork reversal[32]. HLTF has not been studied with the same substrates, but it does exhibit different DNA substrate preferences from SMARCAL1 and ZRANB3[41]. Distinct substrate recognition and protein-interaction domains on these proteins generate these substrate differences[32,33,37,41,44].

Second responder: RAD51

RAD51 binds both ssDNA and dsDNA, and is best known for its actions in catalyzing strand invasion during double strand break (DSB) repair by homologous recombination (HR). RAD51 also has essential functions at replication forks, including in regulating fork reversal, that are genetically separable from DSB repair, and these will be our focus.

RAD51 biochemical characteristics

RAD51 is the eukaryotic ortholog of the *E. coli* recombinase, RecA[45]. Like RecA, RAD51 forms nucleoprotein filaments on ssDNA through a cooperative DNA binding mechanism (Figure 1B)[46,47]. However, RAD51 has modest affinity (K_d~10^{-6}M) for both ssDNA and dsDNA[48,49]. Each RAD51 monomer binds three nucleotides of ssDNA and six monomers generate one turn of a helical RAD51-ssDNA filament[50]. RAD51 is also a DNA-dependent ATPase. It is loaded onto DNA in its ATP-bound form, and after ATP hydrolysis the ADP-RAD51 filament is less stable[51,52]. Thus, by hydrolyzing ATP in response to DNA binding, RAD51 is self-inactivating. This inherent instability of the RAD51-DNA filament is only the first of many regulatory mechanisms that are important to ensure RAD51 function is directed properly.

RAD51 requires positively acting mediator proteins to access RPA-bound ssDNA[53]. In mammalian cells, a primary mediator is the tumor suppressor, BRCA2[53]. Like RPA, BRCA2 uses OB folds to bind ssDNA in addition to a less well characterized second ssDNA binding region[54]. BRCA2 ssDNA binding helps to displace RPA and load RAD51. BRCA2 BRC repeats promote RAD51 filament nucleation by delivering ~4 RAD51 monomers to the DNA as a unit, inhibiting RAD51 ATPase activity, and reducing the association of RAD51 with dsDNA[55-57]. Additional BRC repeats and a C-terminal region help stabilize the RAD51-ssDNA filament. Other RAD51 mediators, including RAD51 paralogs, may cooperate with BRCA2 or act in different contexts to promote RAD51 filament formation and stabilization[53].

RAD51 as a fork reversal enzyme

In *E. coli*, the RecG motor protein acts like SMARCAL1 to reverse replication forks[58]. This function is stimulated by the *E. coli* SSB with exactly the same leading vs. lagging strand preference as SMARCAL1[32,59]. However, the RecA recombinase has also been implicated in fork reversal and can catalyze this reaction *in vitro*, a function that is independent of the RecFOR proteins (BRCA2 orthologs in *E. coli*)[60]. Unlike RecG, RecA catalyzed fork reversal is inhibited by *E. coli* SSB[61].

In human cells, RAD51 is also required for fork reversal[62]. Interestingly, like in prokaryotes, this fork reversal function of RAD51 is independent of the BRCA2 mediator[63,64]. The diffusion properties of BRCA2 and RAD51 suggest that most cellular RAD51 is bound to BRCA2 all the time[65], so it is surprising that BRCA2 is not needed for RAD51-dependent fork reversal. Unlike RecA, RAD51 does not have fork-remodeling activity on its own, although it may stimulate fork reversal by other proteins[66]. These observations raise a number of questions. Does RAD51 bind to the stalled replication fork and drive fork reversal in cells? If so, how does it access the ssDNA without the BRCA2 mediator protein? Are RPA and RAD51 binding the same stalled fork at the same time? How does RAD51 cooperate with motor proteins like SMARCAL1 and ZRANB3?

