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Local structural dynamics of Rad51 protomers revealed by cryo-electron microscopy of Rad51-ssDNA filaments

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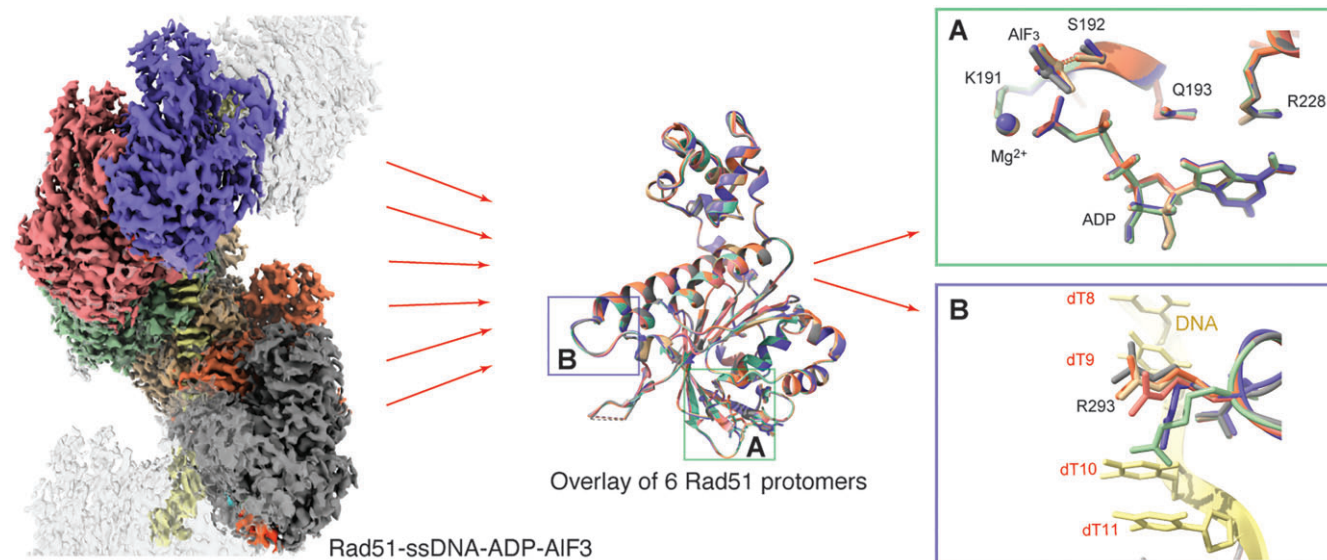
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Abstract

Homologous recombination (HR) is a high-fidelity repair mechanism for double-strand breaks. Rad51 is the key enzyme that forms filaments on single-stranded DNA (ssDNA) to catalyze homology search and DNA strand exchange in recombinational DNA repair. In this study, we employed single-particle cryogenic electron microscopy (cryo-EM) to ascertain the density map of the wild-type budding yeast Rad51-ssDNA filament bound to ADP-AlF₃, achieving a resolution of 2.35 Å without imposing helical symmetry. The model assigned 6 Rad51 protomers, 24 nt of DNA, and 6 bound ADP-AlF₃. It shows 6-fold symmetry implying monomeric building blocks, unlike the structure of the Rad51-I345T mutant filament with three-fold symmetry implying dimeric building blocks, for which the structural comparisons provide a satisfying mechanistic explanation. This image analysis enables comprehensive comparisons of individual Rad51 protomers within the filament and reveals local conformational movements of amino acid side chains. Notably, R293 in Loop 1 adopts multiple conformations to facilitate L296 and V331 in separating and twisting the DNA triplets. We also analyzed the crystal structure of Rad51-I345T and the predicted structure of yeast Rad51-K342E using the Rad51-ssDNA structure from this study as a reference.

Graphical abstract



Introduction

Double-strand breaks, gaps, and interstrand crosslinks (ICLs) in DNA disrupt normal cellular functions and, if left unrepaired, can lead to cell death. Homologous recombination (HR) is a highly conserved pathway that repairs such damages by utilizing an intact DNA template from the sister chro-

matid or homologous chromosome when available [1, 2]. DNA resection, homology search, DNA strand exchange, and new DNA synthesis encompass the central steps of HR, while subsequent steps determine the amount and configuration of new genetic information incorporated into the repaired DNA. Dozens of proteins are involved in HR process and regulation,

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but the key catalytic unit is the filament formed by the essential enzyme Rad51 on single-stranded DNA (ssDNA), which performs the signature reactions of homology search and DNA strand exchange [3, 4].

Nucleotide cofactors directly impact the DNA-binding affinities of Rad51 through the highly conserved ATPase core domain of the Rad51 family of proteins [5]. The ATP-bound form of Rad51 displays high affinity towards ssDNA and forms an ‘active’ nucleoprotein filament by extending B-form-like ssDNA about 1.5 fold to reach a helical pitch of ~95–100 Å [6, 7]. The ADP-bound form of Rad51 forms an ‘inactive’ nucleoprotein filament in a compressed state with a helical pitch of ~75–85 Å [7, 8]. The presence of ATP enhances the assembly of Rad51 filaments on DNA, resulting in enhanced stability and resistance to nuclease digestion [9, 10]. Even though ATP hydrolysis by Rad51 is dispensable for its DNA strand exchange activity, it is important to maintain a stable and readily available protein pool of Rad51. Turnover of ATP and, consequently, Rad51 filaments prevents the formation of overly stable Rad51 filaments and depletion of free Rad51 *in vivo* [11–14]. Likewise, DNA binding has an influence over how Rad51 interacts with ATP. Wild-type Rad51 alone shows minimal ATPase activity, similar to the Rad51-K191A or -K191R mutants in the Walker A box of its ATP binding site. The presence of DNA stimulates the ATP hydrolysis activity of the wild-type Rad51 protein significantly, but it has no effect on the activity of the Rad51-K191A and -K191R mutants [9, 15]. Thus, the interactions of Rad51 with ATP and DNA are interconnected and impactful [5, 11].

Structures of bacteria, archaea, yeast, and human homologs of Rad51 filaments on DNA show highly conserved core domains responsible for ATP, DNA, and protomer-protomer interactions [16–19]. The structures of Rad51-I345T and Rad51-H352Y, two yeast Rad51 mutant proteins that exhibit enhanced DNA-binding activities, have been solved by X-ray crystallography [18, 20]. Rad51-I345T partially bypasses the requirement of Rad51 paralogs, Rad55 and Rad57, through its increased affinity for DNA, even in the presence of RPA [21]. H352 is a key residue at the Rad51 protomer-protomer interface. The Rad51-H352Y mutant forms stable filaments on ssDNA with enhanced resistance to salt disruption but lacks ATP hydrolysis and DNA strand exchange activities [20, 22]. Surprisingly, both Rad51 mutant structures lack DNA and nucleotide cofactors, even though they bind DNA more tightly than the wild-type protein. Additionally, the Rad51-I345T filament exhibits a three-fold symmetry with a dimeric building block, while the Rad51-H352Y filament presents a six-fold symmetry and uniform protomer-protomer interfaces with a monomeric assembly unit. A high-resolution structure of wild-type yeast Rad51 filaments with discernible DNA and nucleotide cofactors is needed to resolve conflicts of the Rad51–Rad51 interface and advance our understanding of the interplay between ATP and DNA and their impact on Rad51 conformation.

Cryogenic electron microscopy (Cryo-EM) has emerged as a promising technique for achieving atomic resolution in the study of macromolecules. Compared to traditional X-ray crystallography methods, cryo-EM offers the advantage of preserving macromolecules in a more native environment. In this study, we utilized cryo-EM single-particle image analysis to obtain a high-resolution structure of a budding yeast Rad51 filament bound to ssDNA and the non-hydrolysable transition-state ATP analog ADP-AlF₃ without imposing he-

lical symmetry. Our analysis of the Rad51 helical filament provides insights into the conformational dynamics of amino acid side chains through the comparison of individual Rad51 protomers within the filament. Notably, we observed movements of S192 and R293 associated with ATP hydrolysis and DNA binding, as well as amino acids involved in protein binding. Furthermore, we integrated molecular modeling with AlphaFold predictions to elucidate the structural basis for the altered activities of Rad51 mutants, including yeast Rad51-I345T and Rad51-K342E.

