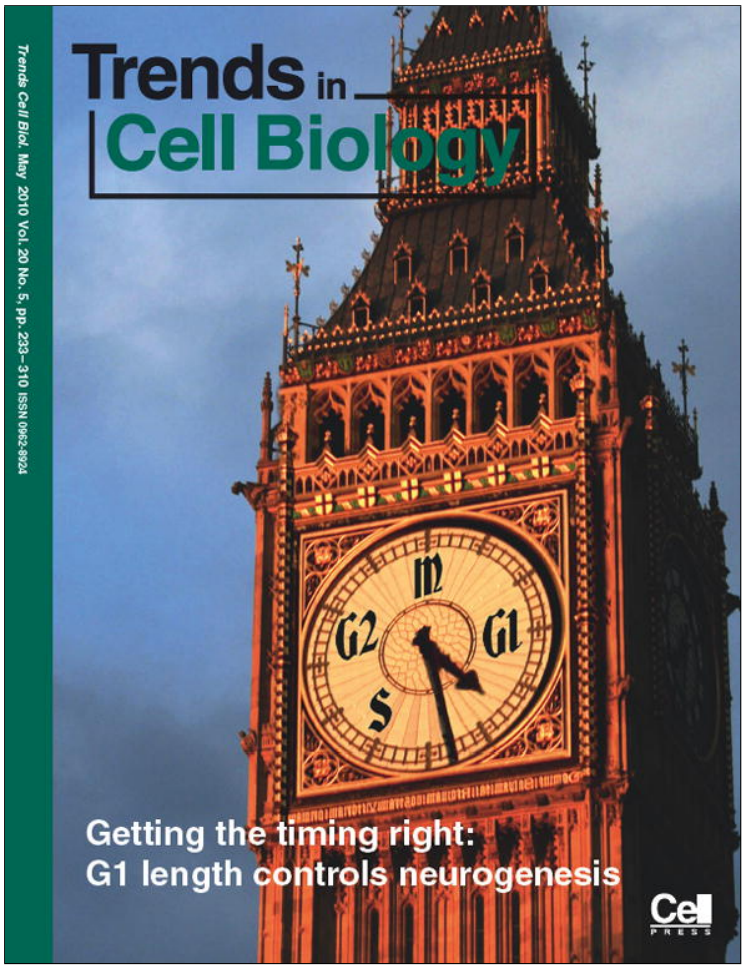

Review

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Single-molecule imaging brings Rad51 nucleoprotein filaments into focus

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The Rad51 protein is essential for DNA repair by homologous recombination. After DNA damage, Rad51 localizes to nuclear foci that represent sites of DNA repair *in vivo*. *In vitro*, Rad51 self-assembles on single- or double-stranded DNA to form a nucleoprotein filament. Recently, the merging of innovative single-molecule techniques with ensemble methods has provided unique insights into the dynamic nature of this filament and its cellular function. The assembly and disassembly of Rad51 nucleoprotein filaments is seen to be regulated by recombination accessory proteins. In this regard, the BRC repeats of the BRCA2 protein were shown to modulate the DNA binding selectivity of Rad51. Furthermore, single-molecule studies explained the need for a DNA translocase, Rad54 protein, in the disassembly of Rad51 double-stranded DNA filaments.

DNA repair and homologous recombination

Cellular DNA damage can arise from external factors such as radiation, or from internal agents such as free radicals produced by metabolism. All organisms possess evolutionarily conserved mechanisms that correct such impaired DNA. Consequently, viruses, bacteria, archaea and eukaryotes have been used as complementary systems to examine the biology and mechanism of DNA repair. One type of potentially lethal DNA damage is the double-strand break (DSB), where both strands of DNA are cut. DSBs can be repaired through a relatively error-free pathway termed homologous recombination (Box 1, Figure I) [1,2].

A decade of microscopic analyses *in vivo* revealed that when DNA is damaged, a variety of repair proteins localize to nuclear foci that represent the sites of DNA repair [3,4]. Identification of a marker for DNA breaks, phosphorylated histone variant H2AX (γH2AX), suggested that the nuclear accumulation of homologous recombination proteins into visible foci is coincident with and localized to places of DNA damage [5]. By observing the kinetics of nuclear focus formation, the temporal order of the recruitment of DNA repair proteins to these sites was elucidated (Figure I) and the dynamic nature of these complexes was visualized. This dynamic behavior is an essential characteristic that enables cells to efficiently deal with many different types of DNA damage at potentially any location in the chromosome [6].