While some models have RAD51 binding to the parental ssDNA to catalyze fork reversal, thinking of fork reversal as a dynamic process with the reversed fork in equilibrium with the restored fork suggests a second possibility (Figure 2C). When the fork reverses because of a leading strand lesion, the expectation is that there will be ssDNA in the reversed arm since the lengths of the nascent leading and lagging strands are different. RAD51 binding to that ssDNA end could capture it and drive the equilibrium towards fork reversal, thus explaining the genetic requirement for RAD51 to observe reversed forks. This idea fits with the ability of RAD51 to inhibit fork restoration in some contexts[66]. Perhaps, the initial extruded ssDNA tail is too small (<8nts) for the 70AB domains of RPA to bind – thereby precluding the need for displacement by BRCA2. Alternatively, there could be another mediator protein, such as MMS22L-TONSL complex that is important for the fork reversal functions of RAD51[67-69], or it is even possible that RAD51 dsDNA binding is involved in fork reversal. Biochemical reconstitution of these reactions and additional analysis of where and when RAD51 binds in comparison to RPA will be needed to test these ideas.

Excessive fork reversal can be detrimental to genome stability

Fork reversal preserves genome stability. However, as is typical of many DNA repair and tolerance mechanisms, this intermediate in DNA processing can be deleterious if not properly controlled. For example, reversed forks have been implicated as the cause of DSBs associated with transcription-replication collisions[59], UV-induced DNA damage[70], and in bacterial strains deficient in the helicase DnaB[29]. Additionally, reversed forks may cause the genome instability associated with the rDNA region in *S. cerevisiae*[71,72] and the hyper-recombination in the *E. coli* termination region[73]. In mammalian cells, deregulation of the fork remodeler, SMARCAL1, either by overexpression[22] or by ATR inhibition[36], causes increased genome instability and breaks.

These deleterious consequences of fork reversal can be traced to its 4-way junction structure with an exposed DNA end. The junction itself can be targeted by structure-selective endonucleases (Figure 2B). Cleavage would generate a true DSB that would then require recombination for repair. While regulated recombination may be beneficial to resume replication, it can also lead to chromosomal rearrangements and instability through inappropriate recombination and microhomology mediated recombination events[74]. The reversed nascent-nascent duplex could also be bound by DSB repair proteins like KU70/80 or by telomerase if it contains telomeric sequences[75]. These proteins may interfere with fork restoration or cause telomere dysfunction, respectively. Finally, the end of the reversed arm is an access point for nucleases like MRE11 as part of the MRE11-RAD50-NBS1 (MRN) complex. MRE11 could remove end-binding proteins to facilitate fork restoration; however, excessive nuclease activity at persistently stalled forks can be problematic.

RAD51 as a fork protection enzyme

As first described for RecA in *E. coli*, RAD51 binding to the reversed arm of the chicken foot protects it from excessive degradation mediated by exonucleases. This process is called fork protection and is dependent on BRCA2-mediated stabilization of RAD51-ssDNA filaments[76,77]. Thus, *BRCA2*-deficient cells exhibit fork instability or nascent strand degradation. A BRCA2 C-terminal region that binds and stabilizes RAD51 filaments is required for fork protection even though it is dispensable for repair of site-specific DSBs[76]. Fork degradation in BRCA2-deficient cells can be restored by overexpression of a mutant RAD51 protein that cannot hydrolyze ATP and forms hyper-stable filaments. RAD51 binding can also protect DNA from MRE11-dependent degradation *in vitro*; a function not shared by RPA[63].