Materials and methods

DNA and protein purification

The 5′-biotinylated 100 mer used in this study was described previously with the following sequence, 5′-biotinylated AACGACGTTTGGTCAGTTCATCAACATCATAGCCAGATGCCAGAGATTAGAGCGCATGACAA GTAAAGGACGGTTGTCAGCGTCATAAGAGGTTTTAC-3′ [23]. Native untagged budding yeast Rad51 protein was purified as described [24, 25].

DNA-binding assay

Fifteen µL reactions containing 0, 250, 500, 1000, and 2000 nM Rad51 were incubated with 3 µM nt φX174 ssDNA (5 386 nt; NEB) in buffer containing 2 mM nucleotide (where indicated), 35 mM Tris-acetate pH 7.5, 7 mM magnesium acetate, 50 mM NaCl, 1 mg/mL BSA, and 1 mM DTT for 30 min at 30°C. Reaction products were separated on 0.7% agarose gel in TA buffer (40 mM Tris-acetate pH 8) at 4°C and visualized by Sybr Gold staining (Thermo Fisher Scientific).

Cryo-EM sample preparation and data acquisition

Fifteen µM Rad51 was incubated with 45 µM nt of the 5′-biotinylated 100mer in in buffer containing 35 mM Tris-acetate pH 7.5, 7 mM magnesium acetate, 50 mM NaCl, 5% glycerol, 1 mM ADP-AlF₃, and 1 mM DTT for 15 min at 30°C to form nucleoprotein filaments before freezing. 5 µL of Rad51-ssDNA complexes was deposited on thin carbon-coated Quantifoil grids and incubated for 1 min before vitrification using a Vitrobot Mark IV (Thermo Fisher Scientific). Cryo-grids was initially screened using a Thermo Fisher Glacios equipped with Gatan K3 direct electron detector. A large-scale cryo-dataset was collected using a Titan Krios microscope (Thermo Fisher Scientific) equipped with a K3 detector and a BioQuantum energy filter at the Stanford-SLAC Cryo-EM Center (S²C²) [26]. In all, 25 659 movies were collected using SerialEM at a nominal magnification of 130 000, corresponding to a pixel size of 0.68 Å. The movies were collected with an exposure mode of 40 frames in 1.85 s to reach a total dose of 60 e[−]/Å² and a defocus range of −1.2 to −2.2 µm.

Cryo-EM image processing and 3D map reconstruction

The image processing procedure using CryoSPARC is summarized in [Supplementary Fig. S1](#) [27]. The original movies were imported into a CryoSPARC database for motion correction, and dose-weighted/non-dose-weighted micrographs were generated before the contrast transfer function (CTF) estimation. Low-quality micrographs were eliminated after

manual inspection, and particles were picked automatically with a template picker from selected micrographs. Particles were extracted with a box size of 448 by 448. Iterative rounds of 2D classification were performed to remove junk classes and associated poor-quality filaments. A final particle set containing 2 941 384 filaments were used to create three *ab initio* models for following 3D homogeneous refinement. After multiple runs of 3D refinement and CTF refinement, 1 901 160 out of 2 941 384 particles were included to reconstruct the final density map of Rad51-ssDNA filaments at a resolution of 2.35 Å based on the Fourier shell correlation (0.143 Å) criterion. A second species of Rad51 filaments were reconstructed to a 3D map at 2.52 Å, using 754 384 particles.

Model building and refinement

The previously published yeast Rad51-I345T crystal structure was used to generate an initial atomic model that was docked into the EM map by using PHENIX [28]. The ADP-AlF₃, magnesium ion and ssDNA were manually fit into the density map as ligands in COOT [29, 30]. Based on the sequence-nonspecific DNA-binding behavior of Rad51, we used a polydT sequence to build the ssDNA in the atomic model. Multiple building and refinement were performed in PHENIX and COOT to improve model accuracy with no imposed symmetry. Final atomic models were visualized and analyzed in CHIMERA [31].

Structural comparison and modeling

To create the model of the yeast Rad51-dsDNA filament structure, we superimposed the yeast Rad51-ssDNA model from this study with the human RAD51-dsDNA cryo-EM structure (7EJE) in CHIMERA. Based on the almost perfect superimposition of the ssDNA in the yeast Rad51-ssDNA structure and one of the strands from the dsDNA in the human RAD51-dsDNA structure, we replaced the ssDNA with the dsDNA to create the yeast Rad51-dsDNA filament model.

The structural comparisons of the yeast wild-type Rad51 structure (PDB: 9B2D) with the Rad51-I345T (PDB: 1SZP) and Rad51-H352Y (PDB: 3LDA) mutant proteins were performed in CHIMERA. The dimeric structures of the yeast Rad51-I345T and Rad51-K342E were predicted and generated by AlphaFold3, which was further compared to the wild-type Rad51 structure (PDB: 9B2D) in CHIMERA. In general, Rad51-3 and Rad51-4 from the wild-type Rad51 structure were used for alignment with the predicted dimer structures.

Sequence alignment

Alignments of Walker A and B nucleotide binding motifs, and the regions around R293 and V331 were generated using Clustal Omega. Abbreviations: *Ec*: *Escherichia coli*, *Sc*: *Saccharomyces cerevisiae*, *Sp*: *Schizosaccharomyces pombe*, *At*: *Arabidopsis thaliana*, *Ce*: *Caenorhabditis elegans*, *Dm*: *Drosophila melanogaster*, *Dr*: *Danio rerio*, *Mm*: *Mus musculus*, *Hs*: *Homo sapiens*.

Results

Cryo-EM structure of Rad51-ssDNA filament with ADP-AlF₃.

We purified yeast Rad51 protein and screened Rad51-ssDNA filament formation using different nucleotides (Fig. 1A and

B). To achieve optimal protein-DNA complex formation, we incubated Rad51 with a mixed sequence 100-mer ssDNA (biotinylated at its 5' end) and ADP-AlF₃, which enabled us to obtain high-quality micrographs for cryo-EM studies. Instead of conventional helical filament analysis, we employed single-particle image analysis with no imposed symmetry to obtain cryo-EM structures of the Rad51-ssDNA filaments in the presence of ADP-AlF₃ at resolutions of 2.35 Å (Fig. 1C) and 2.52 Å (Supplementary Fig. S1). The parameters of data collection, refinement, and model validation were listed in Supplementary Table S1. Our study yielded two almost identical right-handed helical structures with a 100 Å pitch and ~6.3 Rad51 protomers per turn (Supplementary Fig. S2A and B). The reconstructed maps were subjected to an automatic symmetry search, which revealed the presence of 6.32 Rad51 protomers per turn in the higher-resolution map and 6.31 Rad51 protomers per turn in the lower-resolution map (Supplementary Fig. S2). The spatial forms and helical parameters of our reconstructed maps are similar to those of previously published human RAD51-DNA cryo-EM structures [32, 33].

We built an atomic model of the Rad51-ssDNA filament using the 2.35 Å map, containing six Rad51 protomers that are bound to the nucleotide cofactor ADP-AlF₃ and 24 nucleotides of ssDNA, which are modeled as polydT (Fig. 1D and E). The ADP-AlF₃ and magnesium ions are positioned at the interface between two Rad51 protomers, surrounded by the Walker A and Walker B motifs from one Rad51 protomer and one alpha-helix (α 15) and one loop from the neighboring Rad51 (Fig. 2A and B). The fits of the modeled ADP-AlF₃, magnesium ion, and ssDNA nucleotides with the well-resolved density map are illustrated in Figs 2C and 3D. We were unable to observe the densities of the first 78 amino acids at the N-terminus and several amino acids in the flexible DNA-binding Loop 2, indicating that these regions are highly dynamic. These observations are consistent with the yeast Rad51 crystal and human RAD51 cryo-EM structures [18, 20, 32, 33]. We constructed a second atomic model of the Rad51-ssDNA filament using the 2.52 Å map, which overlaps well with the first model at higher resolution showing only slight differences in twist (Supplementary Fig. S2C and D).

