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Insights into the control of RAD51 nucleoprotein filament dynamics from single-molecule studies

Florian Morati and Mauro Modesti

Genomic integrity depends on the RecA/RAD51 protein family. Discovered over five decades ago with the founder bacterial RecA protein, eukaryotic RAD51 is an ATP-dependent DNA strand transferase implicated in DNA double-strand break and single-strand gap repair, and in dealing with stressed DNA replication forks. RAD51 assembles as a nucleoprotein filament around single-stranded DNA to promote homology recognition in a duplex DNA and subsequent strand exchange. While the intrinsic dynamics of the RAD51 nucleoprotein filament has been extensively studied, a plethora of accessory factors control its dynamics. Understanding how modulators control filament dynamics is at the heart of current research efforts. Here, we describe recent advances in RAD51 control mechanisms obtained specifically using fluorescence-based single-molecule techniques.

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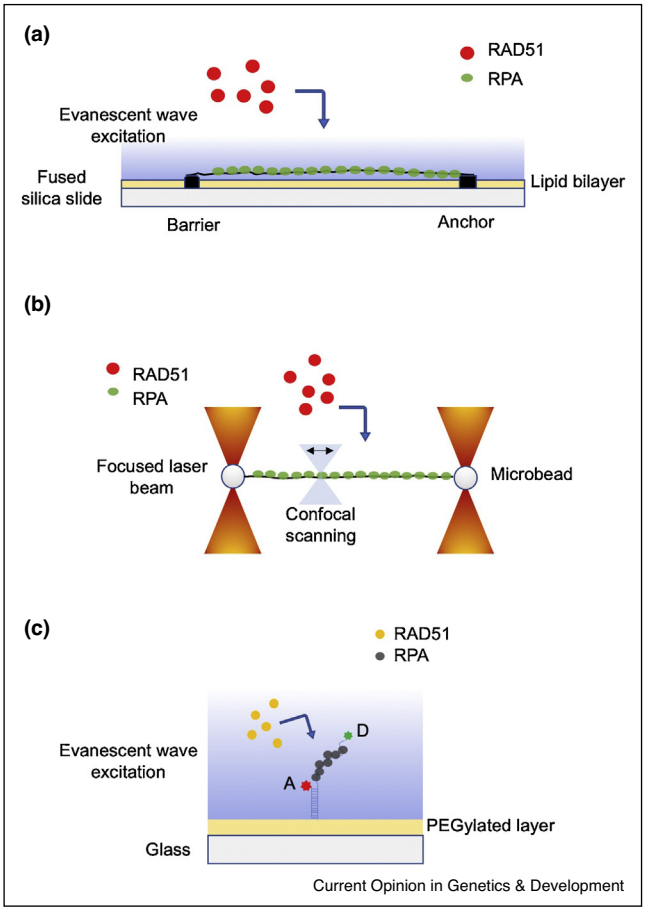
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Introduction

RAD51 filament dynamics consists of nucleation, polymerization and dissociation phases, coupled to structural rearrangements. RAD51 protomers nucleate and assemble into a right-handed helical nucleoprotein filament around single-stranded DNA. An ATP nucleotide cofactor intercalates at each RAD51-RAD51 monomer-monomer interface to cement the filament. The ATP coordinated filament adopts an extended configuration competent for promoting homology recognition and strand exchange catalysis. Upon ATP hydrolysis, the filament is prone to change configuration from extended to a more contracted, catalytic inactive configuration, where monomers are inclined to dissociate. Numerous biochemical and high resolution structural studies have contributed to this two-state picture of RAD51 filament behavior. However, detailed understanding of the intrinsic RAD51 filament dynamics were obtained using single-molecule approaches as they have provided quantitative readouts for nucleation, polymerization and dissociation rates under various buffer conditions, stoichiometries, visualization of movement, pairing interaction dwell times and mechanical properties [1]. Of note, more recent single-molecule studies from the Spies group (University of Iowa, U.S.A.) revealed that intrinsic human RAD51 filament nucleation on single-stranded DNA occurs through dynamic binding and dissociation of RAD51 dimers without cooperative interactions [2], underlining the essential role of modulators in assisting assembly of stable continuous filament in cells.