Rad51 is one of many proteins that form discrete nuclear foci in response to DNA damage [7]. The use of techniques such as fluorescence loss in photobleaching (FLIP) and fluorescence redistribution after photobleaching (FRAP) in mammalian cells revealed that, upon DNA damage, Rad51 relatively stably accumulates at sites of damage, whereas Rad52 and Rad54 are more mobile and undergo constant redistribution within the nucleus [3]. In addition, the Rad51 nuclear foci were seen to colocalize with BRCA2 (breast cancer susceptibility gene 2) foci after DNA damage [8]. Furthermore, the Rad51 foci were dependent on BRCA2, implying a causal relationship [9,10]. Another important example of *in vivo* imaging was performed in yeast cells lacking Rad54; in these cells, Rad51 foci formed but persisted much longer than in wild-type cells, suggesting that Rad54 is involved in Rad51 nucleoprotein filament disassembly [11].

Genetic studies have defined Rad51 as essential for the repair of DNA damage by homologous recombination. *RAD51* knockout mice show embryonic lethality, and deletion of *RAD51* in various cell lines results in extreme sensitivity to DNA- damaging agents, defective DSB repair and, ultimately, cell death [12,13]. The assembly of a catalytically active Rad51 nucleoprotein filament on single-stranded (ss)DNA is required for homologous recombination (Figure I). Like its prokaryotic homolog, RecA, Rad51 nucleoprotein filament assembly occurs in two steps: nucleation and growth [14–16]. Nucleoprotein filament formation results in the extension of DNA by ≈50% relative to B-form length [17]. Also similar to RecA [18–20], studies using mutant proteins defective in ATP hydrolysis, non-hydrolysable ATP analogs, and calcium ions substituted for magnesium ions, revealed that ATP stabilizes Rad51 on ssDNA [21–23]. The product of ATP hydrolysis, ADP, induces disassembly of the nucleoprotein filament [21,23]. As discussed below, single-molecule experiments now permit visualization of individual Rad51 nucleoprotein filaments as they assemble onto and disassemble from single DNA molecules. Owing to space limitations, we have focused on work pertaining only to Rad51, despite a growing and rich single-molecule literature on RecA [15,16,24–32].

Efficient assembly of Rad51 on ssDNA that is complexed with RPA involves the assistance of other proteins such as BRCA2 (Figure I). *BRCA2* encodes a large (3,418 amino acids) protein that is localized to the nucleus [33] and is required for DNA repair by homologous recombination [34]. Studies involving genetics, cell biology and peptide chemistry have unveiled key features of the BRCA2-Rad51 interaction. The discovery of a highly conserved domain containing eight copies of a 30–40 amino acid sequence,

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Box 1. Schematic model of homologous recombinational DNA repair in eukaryotes

DNA repair by homologous recombination can be loosely organized into four steps, each of which requires the concerted activity of several proteins: (i) initiation, (ii) homologous pairing and DNA strand exchange, (iii) DNA heteroduplex extension, and (iv) resolution (Figure I). The initiation step involves the processing of the dsDNA break by nucleases and helicases to produce a 3′ tail of ssDNA at each end of the break. An ssDNA binding protein, replication protein-A (RPA), functions to protect the ssDNA and to remove any secondary structure. Next, with the aid of mediator proteins such as BRCA2 and the Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3 in humans or Rad52 and Rad55/57 in yeast), Rad51 assembles onto the ssDNA to form a nucleoprotein filament called the presynaptic filament. The Rad51 nucleoprotein filament is stabilized by Rad54. This filament then searches for a homologous sequence within the dsDNA and promotes invasion of the ssDNA into the homologous dsDNA target to form a joint molecule. Further processing of the DNA intermediates requires the removal of Rad51 by Rad54. DNA polymerases are next required to synthesize DNA using the intact DNA template [Figure I; light blue (newly synthesized) and blue (template) strands of DNA]. Subsequently, two DNA crossover structures known as Holliday junctions are formed after capture of the second processed DNA end. The next step, DNA heteroduplex extension and branch migration, involves Rad54 and/or a specialized class of motor proteins to catalyze translocation of the DNA heteroduplex joints. The fourth and final step is either resolution or dissolution of Holliday junctions. Both alternatives involve the separation of the crossover structures to produce two intact repaired dsDNA molecules. Resolution through nucleolytic cleavage events can yield either crossover or non-crossover products. Dissolution of double Holliday junctions requires the combined actions of a helicase and topoisomerase to convergently migrate the junctions and yields only the non-crossover product.