The binding and stretching of ssDNA by Rad51, which occurs in a nucleotide triplet pattern (Fig. 3B–E), is similar to that of *E. coli* RecA and human RAD51 [32–34]. Our analysis identified key amino acid residues within the DNA-binding Loop 1, specifically R293, L296, and S297, as well as V331 in the DNA-binding Loop 2 of the neighboring Rad51, which play a crucial role in partitioning nucleotide triplets (Fig. 3E). These residues are highly conserved in eukaryotic Rad51 proteins (Fig. 3A). The positively charged channel, positioned along the interior of the Rad51 filament, binds to the negatively charged phosphodiester backbone (Supplementary Fig. S3A and B). L296 and V331 from two adjacent Rad51 protomers were inserted between two nucleotide bases to form the unique nucleotide triplet structure (Supplementary Fig. S3C and D). Additionally, these two bases are also pushed by R293 and S297, for instance, dT9 and dT10 or dT12 and dT13, as illustrated in Fig. 3E. The combined efforts of these four amino acids result in an extension and separation of the triplets by breaking base stacking, compared to B-form DNA, as illustrated in Fig. 3E and Supplementary Fig. S3D. The action of these four amino acids

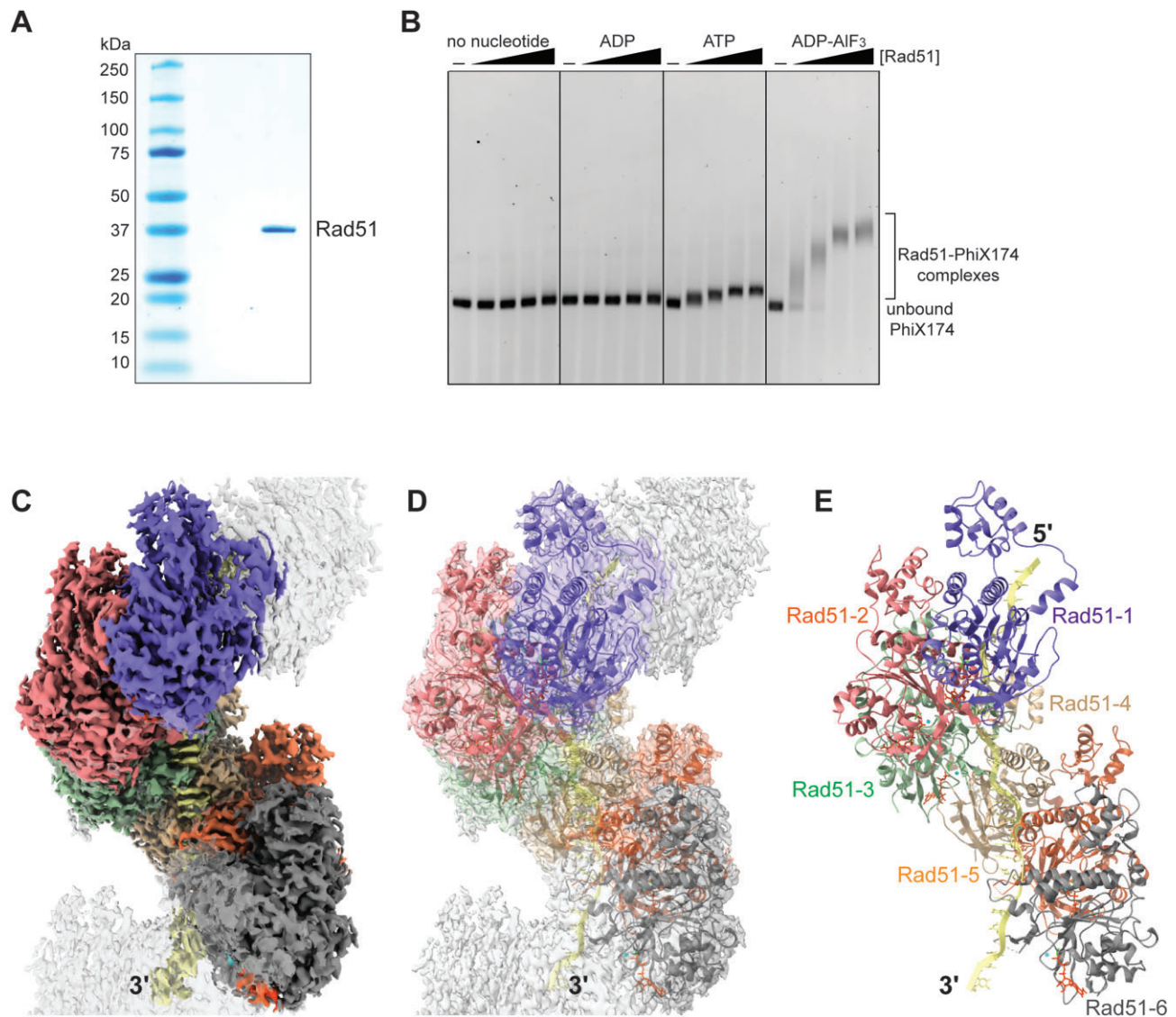


Figure 1. A cryo-EM Structure of yeast Rad51-ssDNA filament with ADP-AlF₃ at 2.35 Å resolution. **(A)** Around 1 µg of purified yeast Rad51 visualized on a 4–20% gradient SDS-PAGE stained with Denville Blue. Protein standards were labeled on the left. **(B)** EMSA of Rad51 binding on ϕ X174 ssDNA with no nucleotide, 2 mM of ADP, ATP, or ADP-AlF₃. **(C)** Density map of Rad51-ssDNA filament with six color-coded Rad51 protomers and ssDNA in yellow. **(D)** Overlay of density map in **C** with the molecular model in **E**. Rad51 protomers are individually labeled in **E**.

is highly conserved in the active human Rad51-ssDNA cryo-EM structure (Supplementary Fig. S4A).

The first high-resolution structure of yeast Rad51 was the crystal structure of the Rad51-I345T mutant filament which exhibits a three-fold helical symmetry and comprises two types of protein–protein interfaces [18]. These interfaces stem from two distinct Rad51-I345T protomers, which display slight movement and twist at the N-terminal domains using the C-terminal ATPase core domain as a reference (Fig. 4A [18]). However, when all six Rad51 protomers from this study align for comparison, we do not observe different types of protein–protein interfaces (Fig. 4B). This finding is similar to the uniform protomer–protomer interface observed in the crystal structure of the yeast Rad51-H352Y protein [20]. From these observations, we conclude that wild-type Rad51 forms DNA filaments with single protomer building blocks and that the dimeric building block structure of the Rad51-I345T ssDNA filament is specific to this mutant (see also below).

Local dynamics of amino acids of Rad51 involved in ATP-binding

We noticed that the AlF₃ were trapped at different positions, despite the remarkable similarity between ADP and the protein backbone in the superimposition of six Rad51 protomers (Fig. 4C). We entertained the idea of whether reaction intermediates could be captured by aligning all the six Rad51 protomers, with a focus on amino acid side chains. Indeed, we discovered the side chain of S192 could interact and pull AlF₃ away from ADP (Fig. 4C and D). The varied positions of S192 and AlF₃ suggest how the movement of S192 might work with the γ -phosphate to facilitate K191 in catalyzing ATP hydrolysis. Interestingly, K191 does not show any movement, but that may be due to the presence of the non-hydrolysable transition state analogue, ADP-AlF₃, used in the filament assembly. The quality of fitting modeled K191 and S192 into the density maps is illustrated in Supplementary Fig. S3E, across all six Rad51 protomers.