The effort is now aimed at understanding how a constantly growing list of modulators control RAD51 by influencing its intrinsic dynamics [3–5]. The use of fluorescence-based single-molecule techniques to study these mechanisms has been instrumental in this respect. They come in many flavors including DNA curtain, dual trap optical tweezers and single-molecule fluorescence resonance energy transfer (smFRET) technologies (Figure 1) [6,7]. In recent studies of RAD51 control, the DNA curtain and the dual trap optical tweezers technologies have come of age. Both methods are similar as they each allow real-time visualization of fluorescently labeled protein during their interaction with individual DNA molecules. In the case of DNA curtains, DNA molecules are attached via their extremities to a passivated glass surface and the interactions of fluorescently labeled proteins are monitored by Total Internal Reflection Fluorescence microscopy (TIRFM, evanescent wave excitation) (Figure 1). Similarly, with dual trap optical tweezers individual DNA molecules are attached from their extremities to microbeads that are held and manipulated with optical traps away from the surface, and fluorescence is detected by high-speed confocal microscopy in the plane of the DNA molecule (Figure 1). In smFRET assays (Figure 1), DNA substrates on which FRET donor and acceptor dyes are inserted are attached to a passivated glass surface. FRET time trajectories in the presence of proteins under different reaction conditions are recorded to capture and extract information on interaction dynamics down to single protein monomer resolution [7]. Finally, an interesting single-molecule approach has been developed by the Wyman group (Erasmus MC, The Netherlands) that combines scanning force microscopy with TIRFM to study RAD51 control, providing static topographic images

Figure 1



Schematics for the principle of single-molecule approaches that combine fluorescence detection and handling of individual DNA molecules to monitor protein-DNA transaction. **(a)** DNA curtain technology [13]. **(b)** Dual trap optical tweezers [12]. **(c)** Single-molecule fluorescence resonance energy transfer assay [7]. A = acceptor, D = donor.

of protein-DNA complexes in which the constituents are identified through fluorescent labeling [8].

Below we will highlight very recent studies that have provided new insights into the mechanisms of RAD51 control by using *in vitro* fluorescence-based single-molecule techniques. Table 1 catalogs the proteins and the RAD51 modulators from the different model systems that were the subject of these recent studies; and provides an overview of the new mechanistic insights that have been obtained.

Control of RAD51 filament assembly by mediators and chaperones

RAD51 filament assembly on single-stranded DNA is rate limited at the initial nucleation step and is in competition with RPA, an abundant single-stranded DNA binding protein complex. In mammalian cells, BRCA2 mediates filament nucleation by enhancing RAD51 selectivity for single-stranded DNA and helping RPA displacement. Assembly of catalytic competent filaments depends in addition on the RAD51 paralog proteins, a set of five proteins (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) that belong to the RecA/RAD51 family. They have evolved after duplication of ancestral RAD51 and have apparently lost RAD51 DNA strand exchange enzymatic activity. They are believed to function in two distinct complexes, RAD51B-RAD51C-RAD51D-XRCC2 and RAD51C-XRCC3, to promote the formation of active RAD51 filaments. However, the mechanisms by which the RAD51 paralog complexes act on RAD51 filament dynamics have remained elusive. Two recent single-molecule studies using purified proteins from model systems provided mechanistic insights on the mechanism of action of RAD51 paralogs during filament assembly [9••,10••,11]. To achieve this, both groups worked out clever ways to generate and manipulate individual single-stranded DNA molecules and coat them with fluorescently labeled RPA [12,13] in order to visualize the dynamics of individual RAD51 filament assembly and the relative localization of modulators. Using purified nematode RAD-51, BRC-2 and RFS-1/RIP-1 protein complex (respectively equivalents to mammalian RAD51, BRCA2 and RAD51 paralogs) and monitoring fluorescent RPA eviction from individual single-stranded DNA molecules held with dual optical tweezers, the Boulton group (The Francis Crick Institute, U.K.) in collaboration with the Rueda group (Imperial College London, U.K.) found that BRC-2 and RFS-1/RIP-1 synergize to promote RAD-51 filament assembly. Similar experiments on DNA molecules with a single-stranded gap of defined polarity allowed to determine filament growth polarity. As for mammalian BRCA2, BRC-2 favors initial nucleation, presumably binding to the 3′ end of nascent filaments. RFS-1/RIP-1, instead binds the filament 5′ end, and in the presence of BRC-2 net filament growth in the 3′ to 5′ direction is observed. The authors further generated functional fluorescent variants of RAD-51 and RFS-1/RIP-1 to monitor DNA interaction dwell times and relative colocalization. Remarkably, they found that RFS-1/RIP-1 shuts down RAD-51 disassembly while transiently interacting with filament 5′ end and dependent on ATP hydrolysis, thus acting as a dynamic chaperone for filament growth.