Many additional HR proteins, including BRCA1, the RAD51 paralogs and the Fanconi Anemia (FA) proteins as well as proteins implicated in chromatin regulation like the histone methyltransferase EZH2 are also needed to prevent fork degradation (see Box 1 and Table 1). Adding to the complexity is that multiple nucleases are implicated in the degradation. While BRCA1/2 and the FA pathway prevent MRE11-dependent degradation[76-78], other factors such as BOD1L prevent DNA2-mediated degradation, also by stabilizing RAD51 on ssDNA[79]. Thus, RAD51 loss can cause MRE11 or DNA2 dependent fork degradation. Other nucleases like EXO1 and MUS81 may also be involved in some cases[80,81]. The mechanisms governing the nuclease choice are unknown. Since both the leading and lagging nascent strands are degraded, one might expect two nucleases of different polarities (5′-3′ and 3′-5′) to be involved. Finally, there are RAD51-independent pathways that prevent fork degradation, raising the possibility that fork protection actually involves multiple different processes[82].

In all cases where it has been examined, a reversed fork is the entry point for nucleases to degrade the newly synthesized DNA. Thus, silencing any one of several fork reversal enzymes including SMARCAL1, ZRANB3, HLTF and even RAD51, restores fork protection to BRCA2-deficient cells[63,64,83,84]. Intriguingly, while nascent strand degradation removes thousands of bases, only small ssDNA gaps are visible by electron microscopy and degraded forks rapidly restart once the replication block is removed[63,64,76].

To explain these observations, we suggest the following model (Figure 3): Upon encountering a replication challenge that exposes leading template strand ssDNA, RPA directs SMARCAL1 to initiate fork reversal. Often, this reversal will be transient and fork restoration will then lead to fork restart or a converging fork will complete DNA synthesis. Alternatively, RAD51 might capture the nascent lagging strand ssDNA that is exposed. If the ssDNA overhang is not large or if polymerases fill it in, the duplexed DNA end could be captured by end-binding proteins like KU[85]. Just as it does at DSBs, MRE11 may act to remove these proteins to allow fork restoration. Fork processing by polymerases and nucleases combined with post-translational modifications to replisome proteins like PCNA ubiquitylation, may generate good substrates for the other fork reversal enzymes like ZRANB3 and HLTF. If the replication blockage is persistent, then a more extensive RAD51-ssDNA filament may stabilize the reversed fork. However, in the absence of BRCA2, progressive rounds of reversal and nuclease action could slowly degrade large amounts of nascent ssDNA. Each time this happens, there is a chance that incorrect processing or premature entry into mitosis could generate the gaps, breaks, and chromosomal aberrations associated with nascent strand degradation. Nonetheless, most forks would be capable of rapidly resuming DNA synthesis once the replication stress challenge is removed.

Differential requirements for RAD51 in fork reversal, protection and HR

Since RAD51 is needed for fork reversal and fork degradation proceeds from the reversed fork, RAD51-deficient cells should not exhibit fork degradation because the substrate, the reversed fork, should not form. Indeed, silencing RAD51 by siRNA was reported to prevent fork degradation[64,83]. However, cells expressing a partial loss of function mutant RAD51 (T131P) and cells treated with a RAD51 inhibitor do exhibit fork degradation[63,84,86]. One possible explanation for this apparent discrepancy is that fork protection may be more sensitive to RAD51 inhibition than fork reversal because less RAD51 may be needed to perform reversal as compared to protection (Figure 4A). Consistent with this hypothesis, we found that at high concentrations, two potent RAD51 siRNAs prevented fork degradation. However, at intermediate concentrations of the same siRNAs, we observed nascent strand degradation (Bhat and Cortez, unpublished). This differential requirement of RAD51 for reversal vs. protection is also consistent with the need for BRCA2 for fork protection but not fork reversal and the observation that overexpression of a RAD51 antagonist, RADX, also causes fork degradation[87].

While RAD51 is required for replication and repair, too much RAD51 can be detrimental to cells. Indeed, RAD51 overexpression causes genome instability[88,89] and RAD51 is frequently overexpressed in cancers[90-92]. Similarly, RecA overexpression is also detrimental to *E. coli*. RecA activity is regulated by RecX, a protein that caps RecA-ssDNA filaments[93]. RADX may be a functional analog of RecX and may operate with additional RAD51 antagonists like RecQ helicases and PARI to prevent inappropriate RAD51 activity[94].