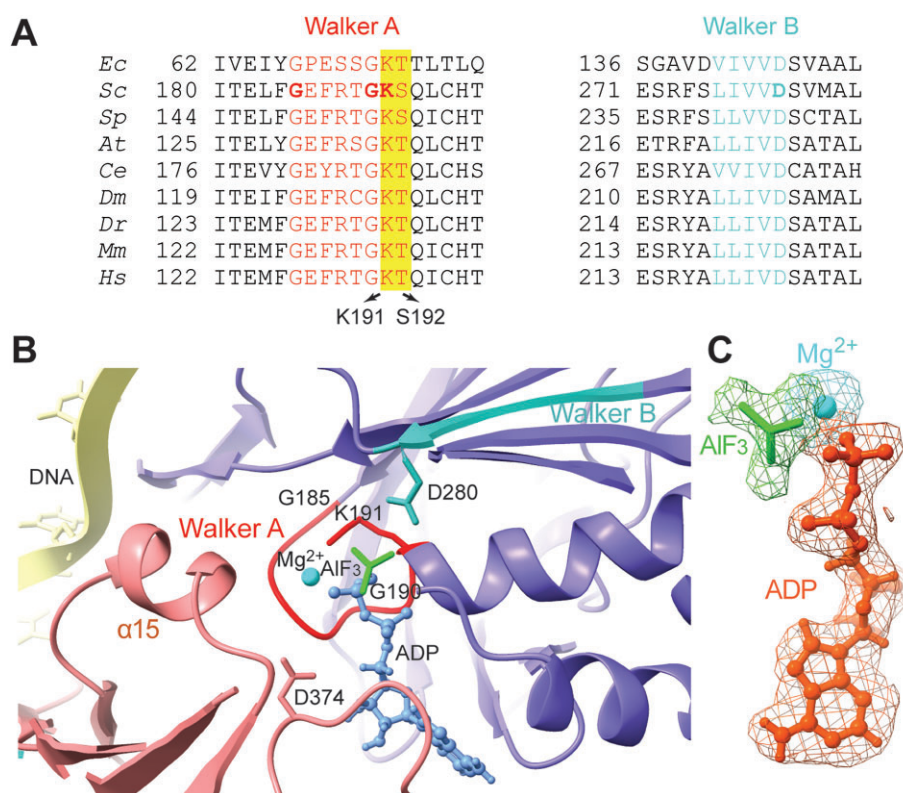


Figure 2. ADP-AIF₃ binding site is located at the Rad51-Rad51 interaction interface. **(A)** Sequence alignment of Walker A and B nucleotide-binding motifs. **(B)** Positions of Walker A and Walker B motifs around ADP-AIF₃ and Mg²⁺. **(C)** Quality of ADP-AIF₃ fit to density map.

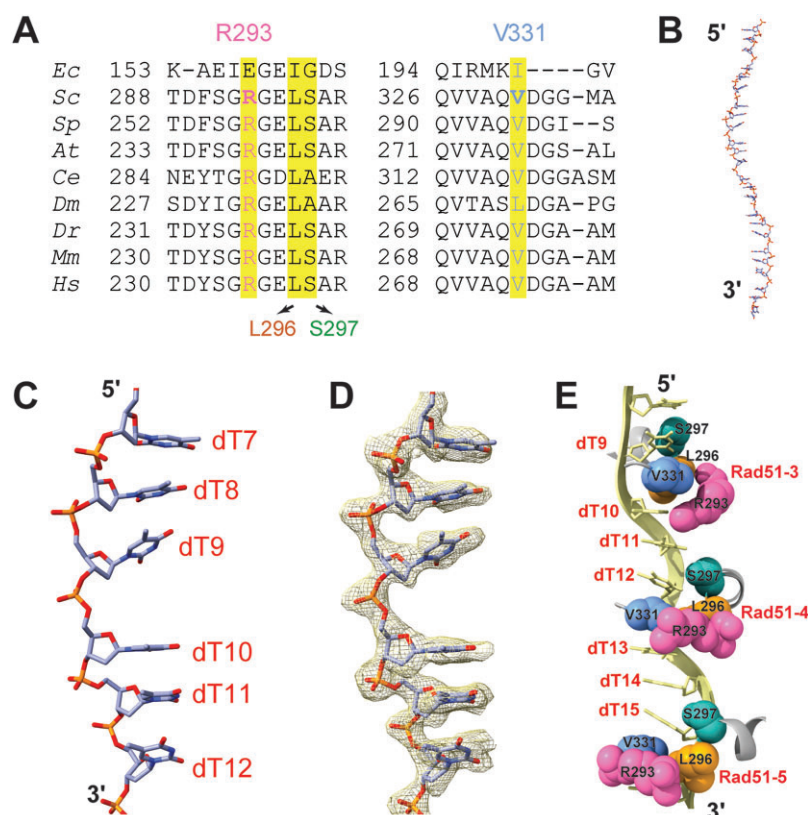


Figure 3. ssDNA triplets and the interacting amino acids. **(A)** Sequence alignment of R293 in the Loop 1 region and V331 in the Loop 2 region. **(B)** A total of 24 nts were mapped in the ssDNA. **(C)** Two ssDNA triplets illustrating the twist of the bases. **(D)** Quality of ssDNA fit to density map. **(E)** Role of R293, L296, S297, and V331 in twisting and unstacking of the DNA bases.

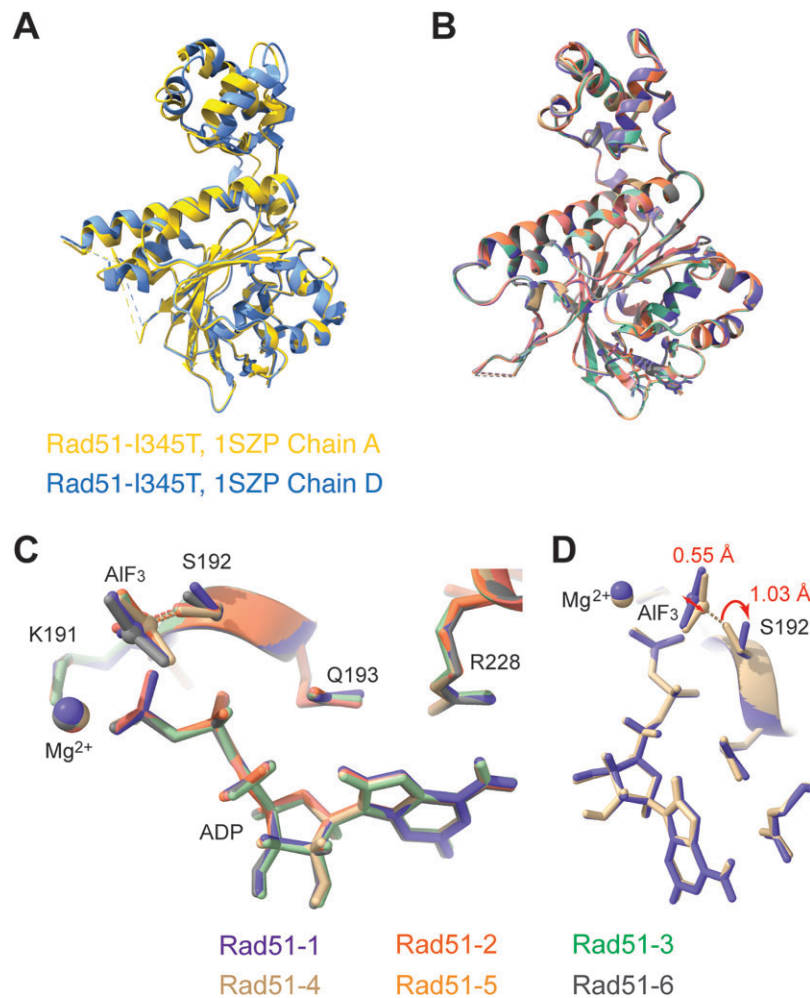


Figure 4. Identical binding interface and individual Rad51 protomer analysis showing movement of key residues during ATP hydrolysis. **(A)** An overlay of Chains A and D protomers of Rad51 in the yeast Rad51 crystal structure (PDB: 1SZP). **(B)** An overlay of six individual Rad51 protomers (color-coded) from **Fig. 1E**. **(C)** S192 pulls AlF₃ away from ADP to facilitate ATP hydrolysis in the enlarged view of the bound ADP-AlF₃ from **(B)**. **(D)** The maximal movement of S192 in two different Rad51 protomers is shown in purple and tan.