A similar behavior of the yeast RAD51 paralog complex, Rad55-Rad57, was observed by the Greene group (Columbia University, U.S.A) using single-stranded DNA curtains. By direct visualization of fluorescently labeled Rad55-Rad57, they found that the yeast RAD51 paralog complex interacts with Rad51 filaments in a transient burst during assembly, promoting at the same time RPA eviction from single-stranded DNA, and needing ATP hydrolysis. Interestingly, the authors also

Table 1

Human	Worm	Yeast	Model system New mechanistic insight (comments)
RAD51	**RAD-51**	**Rad51**	(Ubiquitous RecA-like ATP-dependent recombinase)
DMC1		**Dmc1**	(Meiosis-specific RecA-like ATP-dependent recombinase)
RPA(1-2-3)	RPA(1-2)	**Rpa(1-2-3)**	(ssDNA binding multimeric protein complex)
		Hed1	(meiosis-specific Rad51 inhibitor)
RAD51B RAD51C RAD51D XRCC2 XRCC3	**RFS-1/RIP-1**	**Rad55-Rad57**	**RFS-1/RIP-1** and **Rad55-Rad57** are dynamic filament assembly chaperones [9••,10••] **Rad55-Rad57** mediates filament formation on RPA-ssDNA without Rad52 [10••]
BRCA2	**BRC-2**	Rad52 (analog of BRCA2)	**BRCA2** is a **RADX** antagonist [34••] **BRC-2** together with **RFS-1/RIP-1** controls polarity of filament growth [9••]
BLM	BLM-1	**Sgs1**	**Sgs1** has Rad51 anti-recombinase activity without displacing RPA [20•] **Sgs1** is inhibited by the Dmc1 [22] recombinase **BLM** unwinds dsDNA [24•] **BLM** has no effect on RPA or RAD51 coated ssDNA [24•]
		Srs2	**Rad55-Rad57** antagonizes the anti-recombinase activity of **Srs2** [10••]
RECQ5	RCQ-5		**RECQ5** has anti RAD51 and DMC1 recombinase activity [23•]
RAD54	RAD-54	**Rad54**	**Rad54** translocase switches the intrinsic diffusion-based homology search process of the Rad51 filament to an active 1-D motor-driven search process [26••]
RAD54B	RAD-54.b	**Rdh54**	**Rdh54** is an accessory factor for **Rad54** [27•]
RADX			**RADX** blocks RAD51 filament assembly by forming clusters with RPA on ssDNA [34••] **RADX** displaces RAD51 from ssDNA [33•,34••]

Proteins indicated in bold have been the subject of the recent studies described in this review. Orthologs of the different model systems are indicated when known. Note that Rad52 is not an ortholog of BRCA2 but plays the same function in mediating Rad51 nucleoprotein filament nucleation on RPA-coated ssDNA. ssDNA = single-stranded and dsDNA = double-stranded DNA.

found that Rad55-Rad57 was efficient at mediating Rad51 filament formation without synergizing with Rad52. This is a surprising result as up to now, Rad52 has been considered as the only activity capable of mediating Rad51 filament on RPA-coated single-stranded DNA in the yeast model system [14].

These two recent single-molecule studies of yeast and nematode model systems provide a framework for understanding the mechanism of action of mammalian RAD51 paralog complexes and the interplays with other RAD51 filament modulators such as the BRCA2-PALB2-BRCA1-DSS1 network.

