International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Impact Factor 2025: 7.089

# Single-Molecule Visualization of Srs2-Mediated Rad51 Filament Disassembly During DNA Repair

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**Abstract:** ***In eukaryotic cells, replication protein A (RPA) binds rapidly to exposed single-stranded DNA (ssDNA) gaps to prevent secondary structure formation before passing the substrate to Rad51. Rad51 and replication protein A both play a role in maintaining genome stability during DNA replication. The balance between replication protein A displacement and Rad51 assembly is constantly scrutinized by the translocase Srs2. Srs2 plays a crucial role by translocating progressively from the 3' to the 5' end of the ssDNA, following the same orientation as Rad51 loading. We used single-molecule total internal reflection fluorescence (TIRF) microscopy to evaluate the fate of displaced Rad51 monomers during Srs2-mediated stripping. Rather than being released entirely into free solution, stripped Rad51 molecules are directly replaced by incoming replication protein A molecules tracking immediately behind the moving helicase. This coordinated translation prevents the exposure of naked single-stranded DNA, which protects the genomic template from endonucleolytic cleavage. Additionally, this single-molecule analysis reveals a cohesive, multi-protein recycling pathway that is important for maintaining single-stranded DNA homeostasis during active filament disassembly.***

**Keywords:** Srs2; Rad51; Replication Protein A; Homologous Recombination; Single-Molecule Imaging; DNA Repair; TIRF Microscopy; Genome Stability

## Core Principles and Terminology of Rad51 Filament Dynamics

In reality, the preservation of eukaryotic chromosomal architecture relies on a highly competitive biophysical equilibrium, where structural modifications dictate whether a DNA lesion is steered toward repair or pathway termination. This competitive environment is fully visible during homologous recombination, the cell's primary high-fidelity pathway used to resolve double-strand breaks without introducing mutagenic deletions. The structural centerpiece of this mechanism is the presynaptic filament- in other words, a dense nucleoprotein structure constructed by a Rad51 recombinase coat over long gaps of single-stranded DNA. Before Rad51 can polymerize, however, this single-stranded DNA must be managed by the first-responder complex, Replication Protein A (RPA). RPA binds the exposed nucleic acid strand with extreme affinity, smoothing out secondary configurations to keep the template strand structurally straight.

The transition from a protective RPA coating to a functional Rad51 lattice requires help from recombination mediators that accelerate the nucleation of Rad51 monomers. Once a small piece of Rad51 is established, it undergoes rapid cooperative elongation, driving the displacement of the underlying RPA molecules. The resulting Rad51 nucleoprotein filament alters the physical parameters of the template DNA, untwisting the backbone and stretching it by half its original length to optimize the exposure of genetic bases for subsequent homology searching. However, since the cooperative forces holding the Rad51 polymer together make it incredibly stable, an unmanaged filament can quickly become an obstacle, forming a rigid barrier that blocks replication forks and causes chromosomal structural variations.

To neutralize this high thermodynamic stability, the cell uses the Srs2 translocase—an enzyme that serves as a molecular quality-control supervisor. Actively recruited to troubled replication forks through structural interactions with sumoylated proliferating cell nuclear antigen (PCNA) clamps, Srs2 acts as a directional motor protein. By coupling ATP hydrolysis to directional 3′ to 5′ movement along the single-stranded DNA backbone, Srs2 functions as an active "strippase" that mechanically dislodges Rad51 subunits from the nucleic acid track. This targeted disassembly destabilizes the remaining filament structure, eventually causing a rapid collapse of the polymer and restoring single-stranded DNA homeostasis so the cell can recycle the repair factors.

## Single-Molecule Methodologies and Real-Time Stripping Dynamics

While traditional biochemical assays successfully identify Srs2 as an antirecombinase, they inherently average out the behavior of thousands of interacting proteins simultaneously, effectively masking transient intermediate states, pausing intervals, and individual motor kinetics. To overcome this limitation and observe these hidden behaviors, recent biophysical studies have deployed single-molecule Total Internal Reflection Fluorescence (TIRF) microscopy combined with microfluidic DNA curtains. By fastening long strands of single-stranded DNA to a chemically stabilized glass surface under constant microfluidic flow and labeling Rad51, RPA, and Srs2 with distinct, highly bright quantum dots, researchers can directly watch individual Srs2 motor proteins clear the recombinase coat in real time. These high-resolution traces show that Srs2 does not assemble into massive, multi-subunit rings to exert mechanical force; rather, a single monomer is entirely sufficient to disrupt the stable Rad51 lattice.