RADX is a ssDNA binding protein that contains three RPA-like OB-fold domains and is required to prevent fork cleavage by the MUS81 endonuclease[87]. This function is tied to RAD51 regulation since RADX-deficient cells accumulate excessive fork-bound RAD51 that is hypothesized to cause inappropriate fork reversal even in the absence of exogenous

replication stress (Figure 4B). RAD51 and fork reversal might be a threat to normal elongating forks because leading and lagging strand polymerases may not always be coordinated and ssDNA intermediates could form even at active forks, as shown recently for the *E. coli* replisome[95]. If this is true in vertebrates, RADX may be needed to counteract RAD51 in the absence of stress. Indeed, reducing RAD51 levels suppresses the fork breakage phenotype of RADX-deficient cells[87].

RADX functionally acts as a buffer for RAD51, supplementing its self-inactivating ATPase activity and other antagonists to prevent inappropriate RAD51 action at replication forks. Silencing RADX increases the amount of RAD51 that can access forks in cells even when RAD51 mediators like BRCA2 are inactive. Therefore, RADX depletion rescues the fork degradation observed in BRCA2 deficient cells[87]. This correlates with an increase in viability and partial resistance to PARP inhibitors. Consistent with only a partial effect on RAD51, RADX deficiency is unable to overcome the requirement for BRCA2 in HR and overexpression only modestly reduces HR efficiencies although it can cause fork degradation[87]. These data further argue that the three functions of RAD51 in fork reversal, fork protection and HR repair require increasing amounts of RAD51 function (Figure 4C). An important determinant of the differential requirements of RAD51 might be the amount or persistence of ssDNA. End resection at a DSB can yield long stretches of ssDNA (several hundred to thousands of nucleotides)[96] while the ssDNA at a stalled fork averages less than 100 bases[62].

Summary and Outlook

RAD51 and RPA have different biochemical characteristics and functions. However, both are essential regulators of replication fork stability through management of ssDNA. The ability of both proteins to promote fork reversal facilitates responses to replication challenges but is also dangerous since reversed forks are access points for nucleases. As with most DNA repair mechanisms, the management of intermediates is essential and failures can generate worse outcomes than not attempting the repair at all. Many unanswered questions about mechanisms remain, but the explosion of interest in replication stress responses will certainly speed our understanding of these complex and critical genome maintenance processes.

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Box 1

Replication fork protection

While many proteins have been implicated in fork protection, multiple conceptual questions about this genome maintenance pathway remain to be answered.

First, is fork protection a single pathway or an assembly of multiple distinct mechanisms? Until recently, the unifying theme of fork protection was the need to stabilize a RAD51-ssDNA filament to prevent nuclease action. However, there are reports of RAD51-independent mechanisms of fork protection[82]. Combined with the involvement of different nucleases in different contexts, this observation suggests that the fork protection assay may be monitoring more than one type of fork processing.

Second, just about every double-strand break repair protein examined seems to regulate fork protection. Is the end of the reversed arm of the chicken foot subject to the same processing events as every other type of double strand break or are there specific mechanisms that discriminate these types of reversed fork ends? Also, what does the end look like? How much ssDNA is there and does it change during processing?

Third, how is fork protection physiologically important? The fork protection assay requires stalling all forks for extended periods of time, and even in these circumstances removal of the replication challenge leads to rapid fork restart. The non-physiological experimental perturbation nevertheless correlates with physiological significance since a patient-derived RAD51 mutant (T131P) is defective in fork protection but not HR[86], BRCA1 heterozygosity causes fork degradation and may promote tumorigenesis[97], and genetic backgrounds that restore fork protection to BRCA2-deficient cancer cells without restoring HR cause chemotherapy and PARP inhibitor resistance[98]. Further studies are needed to understand the importance of fork protection to determining cancer etiology and therapeutic response.