Control of RAD51 filament dynamics by motor proteins

There are a number of helicases/translocases, ATP fueled motor proteins, implicated in the control of RAD51 filament dynamics [15,16]. Using the DNA curtain single-molecule approach, the Greene group recently explored the mechanism of action of several motors for their ability to act on RAD51 filaments. In the study introduced above [10••], they have further investigated the yeast Srs2 helicase, an anti-recombinase that dynamically strips Rad51 filaments from single-stranded DNA as determined by smFRET experiments [17]. They focused on the Srs2 interplay with the Rad55-Rad57 complex. Contrary to the expectation that Rad55-Rad57 would block Srs2 anti-recombinase action [18], by directly tracking fluorescently labeled Srs2 and Rad55-Rad57, they found that Rad55-Rad57 instead promotes re-assembly of Rad51 filaments after their disruption by Srs2. The authors propose that Rad51 filaments are in a dynamic state that is controlled by the antagonistic action of assembly chaperones and anti-recombinase motors. This dynamic modulation of Rad51 filaments might be important for channeling intermediates into the appropriate repair pathway.

The RecQ helicase family is important for maintaining genomic integrity and includes five members in mammals [19]. Using the DNA curtain single-molecule approach the Greene group recently characterized the behavior of the yeast RecQ ortholog Sgs1; and of RecQ5 and BLM, two mammalian members of the RecQ helicase family. By monitoring the behavior of Sgs1 on single-stranded DNA covered with RPA, they found using two-color single-molecule imaging that Sgs1 translocases rapidly and with high processivity [20•]. Unlike Srs2 which evicts RPA bound to the single-stranded DNA during translocation [21], Sgs1 appears to function by a different mechanism as it does not displace RPA while translocating. However, Sgs1 was able to displace Rad51 using a processive $3' \rightarrow 5'$ translocation activity, indicating it has an anti-recombinase activity that could be redundant with Srs2 activity. Interestingly, similar experiments using Dmc1, the meiosis-specific recombinase, revealed that Sgs1 similarly to Srs2 [22] is unable to remove Dmc1 bound to single-stranded DNA. Thus, both Srs2 and Sgs1 are anti-recombinase that use different mechanisms to

disrupt Rad51 filaments and are inhibited by Dmc1. These findings are providing insights into how recombination intermediates may be channeled towards different fates during mitotic and meiotic recombination.

Similar single-molecule experiments using DNA curtains have recently been conducted to study RecQ5 [23•] and BLM [24•]. Tracking fluorescently labeled RecQ5 revealed that it can rapidly and with high processivity translocate on single-stranded DNA covered by RPA, RAD51, or DMC1 and strip them from the DNA substrate. RecQ5 appears therefore to be able to act as an anti-recombinase. However, it was not able to translocate past DNA heteroduplex joints. No evidence for an anti-recombinase activity was found for BLM. Instead, BLM robustly unwinds naked double-stranded DNA but exhibits no binding or translocation activity on either RPA or RAD51 coated single-stranded DNA. The authors suggest that there is a division of labor for the various RECQ helicases in that they may preferentially act on specific substrates during DNA repair processes.

The RAD54 translocase acts at multiple steps during DNA recombination processes mediated by RAD51 [25]. A single-molecule study by the Greene group recently revealed that yeast Rad54 can act by propelling the Rad51 nucleoprotein filament along a double-stranded DNA molecule thereby switching the intrinsic diffusion-based homology search process of the Rad51 filament to an active 1-D motor-driven search process [26••]. This behavior could directly be demonstrated by simultaneously tracking the movement of fluorescently labeled RAD54 and a fluorescently labeled Rad51 nucleoprotein filament (label on the DNA) on curtains of double-stranded DNA molecules. The study unveils that Rad54 opens the strands of the double-stranded DNA template and in association with RPA allows sampling of both strands for homology search by the propelled Rad51 nucleoprotein filament. In another work, the group also studied the behavior of Rdh54, a paralog of yeast Rad54, and found that it is unable to promote 1D translocation-based homology search on its own [27•]. Instead, the Rdh54 motor appears to act as an accessory factor for Rad54. An extra level of regulation was studied by analyzing the action of yeast Hed1, a factor that blocks Rad51 activity to allow the Dmc1 recombinase to promote strand exchange during meiotic DNA recombination [28]. Hed1 binds to the Rad51 nucleoprotein filament and prevents the association of Rad54 with Rad51.