The crystal structure of Rad51-H352Y was obtained with six-fold helical symmetry, but multiple conformations of amino acid side chains were still present. Specifically, it has been observed that the Y352 residue adopts two distinct conformations in a 1:1 ratio, as indicated by mixed density [20]. We also examined published cryo-EM structures of human RAD51 structures with ssDNA or dsDNA, derived from imposing helical symmetry, based on the high structure and function conservation of yeast and human Rad51 proteins. Human T134 (yeast S192) also adapts different conformations, pulling Mg²⁺ and the γ -phosphate (**Supplementary Fig. S4B** and **C**) in a similar way to yeast S192 (**Fig. 4C** and **D**). The orientation of K133 in human RAD51 remained unchanged as the equivalent yeast K191 (**Supplementary Fig. S4B** and **C**). This consistency confirms the local dynamics of residues in yeast and human Rad51 involved in ATP hydrolysis.

Local dynamics of amino acids of Rad51 involved in DNA-binding

Next, our investigation delved into the local movements of the amino acids involved in DNA binding. As illustrated in **Fig. 5A**, R293 from different yeast Rad51 protomers exhibit mul-

tiple conformations, distributed within the top and bottom nucleotide bases from two adjacent triplets (dT9 and dT10). The different positions of R293 show a maximal distance of 7.58 Å and suggest a pushing action towards the nucleotide base dT10 (**Fig. 5B**). The dynamic movement of R293 is supported by the good fits into the density maps across all six Rad51 protomers (**Supplementary Fig. S3F**). We further compared the wild-type Rad51 structure from this study with the crystal structures of Rad51-I345T and Rad51-H352Y with a focus on R293 and S192. No density of R293 is observed in the two mutant structures, reflecting a flexible nature of R293, consistent with our analysis (**Fig. 5C**). S192 from the two mutant structures is in a different orientation compared to the wild-type Rad51 structure, considering the similarity of the residing α -helix and neighboring loop (**Fig. 5D**). Interestingly, the two mutant structures have no nucleotide present and side chains of S192 points to a different direction, compared to S192 from the wild-type structure with ADP-AlF₃. Additionally, we compared four known human RAD51 filament cryo-EM structures. R235 from two different human RAD51-ssDNA structures (7EJC and 5H1B) also displayed two conformations within two nucleotide bases with a 3.5 Å distance (**Supplementary Fig. S4D**), supporting a dynamic nature of yeast R293 (human R235).

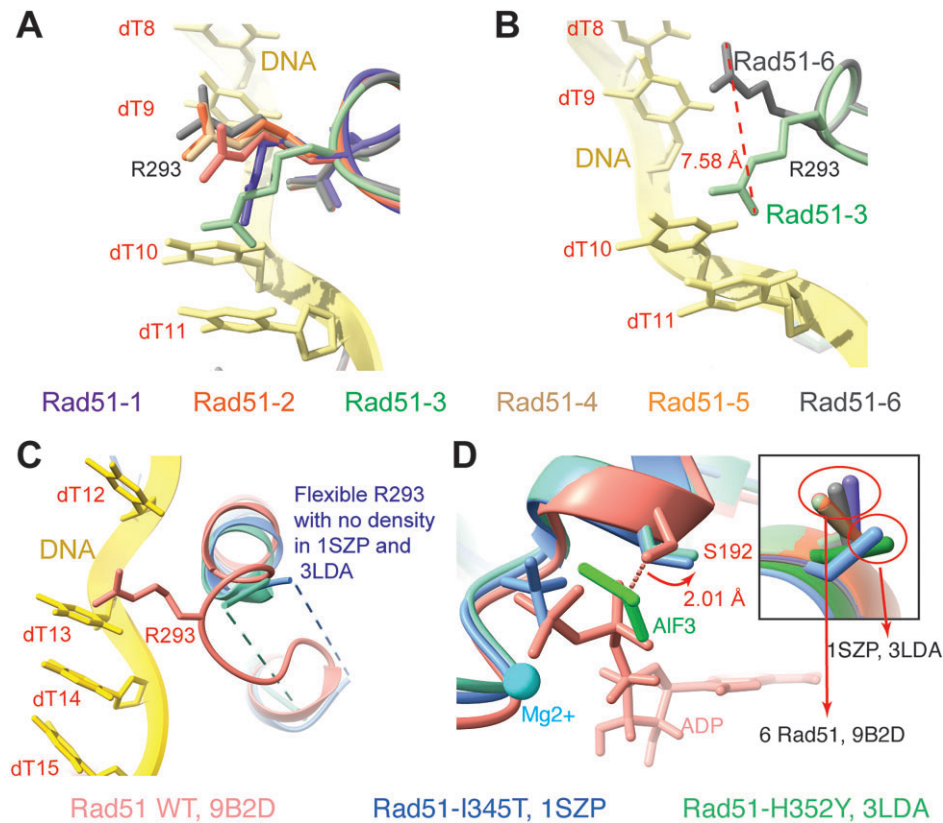


Figure 5. Movement of key residues during ssDNA binding. (A) Multiple conformations of R293 in different Rad51 protomers are illustrated, with the maximal distance shown in B. Amino acids from each Rad51 protomer are labeled as indicated. (B) The maximal distance between the two R293 NH1 from Rad51-3 and Rad51-6 is shown. (C) Positions of R293 in the cryo-EM structure (this study, PDB: 9B2D, salmon) and the published crystal structures of Rad51-I345T (PDB: 1SZP, blue) and Rad51-H352Y (PDB: 3LDA, green) are depicted. (D) Different conformations of S192 are overlaid with the two yeast crystal structures. DNA is shown in yellow with bases labeled.

It is worth noting that in the human RAD51–dsDNA structures, the side chains of R235 and D274 exhibit changes in orientation towards the complementary strand (Supplementary Fig. S4E and F). These consistent movements of yeast R293 and corresponding human R235 may play crucial roles in coordinating the insertion of L296 and V331 (human L238 and V273) to separate neighboring DNA triplets during the presynaptic stage while stabilizing complementary strands in the postsynaptic stage (Figs 3E and 5C and D).

Local dynamics of amino acids of Rad51 involved in protein-interaction

We continued to investigate whether the amino acids involved in self-association or hetero-protein association also exhibit local movements. First, we analyzed the conformations of the amino acids in the α -helix (α 6) located at the Rad51–Rad51 interaction interface (Fig. 6A and B). Of the residues examined, D149, R153, E156, and I158 exhibit multiple orientations, all positioned away from the filament axis and exposed to the solvent (Fig. 6C). Conversely, F144, T146, A147, and H151 of the FxxA motif interact with the neighboring Rad51 protomer and display only one orientation with minimal movements (Fig. 6C, Supplementary Fig. S5E).

Next, we inspected the evolutionarily conserved acidic patch discovered in fission yeast Rad51, which is involved in hetero-protein associations with multiple partners [35]. In budding yeast Rad51, this patch comprises three aspartates (D239, D241, D242) which are highly dynamic and are ori-

ented towards the solvent (Fig. 6D), thereby accommodating a sensing configuration to search and interact with other recombination protein factors such as Rad52, Rad55–Rad57, and Rad54 [35]. Our observations also reveal that the amino acids present on the neighboring β -sheets, such as V247, A248, and Y249, interact with the adjacent Rad51 without exhibiting any alternative conformations, irrespective of their facing direction (Fig. 6E). Similarly, the amino acids on the neighboring α -helix (α 10), from P226 to Q234, also interact with adjacent Rad51 and show minimal movement.