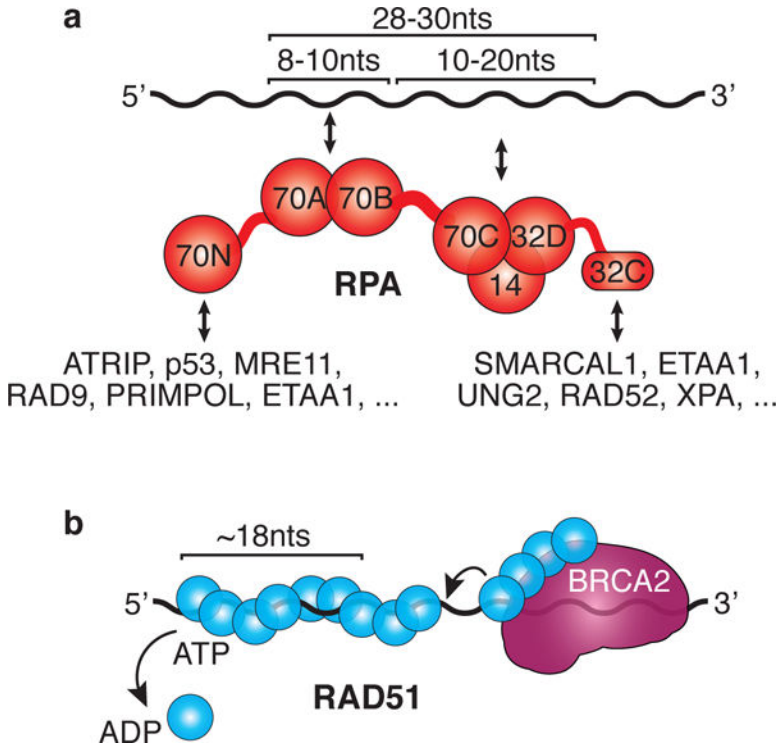

Fig. 1. RPA and RAD51 have different biochemical characteristics

(A) RPA uses modular domain architecture to facilitate dynamic DNA and protein interactions. The size of the ssDNA that is bound by the RPA DNA binding domains is indicated as well as the major protein-protein interaction domains with example binding partners. (B) RAD51 forms a protein-ssDNA filament with the help of BRCA2 and is regulated by its ATPase activity. BRCA2 uses its own ssDNA binding activity to help deliver RAD51 and displace RPA (arrow). ATP hydrolysis promotes filament disassembly.