DNA replication fork remodeling and RAD51 control

The RAD51 recombinase is involved during rescue processes when DNA replication progression is perturbed [29]. It is implicated in fork reversal, protection and restart for which the mechanisms are currently under intense investigation [30]. A framework has been proposed by the Cortez group (Vanderbilt University, U.S.A.) that posits that a gradient of RAD51 levels is engaged during fork rescue. A limited number of RAD51 monomers are required for fork reversal, higher levels are needed for fork protection, while fork restart would need longer RAD51 filaments.

Recent single-molecule experiments using the single-stranded DNA curtain approach have investigated the mechanism of action of human RADX, a negative modulator of RAD51 acting at stalled DNA replication forks [31,32]. By monitoring loss of fluorescent RPA from single-stranded DNA to indirectly measure RAD51 filament assembly, the authors found that the presence of RADX strongly decreases the rate of RAD51 filament assembly but without displacing RPA from single-stranded DNA [33•]. When RADX associates with the RPA-coated substrate very little RPA displacement is observed. In fact, RADX interacts with RPA on the single-stranded DNA forming clusters that compact the substrate and prevents RAD51 loading [34••]. In addition, RADX is also able to displace RAD51 bound to single-stranded DNA [34••]. Interestingly, BRCA2 appears to counteract the negative effect of RADX on RAD51 filament assembly indicating that a delicate balance in the action of negative and positive modulators to control RAD51 levels on single-stranded DNA is involved during fork reversal and protection.

Except for a few studies that used smFRET assays to investigate RAD51 filament disruption by FBH1, an F-box helicase implicated in a pathway of DNA replication fork rescue [35–37], few efforts have been made to reconstitute and study DNA replication fork rescue processes and RAD51 involvement at the single-molecule level. However, the tools that exploit the fluorescence-based single-molecule techniques described above as well as magnetic tweezers are now in place for the field to expand in this area [38,39].

Perspectives

Single-molecule approaches including DNA curtain, dual trap optical tweezers and smFRET technologies are now proven to be ideal to quantitatively study the dynamic DNA-protein transactions governing the behavior and control of RAD51 nucleoprotein filaments. Studies using proteins from model systems have been and will continued to be influential by providing frameworks to study the human system. In fact, there are a number of human RAD51 modulators for which the mechanism of action is not well understood and could be clarified using singe-molecule approaches [3–5]. To name a few, we have the two human RAD51 paralog complexes RAD51B-RAD51C-RAD51D-XRCC2 and RAD51C-XRCC3 that appear to have distinct roles either during DNA double-strand break repair or during stressed DNA replication fork rescue by mechanisms that are not understood

[40,41]; the human SWS1-SWSAP1-SPIDR complex which interacts with RAD51, DMC1 and BLM, and for which the mechanism of action is not determined [42,43]; or the human HELQ helicase which interacts with the RAD51 paralogs and is implicated in homologous recombination through an unknow mechanism [44,45].

The field will likely witness further technological advances such as exploitation of light polarization to gain insight into how molecules move and are organized in space and time [46], combination of topographic imaging by high-speed atomic force microscopy in solution and fluorescence detection [47], or the use of nanochannels to manipulate individual DNA molecules [48]. However, while providing key mechanistic insights on the dynamics and mechanics of protein-DNA transactions, *in vitro* single-molecule approaches by definition convey a reductionist view of the processes that occur inside living cells. Great efforts are now being deployed to study RAD51 control processes in living cells by techniques such as single particle tracking and single molecule localization microscopy [49–51]. These techniques can inform on the mobility and diffusion rates of proteins, their retention to sites of DNA damage, their relative co-localization and the stoichiometry of complexes. The Wyman group (Erasmus MC, The Netherlands) has initiated such an effort aimed at understanding how human BRCA2 controls RAD51 in cells [52,53].

Conflict of interest statement

Nothing declared.

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