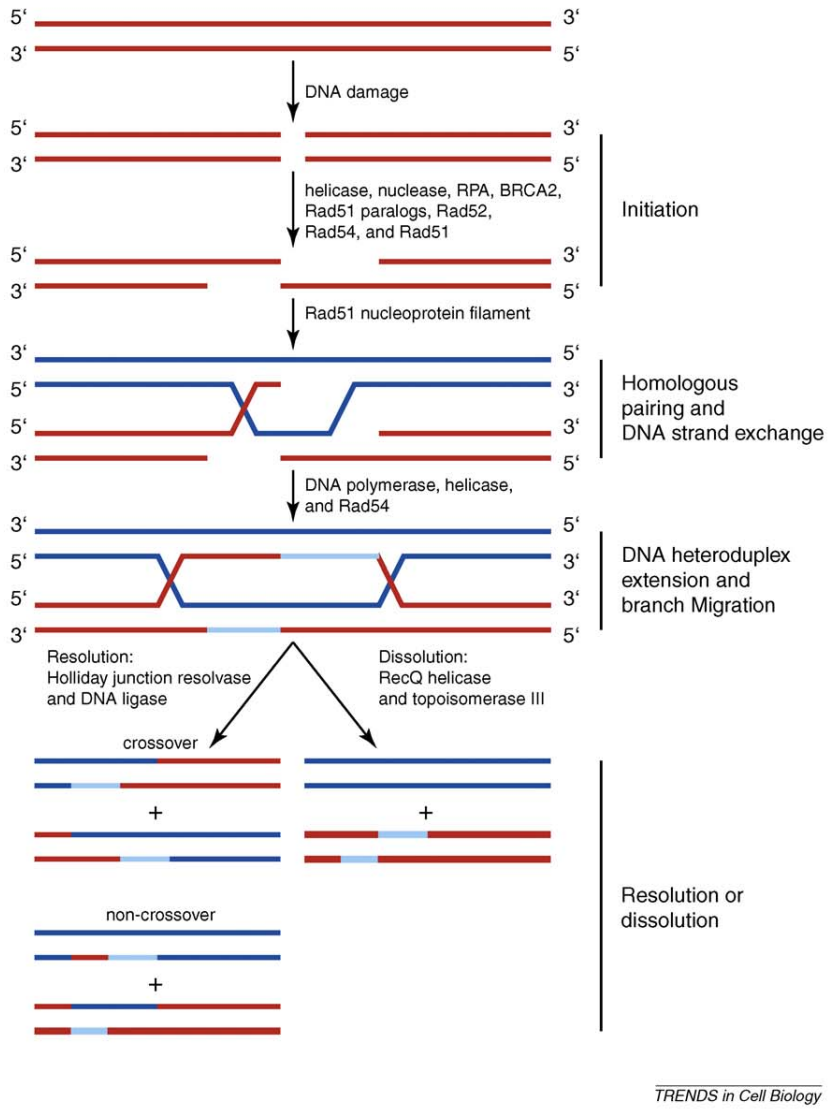

Figure I. Model for recombinational repair of a DNA double-strand break.
The initiation phase involves the resection of the 5′ terminated strand at a DNA double-strand break (red dsDNA molecule). This results in a 3′ terminated ssDNA tail on which the Rad51 nucleoprotein filament is formed. In the next phase (homologous pairing and strand exchange), the Rad51 nucleoprotein filament locates homology, pairs with the homologous dsDNA template (blue) and exchanges DNA strands. Next, synthesis of new DNA (light blue) using the intact homologous DNA (blue) as a template permits formation of two Holliday junctions. DNA heteroduplex extension and branch migration involves movement of the Holliday junctions. The final step (resolution or dissolution) is the separation of crossover structures either by multiple alternate cleavage events, or by convergent migration and associated DNA strand passage of the two Holliday junctions to yield two intact repaired homologous DNA molecules.