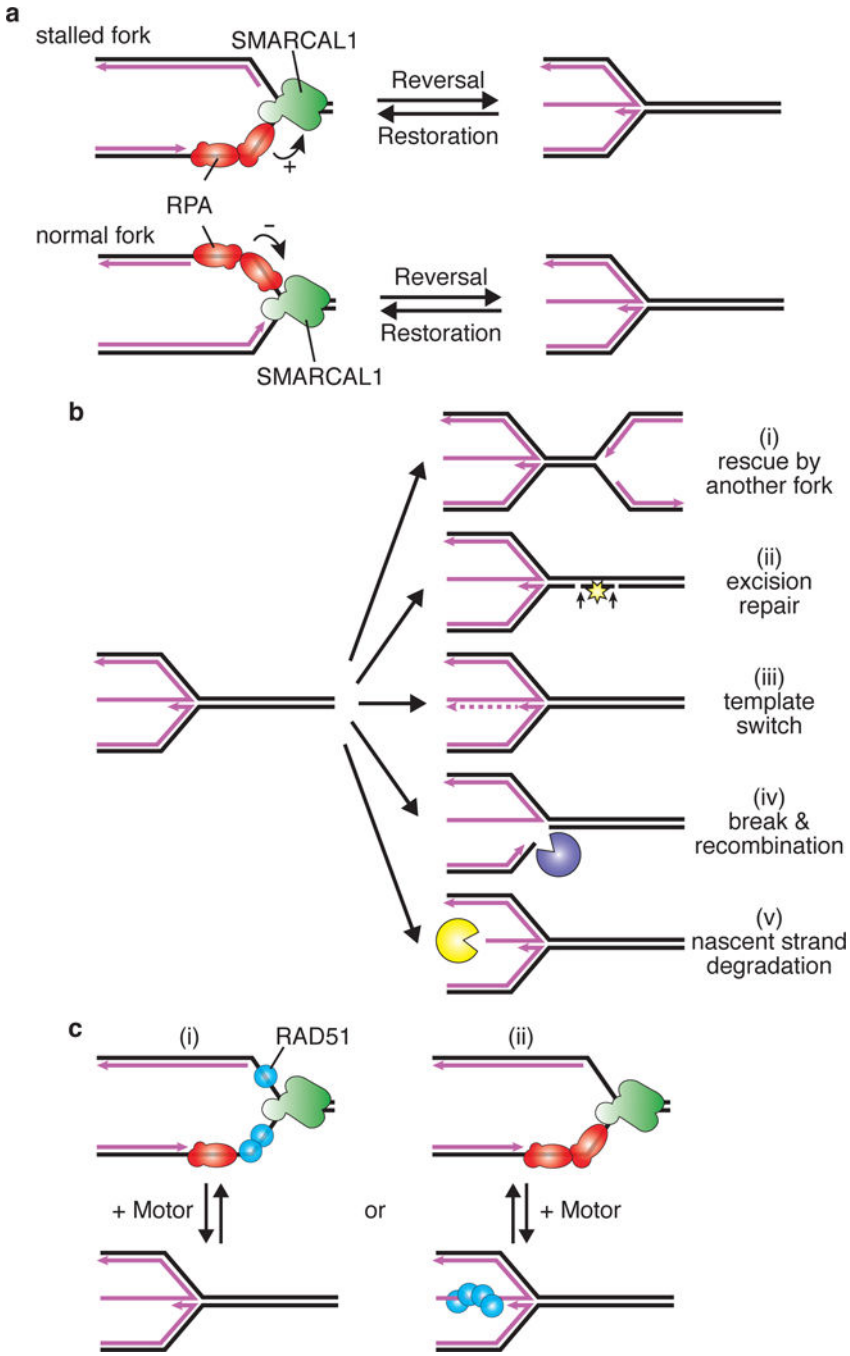

Fig. 2. Replication fork reversal is regulated by RPA and RAD51

(A) RPA stimulates SMARCAL1 to catalyze fork reversal when bound to the leading strand template "stalled fork" but inhibits SMARCAL1 when bound to the lagging strand "normal fork". The polarity difference of RPA bound to these substrates is illustrated since that contributes to SMARCAL1 regulation. Pink lines indicate nascent DNA while template DNA is illustrated in black. (B) Reversed forks are intermediates in fork stabilization and restart mechanisms, but are also susceptible to nuclease processing. Five outcomes of fork reversal are illustrated and described further in the text. (C) Two models for how RAD51

may promote fork reversal. First, RAD51 may bind the template ssDNA of the stalled fork to cooperate with motor proteins like SMARCAL1 and ZRANB3 to drive reversal. Second, RAD51 could capture the reversed fork, driving an equilibrium towards fork reversal.