A 3D model for Rad51 mutant analysis

To evaluate the Rad51 mutants with dsDNA-binding defects, we modeled a Rad51–dsDNA filament structure. First, the yeast Rad51 model was compared with the human RAD51–dsDNA cryo-EM structure (7EJE), and the two structures superimposed well with almost identical protein–DNA contacts (Fig. 7A). Furthermore, the positions of ssDNA backbone and bases in yeast Rad51–ssDNA structure aligned almost perfectly with one strand from the human RAD51–dsDNA (Fig. 7A and B), enabling us to model a yeast Rad51–dsDNA filament (Supplementary Fig. S5A and B). This model holds significant implications for the analysis of Rad51 mutants with dsDNA-binding defects.

Next, we compared our reconstructed Rad51 monomer with the two previously published yeast Rad51 mutant structures (I345T and H352Y). The core domain of four chains from three distinct structures aligns well, with only a minor

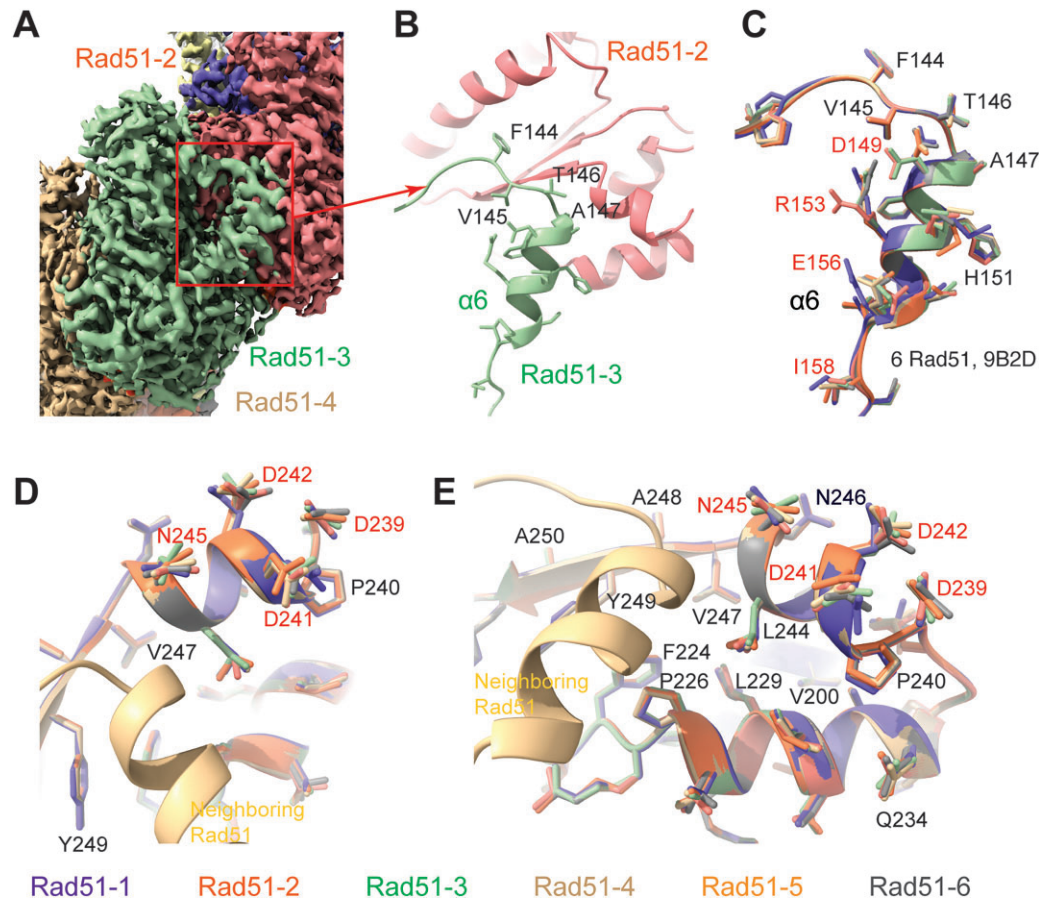


Figure 6. Solvent-exposed residues on alpha-helices at protein-interaction interfaces exhibit flexibility and can adopt multiple conformations. **(A-C)** Conformations of flexible and rigid amino acids at the Rad51-Rad51 interface (FxxA motif). The marked α -helix ($\alpha 6$) and the adjacent loop in **A** were enlarged in **B** and overlaid in **C**. **(D)** Multiple conformations of D239, D241, and D242 within the acidic patch ($\alpha 11$) involved in hetero-protein association. **(E)** Amino acids in the neighboring α -helix ($\alpha 10$) and β -sheet surrounding the acidic patch show minimal movement. Rigid residues are labeled in black, while flexible ones are in red.

structural divergence observed in the N-terminal helical bundle. This divergence is caused by different hinge orientations between the top and bottom domains (Supplementary Fig. S5C). It is most noticeable between the wild-type configuration from this study and the H352Y structure (Supplementary Fig. S5D), supporting the dynamic movement of the helical top as a cohesive unit and the flexibility of the hinge.

Rad51-I345T is a gain-of-function mutant that exhibits increased DNA-binding activity while retaining normal ATPase activity *in vitro* and biological function *in vivo* [21]. Its crystal structure is characterized by a helical filament with a pitch of 130 Å, which is longer than the wild-type structure from our study (Supplementary Fig. S6A–C) and most active Rad51-DNA filaments imaged by EM studies [6, 7, 18, 32, 33, 36]. However, the Rad51-I345T crystal structure lacks the densities of ssDNA and ATP. As a consequence, it does not provide sufficient information to understand how the mutant interacts with ssDNA to increase its binding affinity [18]. To bridge this knowledge gap, we have undertaken a comparison of the Rad51-ssDNA structure from this study with the Rad51-I345T structure to explore the differences and gain a deeper insight into the underlying mechanisms.

We compared wild-type cryo-EM structure with AlphaFold3-predicted and crystal structures of Rad51-I345T and identified Q326 as a key residue with dynamic movements and altered H-bonds (Fig. 7C). In contrast, the

overlay of six protomers of wild-type Rad51 structure show the rigidity of Q326, compared to the different orientations of Q326 in two experimentally determined models (Supplementary Fig. S6D, E). In wild-type Rad51, I345 forms two hydrogen bonds with N325 and Q326, located within a small loop away from ssDNA at distances of 2.95 and 3.30 Å, respectively (Supplementary Fig. S6F). Although T345 is absent from the crystal structure of the Rad51-I345T, the predicted mutant T345 forms tighter H-bonds with N325 (2.83 Å) and Q326 (3.01 Å) (Supplementary Fig. S6G), explaining the different orientations of Q326 as shown in Fig. 7C. Consequently, Q326 in the Rad51-I345T structure (1SZP) forms an altered H-bond with K191 (3.22 Å), compared to the H-bonds formed by Q326 with I345 and E221 in the wild-type structure (this study, Fig. 7D and E). K191 is the catalytic residue for ATP hydrolysis, and the direct H-bond formed by Q326 and K191 in the Rad51-I345T structure (1SZP) is expected to lock the mutant in a high-affinity form leading to enhanced activity and a super extended helical I345T filament with a 130 Å pitch (Supplementary Fig. S6A–C).