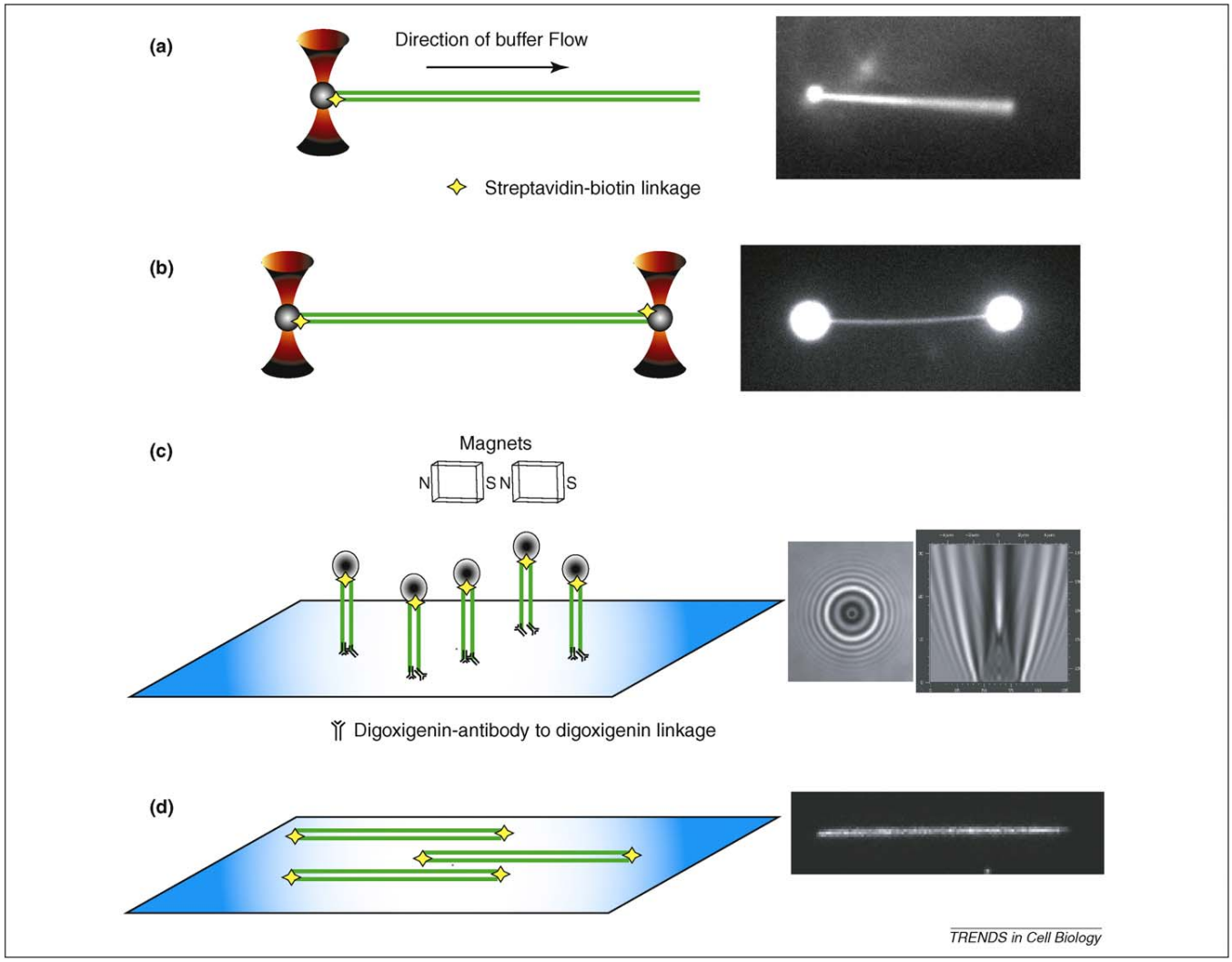

Figure 1. Techniques used to capture and study single molecules of DNA.
(a) Single optical trap (red) captures a bead (gray) that is attached to a single DNA molecule (green). Force generated by the flow of buffer within a flowcell extends the DNA molecule. **(b)** Two optical traps capture beads on each end of a DNA molecule. **(c)** Magnetic tweezer extends DNA attached to the surface of coverglass through antibody–antigen or biotin–streptavidin interactions. The end of DNA distal to the surface anchor is attached to a magnetic bead. **(d)** DNA molecules tethered to the surface of a coverglass within a flowcell are extended by the flow of buffer through the chamber. Modifications on both ends of the DNA result in dual attachment to the surface, and will allow the DNA to remain extended in the absence of buffer flow. Alternatively, if DNA is only tethered to the surface at one end, a continuous flow of buffer is required to maintain extension of the DNA for observation. To the right of each illustration **(a–d)** are actual images obtained with each of the respective techniques.

termed the BRC repeat, was crucial [35]. Biochemical and structural analyses showed that these BRC repeats bind directly to Rad51; this direct interaction meditates the delivery of Rad51 to sites of damage (foci) by BRCA2 and catalyzes the loading of Rad51 onto ssDNA [10,36–39]. Other domains in BRCA2 include oligonucleotide/oligosaccharide binding (OB) folds and a tower domain, which are probably responsible for binding to ssDNA and double-stranded (ds) DNA, respectively [40,41].

Disassembly of Rad51 from heteroduplex DNA is required for later stages of homologous recombination so that downstream proteins can gain access to duplex DNA (Figure I) [42]. Rad54 and its homolog, Tid1, are ATP-dependent dsDNA translocases [43,44]. The translocation activity is used to promote the disassembly of Rad51 from dsDNA after successful homologous DNA pairing and strand exchange [45–47]. In addition to displacing Rad51 from dsDNA, Rad54 possesses the ability to reposition nucleosomes and to stimulate DNA strand exchange [48–50].