These real-time visualizations show that Srs2 translocation is highly processive, strictly unidirectional, and moves exclusively from the 3′ to 5′ end of the single-stranded DNA strand at an average velocity of roughly 50 monomers per second. Surprisingly, the single-molecule trajectories prove that Srs2 is not a passive obstacle that merely blocks filament growth. Instead, it acts as an active, ATP-driven wedge that physically pries open the interface between adjacent Rad51 monomers. Once the terminal "cap" of a Rad51 segment is detached by this action, the cooperative binding network of the remaining polymer segment is broken, initiating a rapid

collapse of the remaining recombinase units down the line like dominoes. Real-time data show that Srs2 specifically docks onto internal gaps or flaws within the Rad51 filament-which are often marked by lone, lingering RPA clusters-rather than binding randomly to a seamless Rad51 coat.

Furthermore, high-resolution single-molecule Förster resonance energy transfer (smFRET) assays have helped analyze the specific structural step size and nucleation limits for this interaction. A single Rad51 monomer occupies an anchor of three nucleotides, while a minimum of six Rad51 monomers is needed to establish a thermodynamically stable, cooperatively nucleating site on a single-stranded DNA template. Srs2 takes advantage of these very same physical constraints. Through ATP-dependent repetitive scrunching of the single-stranded DNA and its use as an antagonist to naked single-stranded DNA, smFRET data indicate that Srs2 continuously cycles short pieces of single-stranded DNA through its motor domain, decreasing the lattice size to below the six-monomer minimum needed to create a stable, cooperative nucleation site for filament elongation.
Additionally, this single-molecule tracking has revealed that the allosteric trigger mechanism is more coordinated than a simple mechanical plow. As Srs2 translocates through the DNA, its C-terminal domain creates a linear protein-protein interaction directly with the neighboring Rad51 monomeric unit. This interaction generates a nucleation point for the internal conformation of Rad51, which results in accelerated intrinsic ATP hydrolysis. Srs2 concurrently stimulates the turnover of ATP within the recombinase, thereby transitioning Rad51 from its high-affinity, ATP-bound conformation to a low-affinity, ADP-bound conformation, causing Rad51 to spontaneously dissociate from the ssDNA. By utilizing the recombinase's own internal chemical clock via this elegant allosteric modification, Srs2 is able to clear a large portion of the genomic landscape utilizing a highly efficient thermodynamic process.

**Biophysical Conflict and Roadblocks by Recombination Mediators**

The specific translocation by Srs2 as a motor along ssDNA tracks is not an isolated process in a vacuum; rather, the Srs2 motor must navigate a highly crowded genomic landscape filled with a variety of DNA-binding proteins and structures. These elements provide competing challenges to the ability of Srs2 to strip DNA. A major biochemical obstacle is presented by the Rad55-Rad57 heterodimer, a highly conserved structural complex that directly binds to the Rad51 helical lattice and interferes with the ability of an Srs2 monomer to translocate along the ssDNA. Using single-molecule real-time tracking and optical trap assays, studies have shown that when an active Srs2 monomer encounters a bound Rad55-Rad57 node, its translocation velocity is greatly reduced. This causes Srs2 to experience extended periods of kinetic stall activity, resulting in long delays while attempting to overcome the region where Rad55-Rad57 blocks the displacement of Rad51. Srs2 does not immediately leave the helicase complex when it encounters a Rad55-Rad57 heterodimer node; instead, it binds at the interface with the Rad55-Rad57 complex and accumulates local mechanical strain over time. These data suggest that a series of competing forces directly affect and define the density of the presynaptic filament through a delicate mechanical tug-of-war between Srs2-driven separation and Rad55-Rad57-mediated stability.

These findings outline that, rather than being simple, passive physical barriers to helicase activity, the regulatory functions of these mediator proteins involve more than just blocking access. After Srs2 successfully passes through these barriers and removes local areas of Rad51, the adjacent Rad55-Rad57 complexes act as “molecular chaperones" to assist in the rapid re-nucleation of free Rad51 monomers from the surrounding nuclear medium directly onto the newly exposed single-stranded DNA track. This rapid replenishment mechanism occurs simultaneously with other specialized co-factors, such as the multi-subunit Shu complex (containing Shu1, Shu2, Psy3, and Csm2), which physically associates with the nucleoprotein filament to lower the disassembly efficiency of the Srs2 motor. The overall result is an ongoing cycle of dynamic, DNA-based biochemical flux wherein single-stranded DNA regions undergo continuous protein removal and rapid restoration. This complex feedback system allows the efficient recycling of transient or uncoupled nucleoprotein clusters while preserving mature, functional presynaptic filaments for downstream strand exchange.