A 3D model facilitating artificial intelligence-guided mutant structure prediction and analysis

Protein structure prediction systems such as AlphaFold play a vital role in creating molecular models of both wild-type

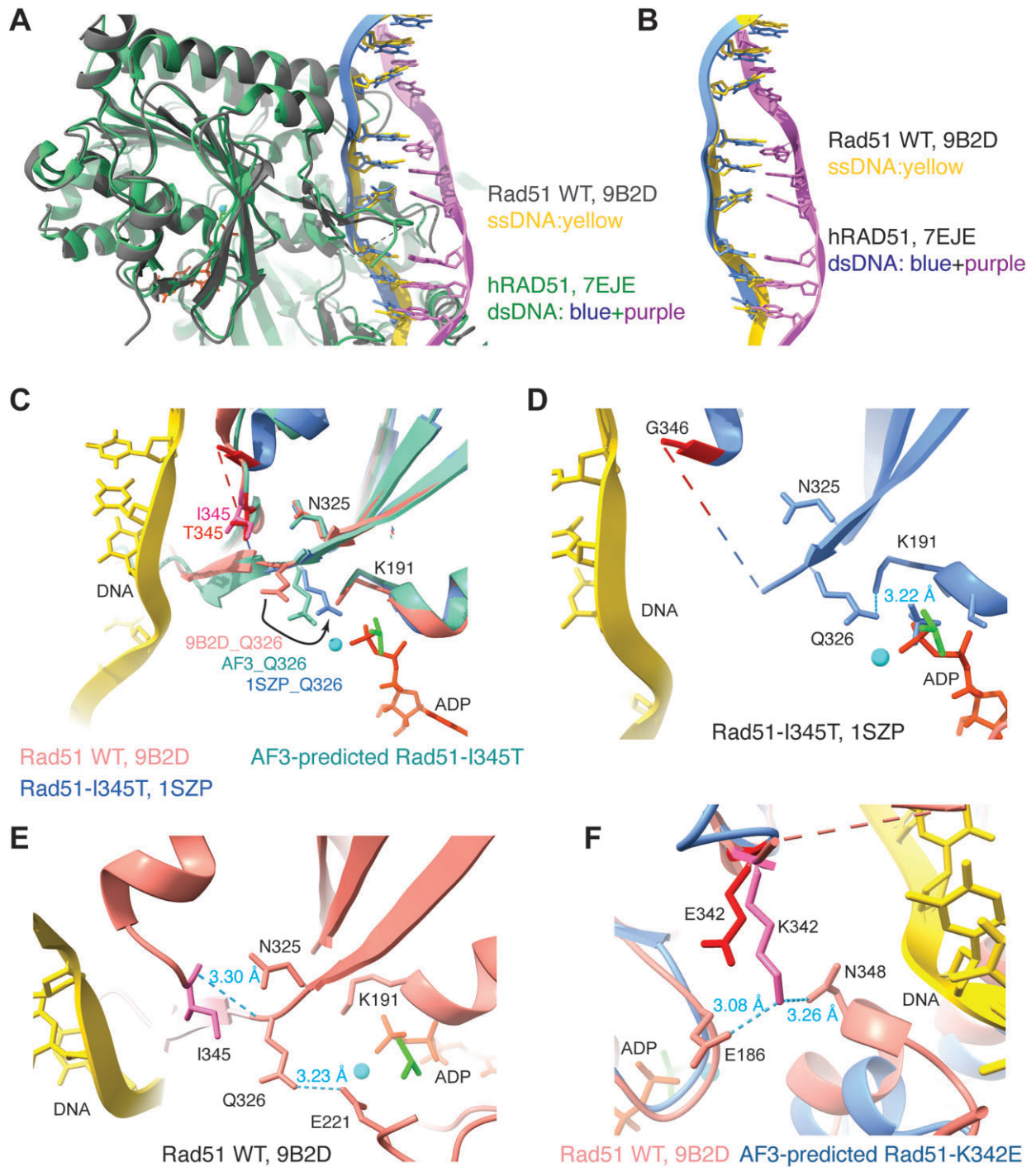


Figure 7. The contacts between Rad51 and DNA, as well as the positions of the DNA backbone and bases, are highly conserved in yeast Rad51-ssDNA and human RAD51-dsDNA complexes. **(A)** Overlay of the yeast Rad51 (gray) -ssDNA (yellow) model from this study (PDB: 9B2D, gray) and the human RAD51-dsDNA cryo-EM structure (PDB: 7EJE, green). **(B)** A detailed view of ssDNA (yellow) with dsDNA (blue and purple) from **A**. **(C)** Overlay of the WT Rad51-ssDNA cryo-EM model (PDB: 9B2D, salmon), AlphaFold3-predicted model (green) and crystal structure (PDB: 1SZP, blue) of Rad51-I345T demonstrates the movement of Q326. **(D)** Q326 forms an H-bond with K191 in the Rad51-I345T structure (PDB: 1SZP). **(E)** Q326 forms alternative H-bonds with E221 and I345 in the WT Rad51-ssDNA (PDB: 9B2D). DNA from **C** is shown in yellow and included as a reference in **D-E**. **(F)** Overlay of WT Rad51-ssDNA cryo-EM model (PDB: 9B2D, salmon) and AlphaFold3-predicted Rad51-K342E structure (blue) with DNA (yellow).

and mutant proteins. The precise location of DNA and nucleotide cofactor in our Rad51 structure, in combination with local amino acid dynamics, enables us to improve the analysis of predicted mutant structures. We conducted an analysis of the artificial intelligence (AI)-predicted structure of the yeast Rad51-K342E mutant. This mutant exhibits significant defects in dsDNA-binding, while demonstrating DNA-independent ATPase activity that is sensitive to ADP inhibition. Furthermore, the K342E mutant forms protein filaments in the absence of DNA [37]. K342 is distributed along the DNA within the helical filament and is situated between ADP- AlF_3 and DNA (Supplementary Fig. S7A–C). In the wild-type filament, K342 forms hydrogen bonds with E186 at 3.08 Å and N348 at 3.26 Å, both of which are lost by the E342 mutation (Fig. 7F). N348 interacts with DNA backbone and E186 is within the nucleotide-binding Walker A motif, providing a structural basis for the loss of coordination of nucleotide- and DNA-binding in the K342E mutant, as well as the observed defects in DNA-binding.

Discussion

Helical symmetry has been widely imposed on 3D reconstructions of the negative-stained or cryo-EM density maps of Rad51 filaments on ssDNA and dsDNA in order to generate different views of a single protomer and thus improve structural resolution of the general Rad51 filament structure [7, 32, 38]. However, helical reconstructions eliminate the side chain variation that may exist between protomers within a Rad51 filament at high resolution. For the first time, we performed single-particle analysis without imposed helical symmetry and reconstructed a high-resolution map of Rad51-ssDNA filaments at 2.35 Å. The molecular model of the filaments, captured with the bound ligand ADP- AlF_3 and ssDNA, is consistent with previous cryo-EM studies of human RAD51 filaments with DNA in a high-affinity state [32, 33]. Comparisons of individually reconstructed protomers lead to the discovery of local structural movements of amino acid side chains within the overall highly conserved and rigid Rad51 nucleoprotein filaments. The observation that these residues in each protomer could be iteratively refined to obtain high-quality density maps indicates the high probability of these residues holding the corresponding conformation in the short repetitive filament segment. The key to achieving high-quality maps for identifying different orientations of flexible residues, such as those found in S192 and R293 in different Rad51 protomers, lies in utilizing a large set of selected particles with high homogeneity. In contrast, the mixed sequence of a 100-mer ssDNA substrate poses challenges for image processing due to the averaging effects of pyrimidines and purines. This limitation constrains the high resolution primarily to pyrimidines and restricts the modeling of nucleotides to thymidines (Supplementary Fig. S3F).