Single-molecule techniques to analyze Rad51 filament dynamics

Figure 1 illustrates several configurations of instrumentation currently being used to characterize the dynamic behavior of proteins acting on single DNA molecules. Depending on the configuration, high spatial (nanometer) and temporal (millisecond) resolution can be achieved. The tracking of DNA and/or protein permits real-time determination of reaction intermediates, reaction rates and forces generated, which might otherwise be obscured in ensemble measurements as a result of averaging of a heterogeneous population. All of the methods discussed here involve tethering or immobilizing single DNA molecules within the confines of a flowcell (a micro-fluidic device usually created by sandwiching a spacer between a glass microscope slide and coverglass

[51]), combined with imaging the protein, DNA or a particle; however, other methods, notably Förster resonance energy transfer (FRET), can also be used [32].

When individual DNA molecules are immobilized by trapping techniques, a bead is attached to one or both ends of a linear DNA molecule. The bead is then immobilized by an optical tweezer [15,25,26,52–57] or a magnetic field [27–31,58–61] (Figure 1a–c). If a single bead/trap is used, then the DNA molecule can be extended by the force of fluid flow (Figure 1a). If dual traps are used, then the position of the traps can be manipulated to extend the DNA molecule in the absence of flow (Figure 1b). Alternatively, one or both ends of linear DNA can be tethered to a microscope slide or coverglass [62–66] (Figure 1d). A novel variation of this surface tethering technique immobilizes and aligns hundreds of DNA molecules [63,64,66]. Depending on the trapping strategy, the DNA and/or protein are monitored in real time by fluorescence microscopy (Figure 1a, b,d), bright-field microscopy (Figure 1c), force measurements (Figure 1b,c), or a combination of methods. A comparison of the advantages and disadvantages of these many different approaches is beyond the scope of this review. Briefly, the force measuring methods provide high spatial and temporal resolution but are necessarily indirect measures of complex formation or activity; the FRET methods are also indirect, but do not require optical or magnetic tweezers; and finally, the imaging methods are direct but require fluorescent modification of proteins or DNA. However, good strategies exist to overcome limitations and to capitalize on the strengths of each technique. In addition, most studies have used dsDNA, rather than ssDNA, because it is easier to manipulate and analyze at the single-molecule level. The technical problems with ssDNA include non-specific binding to beads and surface; compaction due to folding of DNA secondary structure; and DNA fragmentation. Nonetheless, these technical hurdles are gradually being overcome and, in the case of RecA and Rad51, the assembly and disassembly on individual molecules of ssDNA has been described [31,32,60,61].

Assembly and disassembly of individual Rad51 nucleoprotein filaments

The Rad51 protein, from both yeast and humans, has been the recent focus of many researchers using single-molecule techniques. The association and dissociation of Rad51 can be monitored indirectly by measuring changes in DNA length, or directly by visualizing accumulation or loss of fluorescently modified protein. Visualization of a fluorescent protein also permits direct detection of the nucleation stage of the process. The ability to separate the nucleation and growth stages has revealed key features about filament growth from a nucleus.

Fluorescence microscopy with an integrated optical trap has been used to address several aspects of human Rad51 nucleoprotein filament dynamics [67]. A fluorescently labeled cysteine variant of Rad51 was created to enable direct monitoring of the protein. The use of fluorescent protein allowed distinction of protein-bound and naked segments of DNA. The results of force-extension measurements revealed that the protein-free regions of DNA displayed the typical elastic behavior of naked DNA, whereas the Rad51-coated segments were not stretched further and remained bound in a conformation that extended the DNA by about 50%. Single-molecule surface microscopy was also used to observe the dynamics of Rad51 filaments assembled in the presence of ATP and calcium (to reduce ATP hydrolysis [23]) [65]. Frequent nucleation resulted in multiple Rad51 segments along the dsDNA. After the filaments were formed, the calcium ions were then exchanged for magnesium ions to trigger ATP hydrolysis and filament disassembly. By directly imaging the fluorescent nucleoprotein filaments, the rate of disassembly was measured at 0.02 monomers per second when ATP hydrolysis is activated. This rate is slow, and suggests that *in vivo*, efficient disassembly of a Rad51 filament might require the help of an accessory protein such as Rad54.