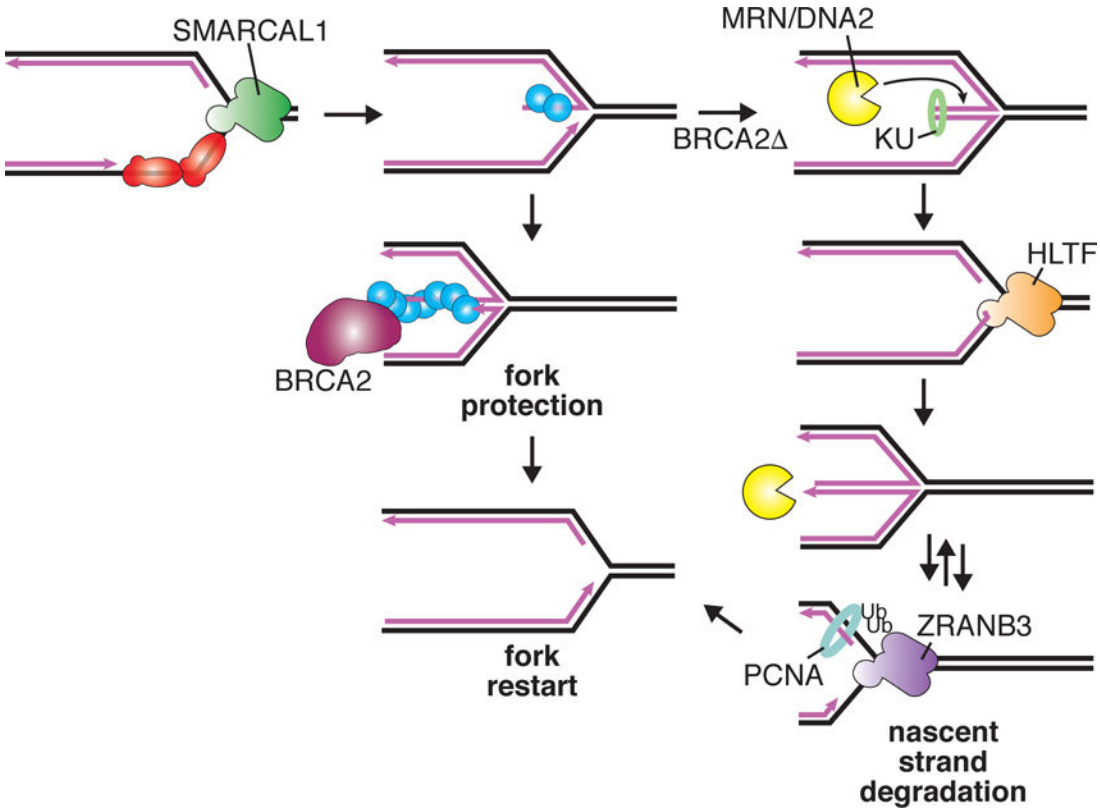

Fig. 3. Nascent strand degradation may be due to multiple rounds of fork reversal and nuclease action

When RAD51-DNA filaments are not stabilized, nucleases including MRN and DNA2 can access the end of the reversed arm. Limited nascent strand degradation could be beneficial to remove end-binding proteins and allow fork restart. However, in the context of a persistent fork block, multiple rounds of end processing and reversal by motor proteins with different substrate specificities yields extensive degradation and repeated opportunities for mistakes that generate chromosomal abnormalities. Depicted is a speculative model of the motor proteins acting sequentially. Ub = ubiquitin.