**Spatial Alignment of the Continuous Post-Stripping RPA Trail**

In addition to dismantling recombinase lattices from damaged replication templates, the cellular environment must carefully organize the byproducts of the dismantling reaction so that vulnerable genetic intermediates are not left exposed. Advanced multicolor Total Internal Reflection Fluorescence (TIRF) microscopy systems utilizing mCherry-tagged Srs2 and GFP-tagged RPA have clearly demonstrated that displaced Rad51 monomers do not randomly shed into the nuclear matrix as disorganized aggregates. Single-molecule imaging shows that Rad51 undergoes a highly synchronized and tightly coupled transfer process from one structural element to another on the single-stranded DNA surface. The fluorescence signal from GFP-RPA intensifies sequentially and directly tracks the advancing mCherry-Srs2 motor along the single-stranded DNA scaffold as RPA continues to bind to the vacant regions of the ssDNA.

This immediate, coordinated substitution of Rad51 with a trailing RPA coat plays an essential homeostatic role in protecting the structural integrity of the genomic template during active repair pathway selection. Exposed, uncoated single-stranded DNA gaps represent highly unstable and dangerous structures that are intensely vulnerable to host endonucleases, which can introduce catastrophic secondary double-strand breaks and large-scale genetic deletions if left exposed. By maintaining a continuous, unbroken protein layer throughout the active stripping process, this multi-protein recycling trail safely shields the single-stranded DNA intermediate from enzymatic degradation. Concurrently, this rapid recruitment serves a critical dual regulatory purpose by managing the global DNA damage checkpoint response. By actively clearing old RPA-Mec1 kinase complexes while immediately laying down fresh RPA, Srs2 effectively controls the downregulation and ultimate termination of the Mec1-dependent checkpoint, allowing the cell cycle to safely resume once the local filament landscape has been cleanly reorganized.

**Implications for Genomic Stability and Pathway Selection**
Srs2 plays a key role in maintaining eukaryotic genomic integrity through its ability to mechanochemically clear filamentous material. As an antirecombinase, Srs2 prevents Rad51 nucleoprotein filaments from assembling inappropriately or excessively, which would otherwise lead to hazardous strand invasion of the genome by aberrant, non-homologous joint types. These aberrant invasion events generate toxic joint molecules that can lead to chromosome rearrangements and entanglements. Single-molecule observations show that Srs2 scans single-stranded DNA substrates, mechanically probing and pruning premature or mismatched Rad51 clusters to prevent their completion. The final result of Srs2 mechanochemically filtering a Rad51 repair intermediate is that it drives the process toward the synthesis-dependent strand annealing (SDSA) pathway—a critical, high-fidelity repair pathway that prevents crossover intermediates, potentially catastrophic mitotic crossovers, and extensive loss of heterozygosity (LOH) in somatic cells.

The Srs2 translocase clears Rad51 tracks in a directionally defined manner under stress conditions, ultimately serving as the master switch between the two major pathways utilized for the repair of damaged DNA: homologous recombination (HR) and alternative post-replication repair (PRR) pathways. Under conditions where replication is significantly disrupted and replication forks stall due to DNA damage, the accumulation of sumoylated PCNA clamps directly recruits Srs2 to the sites of damage. This concentrates Srs2's mechanism to strip Rad51 from the three-way junctions of the stalled fork. Srs2 also removes other proteins during Rad51 clearing, creating a clean single-stranded DNA region devoid of proteins, which alters the local thermodynamic competition for the ssDNA substrate. This change allows error-free and error-prone translesion synthesis (TLS) factors- such as Polymerase Zeta (Rev3/Rev7) and Polymerase Eta (Rad30)—to compete more effectively with the bulky Rad51 machinery for template access. Ultimately, by removing Rad51 from the three-way junction and clearing the local area, Srs2 enables dynamic switching between DNA repair pathways based on the structural characteristics of the lesion, minimizing the collapse of replication forks and preserving genomic integrity in the presence of environmental stress.

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