The use of an optical trap in combination with a microfluidic flowcell was recently used to directly image nucleation, growth and disassembly of human Rad51 filament nucleoprotein filaments [53]. Two complementary approaches were used. The first approach monitored changes in DNA length upon the association and dissociation of unlabeled Rad51; this measurement was accomplished by tracking a fluorescent tag attached to the free end of a DNA molecule. The second approach was to observe directly a fluorescently modified Rad51 during the nucleation, growth and disassembly phases. Nucleation of Rad51 occurred rapidly but, interestingly, the growth of individual filaments was finite. Direct observation of nucleation and growth revealed that 2–3 monomers are required to form a stable nucleation event and that clusters grew to a finite length of $\approx$2 μm ($\approx$6000 base pairs (bp)). Disassembly of the Rad51 nucleoprotein filament exhibited two kinetic steps. The fast step represented the hydrolysis of ATP and the concomitant conversion of the nucleoprotein complex to the compressed ADP-bound form of Rad51; the slow step represented dissociation of Rad51–ADP complexes from the DNA. Importantly, dissociation was incomplete. These findings further supported the need for activity of a motor protein such as Rad54 to accelerate the displacement of Rad51 from dsDNA.

Total internal reflection fluorescence (TIRF) microscopy was recently used to observe indirectly the disassembly of *Saccharomyces cerevisiae* Rad51 from DNA [64]. The DNA molecules were end-labeled with a fluorescent nanocrystal to permit real-time monitoring of changes in dsDNA length as a function of protein dissociation. Free ATP in solution stabilized the nucleoprotein filament five-fold relative to ADP or to cofactor absence. Additionally, Rad51 K191R, a mutant that is deficient in ATP hydrolysis yet retains ATP binding, was examined [46,68]. Although assembly of a complete nucleoprotein filament by this mutant protein was slower than by wild-type Rad51, the mutant nucleoprotein filaments were highly stable once assembled, demonstrating the necessity of ATP hydrolysis in Rad51 nucleoprotein disassembly.

Magnetic tweezer studies were also used to investigate Rad51 assembly and disassembly from both ssDNA and dsDNA [60,61]. Both studies agreed that a nucleus for Rad51 binding was composed of $\sim$5 monomers. Additionally, results showed that Rad51 nucleoprotein filaments consisted of many short patches of filaments made up of

only a few tens of monomers of Rad51 protein [60]. The observed preference for Rad51 to bind to dsDNA rather than ssDNA was shown to be a result of faster depolymerization from ssDNA compared with dsDNA. In fact, Rad51 disassembly did not correlate with ATP hydrolysis [61].

The most recent work made use of dual optical tweezers that permitted fine control of the force applied to a DNA molecule immobilized between two traps [69]. It was discovered that Rad51 filament disassembly from dsDNA is a result of the interplay between ATP hydrolysis and the release of tension stored in the filament. The authors proposed a disassembly model based on the nature of the nucleotide (ATP or ADP) at the terminal end of a Rad51 nucleoprotein filament. If the terminal Rad51 monomer is in the ATP-bound form, then the filament remains stable regardless of the nucleotide state of neighboring internal Rad51 monomers within the segment. Internal ADP-bound Rad51 monomers are stabilized within the filament via protein–protein interactions with neighboring Rad51 monomers. The affinity of a terminal Rad51 for DNA is decreased upon ATP hydrolysis, resulting in dissociation of the last Rad51 monomer and any ADP–Rad51 monomers that are revealed upon dissociation of the previously capping Rad51 monomer.

Collectively, the single-molecule studies of Rad51 revealed that nucleoprotein filament formation on dsDNA