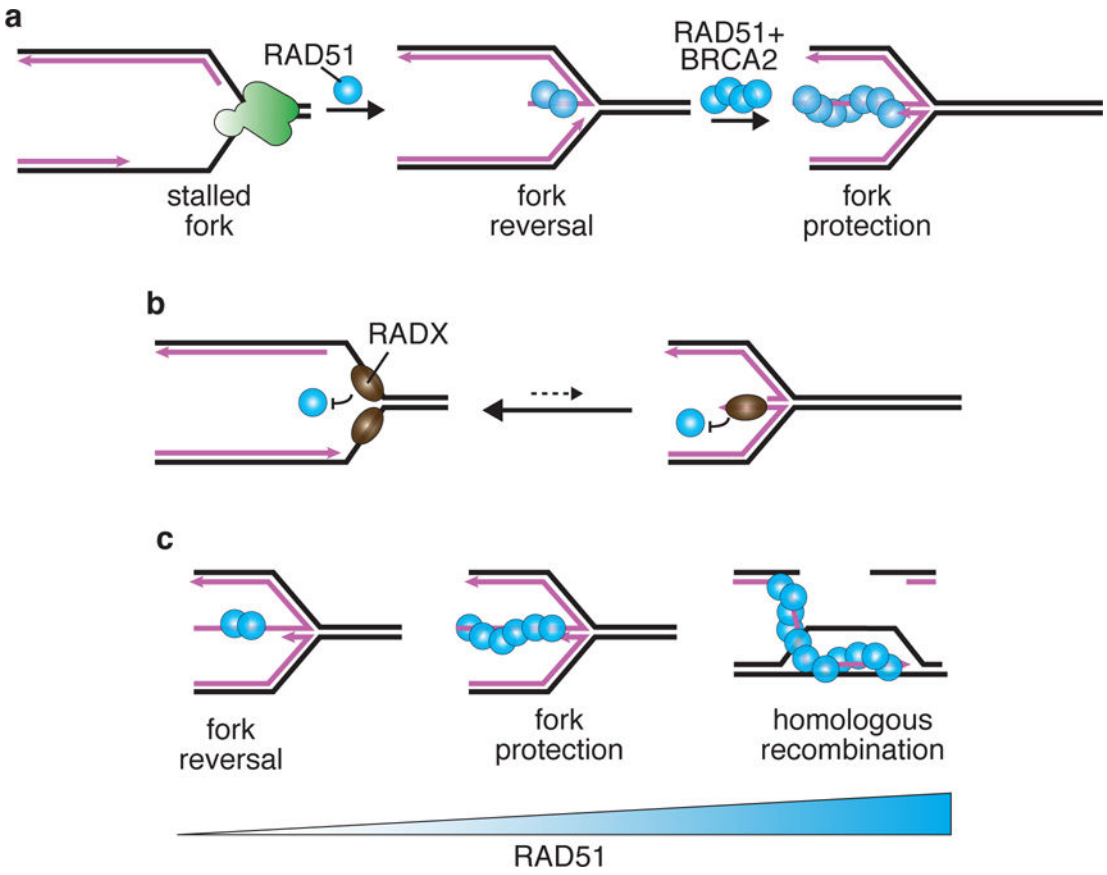

Fig. 4. Different functions of RAD51 may require different amounts of protein and ssDNA

(A) RAD51 is needed for both fork reversal and fork protection. Fork reversal does not require BRCA2 and may require less cellular RAD51 protein than fork protection. **(**B) During normal replication, transient exposures of ssDNA at an elongating replication fork may be shielded from RAD51 by RADX to prevent unnecessary fork reversal. (B) Increasing amounts of RAD51 protein/activity may be needed for fork reversal, fork protection, and HR.

Table 1

List of select proteins involved in fork reversal and fork protection.

Protein	References
Fork Remodelers	
HLTF	41,84
RAD54	66
SMARCAL1	33,63,84
ZRANB3	32,40,42,44,84
Homologous recombination and Fanconi Anemia proteins	
BRCA1	78
BRCA2	76
FANCA, B, D2	78,99
PALB2	100
RAD51	62,76,77
RAD51C, XRCC2, XRCC3	101
RAD52	64
Nucleases and helicases	
BLM	79,99,102
DNA2	79,103
EXO1	81
FBH1	79
MRE11	76,77
MUS81	80
RECQ1	103,104
RECQL5	99
WRN	81,103
Other regulators	
ABRO1	82
ATR	36,105
ATRX-DAXX	106
BOD1L	79
CHD4	98
EZH2	80
MLL2/3	98
MMS22L-TONSL	69
PARI	107
PARP1	104,108
PTIP	98

Protein	References
RADX	87
REV1	109
WRNIP1	110
53BP1	111,112