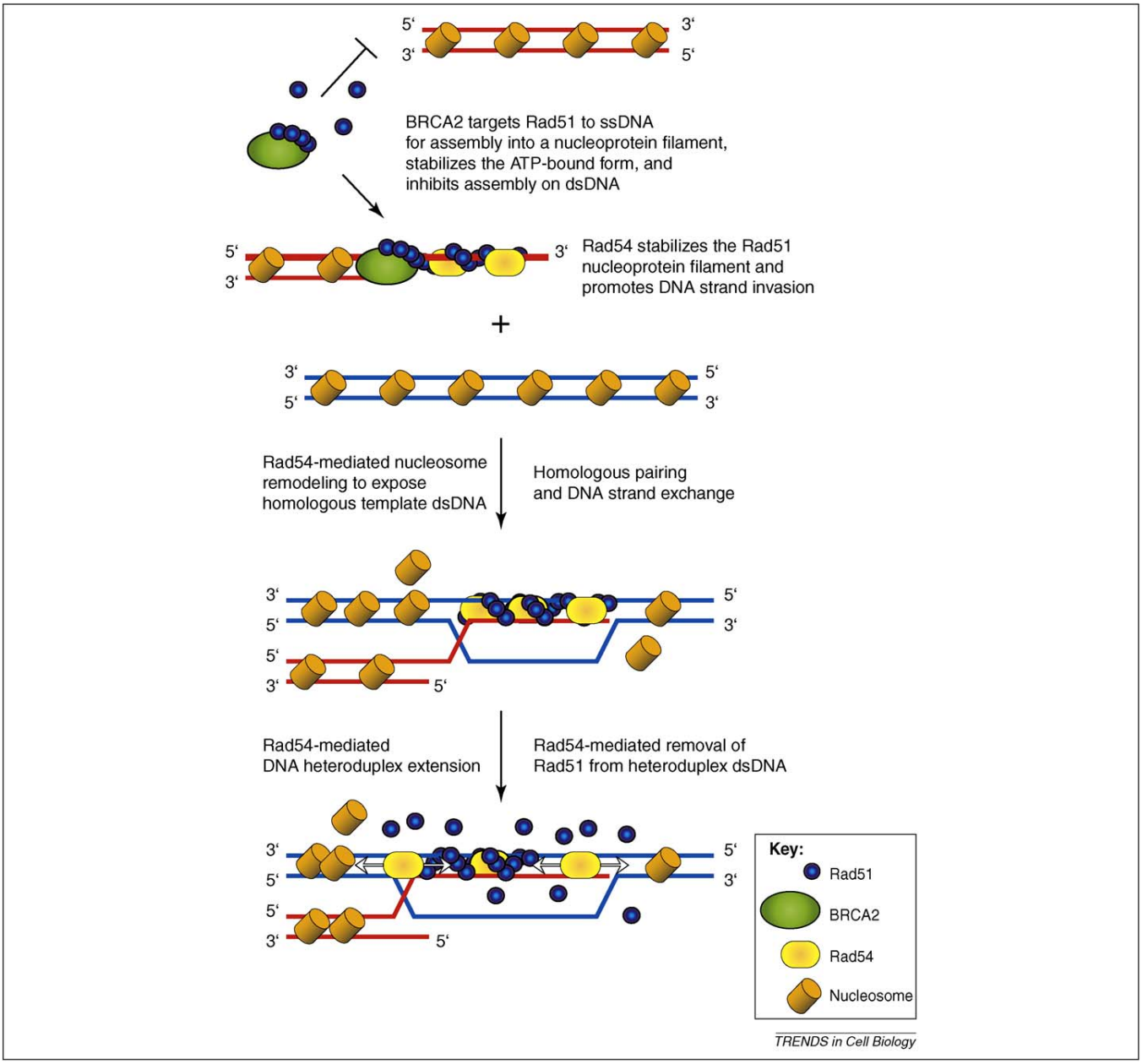

Figure 2. Illustration showing protein-mediated regulation of Rad51 nucleoprotein filament assembly and disassembly. The BRC repeats of BRCA2 (green ovals) promote binding of Rad51 (blue circles) to ssDNA and inhibit binding of Rad51 to dsDNA. The Rad51 filament formed on ssDNA is stabilized by Rad54 (yellow oval). Rad54 also remodels nucleosomal DNA to permit pairing with the homologous target dsDNA. After homologous pairing and DNA strand exchange, Rad51 is bound to heteroduplex dsDNA (red and blue DNA molecules); then Rad54 promotes heteroduplex extension (branch migration) and disassembly of the Rad51–dsDNA filament using its ATP-dependent translocation activity. The Rad54-mediated disassembly of the Rad51-dsDNA filament allows DNA polymerases and other downstream proteins to access the heteroduplex DNA. Subsequent steps (Figure I) complete the process.

occurs via multiple rapid nucleation steps followed by a slower finite growth phase. This process produces a discontinuous filament as a result of nucleation events that occur out of register. Disassembly occurs slowly, requires ATP hydrolysis, and is incomplete, demonstrating the need for an accelerant.

Regulation of Rad51 nucleoprotein filaments

Human Rad51 binds to ssDNA and dsDNA with comparable affinities [70]. This binding of Rad51 to the homologous dsDNA partner inhibits DNA strand exchange [71]. Therefore, it is likely that other recombination proteins play an important role in targeting and loading Rad51 onto ssDNA rather than dsDNA (Figure 2). Such modulation of DNA binding specificity was recently shown to be a function of the BRC repeats of BRCA2 [72]. These BRC repeats had been shown to disrupt Rad51 filament formation on dsDNA [37,73–75], but their effect on ssDNA was not fully examined. A combination of biochemical and single-molecule measurements revealed that both BRC4 and a domain containing BRC repeats 1–8 stimulated the binding of Rad51 to ssDNA, but prevented binding to dsDNA. Stimulation of ssDNA binding by the BRC repeats was accompanied by inhibition of ATP hydrolysis by Rad51. This inhibition served to maintain Rad51 within the ssDNA filament in its ATP-bound form, which is the most stable form of the nucleoprotein filament, and is the species active in DNA strand exchange. Furthermore, the single-molecule analysis showed that the BRC repeats did not accelerate the disassembly of a preformed Rad51 dsDNA nucleoprotein filament, but rather they blocked filament formation by inhibiting nucleation on dsDNA [72]. As a consequence of these interactions, the BRC repeats regulate the DNA binding selectivity of Rad51 by kinetically targeting it to ssDNA and then stabilizing the active filament on ssDNA, which is required for the homology search and DNA strand exchange [76] (Figure 2).

After DNA strand exchange has occurred, the Rad51 filament on the resulting heteroduplex dsDNA product can hinder the activity of proteins (e.g. polymerases, helicases and nucleases; Figure I and Figure 2) that act downstream in the process. Given that dissociation of yeast and human Rad51 from dsDNA is slow and incomplete, additional protein activities must be required to disassemble the Rad51 filament from dsDNA. Because the *in vivo* studies showed that Rad51 foci persist in yeast cells with a *Rad54* deletion, both ensemble and single-molecule approaches focused on Rad54 and its homolog, Tid1 (Rdh54). Using single-molecule techniques, the ATP hydrolysis-dependent translocation of Rad54 was directly observed [43]. Translocation is rapid (~300 bp/second) and processive (~11,500 bp/binding event), and proceeds in either direction along the dsDNA. Single-molecule analysis of yeast Tid1 also revealed that translocation is rapid (~85 bp/second) and processive (~10,000 bp), and occurs in either direction [44]. For both proteins, translocation behavior can be complex, with some molecules displaying pauses, direction reversals, and changes in velocity. Ensemble studies with yeast Rad54 revealed that the ATPase activity of both Rad54 and Rad51 is required for efficient nucleoprotein filament disassembly [46,47]. These findings imply that both translocation by Rad54 and hydrolysis of ATP to ADP by Rad51 (to create the less stable ADP-bound form of the nucleoprotein filament) are needed. Indeed, both Rad54 and Tid1 can disrupt three-strand DNA structures and displace bound protein [44,48,77,78]. Combined, the ensemble results and single-molecule observations reveal a consistent picture of a Rad54/Tid1 function with these motor proteins disassembling Rad51-dsDNA filament, displacing nucleosomes and migrating DNA joint molecules (Figure 2).

Concluding remarks

Single-molecule experiments have already played a crucial role in defining the dynamic behavior of individual Rad51 nucleoprotein filaments. These studies proved to be vital in defining the protein complexes and protein activities that give rise to nuclear focus formation after DNA damage. The results have revealed several key features of Rad51 nucleoprotein filament dynamics, including the number of monomers required for nucleation, rates of filament growth and disassembly, and effects of nucleotide cofactors on the formation and stability of the filament. Additionally, single-molecule and ensemble experiments with other homologous recombination proteins (i.e., BRCA2 and Rad54) have defined regulatory roles in the specificity, formation and stability of the Rad51 nucleoprotein filament.

The high level of resolution, both spatial and temporal, which is attainable by single-molecule techniques permits observation of the actions of many kinds of individual biomolecules. The initial trapping and visualization of a single DNA molecule occurred in 1994 [52,79]. Since then, single-molecule methods have been improved and adapted to investigate the behavior of many different motors and self-assembly systems, both *in vitro* and *in vivo* [80–82]. As illustrated here with Rad51, protein–DNA interactions are ideal candidates for single-molecule analyses [83]. Rates of activity, processivity, forces generated, kinetic/mechanical step size, and sites of interaction are just a few examples of the types of parameters that single-molecule analyses are defining. Now that a variety of inventive single-molecule techniques are available, biochemical systems of increasing complexity can be analyzed with greater sophistication than ever before. The use of more complex DNA substrates, such as nucleosomal DNA or DNA with single-strand gaps, will enable the characterization of additional proteins involved in DNA repair pathways. Similarly, with the selection of fluorescent labels constantly expanding, the ability to simultaneously monitor multiple fluorescent emissions will permit observation of individual components within multi-protein reactions in real-time. Current technologies readily permit simultaneous detection of four different fluorophores. There is no doubt that the reconstitution of more complete systems at the single-molecule level will continue to reveal novel information about these complicated protein–DNA transactions.

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