Our model highlights how structural features of the Rad51 filament could facilitate DNA binding and two notable ways several Rad51 residues distort the ssDNA structure from the expected B-form helix. Along the central axis of the Rad51 filament is a positively charged channel that we propose mediates the initial binding of Rad51 onto the ssDNA backbone (Supplementary Fig. S3A and B). The binding process of Rad51 occurs in a DNA sequence-independent manner and rapidly leads to conformational changes in the Loop 1 and Loop 2 regions. Unlike the flexible R293, L296 from Loop

1 and V331 from Loop 2 are rigid enough to insert between DNA bases and organize the bases into groups of nucleotide triplets. When comparing the structures of human Rad51-ssDNA and RAD51-dsDNA, R235 and D274 exhibit a swing towards the complementary DNA strand (Supplementary Fig. S4E and F). As previously observed in *E. coli* RecA and human RAD51 structures, Loop 2 separates two positively charged channels to bind ssDNA and donor dsDNA, respectively [32, 39]. This suggests that the flexible Loop 1 and Loop 2 play a crucial role in engaging homologous DNA for base pairing and exchange. Furthermore, the DNA structure is strikingly altered compared to B-form DNA with the base insertion of L296 in Loop 1 and V331 in Loop 2 every three nucleotides (Supplementary Fig. S3D). The distinct length extension and base twist of DNA are also facilitated by R293 and S297. The dynamic movement of R293 is in direct contrast to rigid L296 and V331, which pushes the top base in each ssDNA base triplet to break base stacking (Fig. 5A and B). The stability of the ssDNA is significantly weakened by base unstacking, which facilitates the sampling of donor sequences for homology. The role of yeast R293 (human R235) is supported by the severe defects of human RAD51-R235E mutant in dsDNA binding and DNA strand exchange activity and decreased activity in ssDNA binding [40]. These findings suggest that yeast R293 (human R235) is required for the synaptic and post-synaptic activities of Rad51 filaments. In brief, the tight binding of the DNA backbone is responsible for the sequence non-specific binding of Rad51. Meanwhile, the subsequent dynamic movements of the amino acids in Loop 1 and Loop 2 dictate the triplet formation required for homology search and pairing. These local yet dynamic structural changes are exhibited by the protomers in the Rad51-ssDNA filament, akin to a timelapse in frames.

Rapid freezing in cryo-EM preserves target proteins in their native state with an aqueous buffer, providing a unique opportunity to seize enzymes in action. Transient states of enzymes or reaction intermediates can be captured provided the number of single particles is large enough, similar to single molecule studies. K191 in the Walker A motif has been widely characterized as the critical catalytic residue for ATP-binding and hydrolysis [9, 13]. Our analysis shows that K191 is rigid, and this lack of movement probably reflects the non-hydrolysable feature of ADP- AlF_3 . K191 is immobilized by a Mg^{2+} ion and the β -phosphate of the ADP- AlF_3 (Fig. 5A). In addition, S192 was identified here to play a facilitating role in ATP hydrolysis by pulling AlF_3 away from ADP (Fig. 5B). This action is presumed to work with K191 to break the high-energy phosphoanhydride bonds in ATP. Consistent with our analysis, yeast Rad51-S192A and Rad51-S192E mutant cells show hypersensitivities to the DNA-damaging agent methyl methanesulfonate. Purified Rad51-S192A and Rad51-S192E mutant proteins display severe defects in both ATP hydrolysis and ssDNA-binding [41]. Similarly, we noticed the same pattern for human RAD51 that T134 displays a pulling action with three different conformations while K133 almost remains the same conformation (Supplementary Fig. S4B and C). The role of S192 in ATP hydrolysis is supported by the consistency in yeast and human RAD51, validating local amino acid dynamics we observed.

The comparison of individual Rad51 protomers in the filament also reveals local amino acid configurations at the protein-interaction interface. Phenylalanine (F144 in budding yeast Rad51) is an essential residue in the FxxA mo-

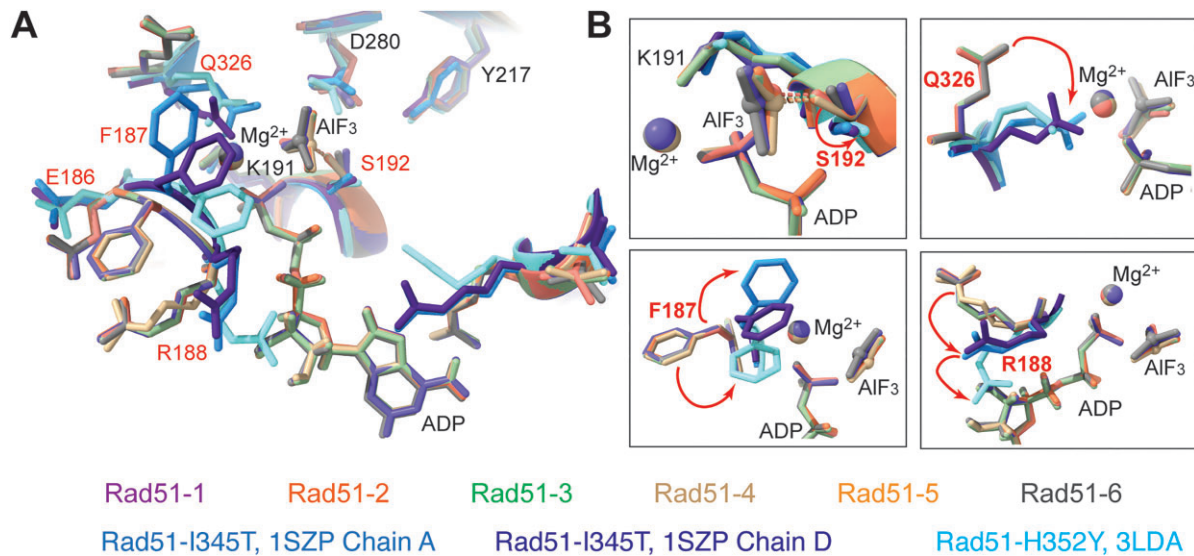


Figure 8. Different configurations of the ATP-binding pocket of the Rad51 protein in its wild-type state and two mutant structures: Rad51-I345T and Rad51-H352Y. **(A)** The ATP-binding pockets from six wild-type Rad51 protomers (this study, PDB: 9B2D, rainbow) are overlaid with Chain A (light blue) and Chain D (purple) from the Rad51-I345T crystal structure (PDB: 1SZP), as well as the Rad51-H352Y crystal structure (PDB: 3LDA, cyan). **(B)** The orientations of F187, R188, S192, and Q326 in Rad51-I345T (light blue and purple) and Rad51-H352Y (cyan) are compared to those in wild-type Rad51 (rainbow).

tif that interacts with the hydrophobic cavity of the neighboring Rad51 protomer (Fig. 3A–C). This important protein–protein interaction motif is responsible for the interaction between human RAD51 and the BRC repeats of BRCA2 as well as RAD51 and SWSAP1, a RAD51 paralog [42–44]. As shown in Fig. 6C, F144 sits at the protomer–protomer interface with V145, T146, and A147, and all four residues across six Rad51 protomers adopt the same conformations including the orientation of side chains. The rigidity of F144, V145, T146, and A147 might lock the neighboring Rad51 in position to form the filament. Interestingly, phenylalanine at other positions also tends to be rigid with no local movements, whether in α -helices or flexible loops. The four amino acids in the false-positive FxxA site, 317–FGVA–320, are located in a similar flexible loop but display no side chain movements. They are buried inside the Rad51 protomer and unlikely involved in self- or hetero-protein interactions of Rad51 (data not shown). Another example is the acidic patch of Rad51 involved in protein interactions with Rad52, Rad55–Rad57, and Rad54 [35]. The three responsible aspartates, D239, D241, and D242, are dynamic, accommodating a sensing configuration to engage other protein factors (Fig. 6D). Similarly, the amino acids in the vicinity but interacting with neighboring Rad51 protomer show minimal movement without alternative conformations (Fig. 6E). Thus, analysis of amino acid dynamics might be effective to identify the key residues in the target motifs for self- and hetero-interactions.

The near atomic-resolution map in this study supports a high-quality fit of a long stretch of ssDNA and the nucleotide cofactor, ADP–AlF₃ (Figs 2 and 3), adding new levels of structural details of the Rad51 nucleoprotein filament. The structural comparisons of the wild-type Rad51 with the previously published structures of the I345T and H352Y mutants [18, 20] reveal the altered interactions of DNA with a single amino acid change and uncover the mechanism behind their gain-of-function mutant behaviors (Fig. 7C–E, Supplementary Fig. S6D–G). The ATP-binding pocket alignment of all three yeast Rad51 protein species reveals a noteworthy general move-

ment of critical residues towards the pocket, as illustrated in Fig. 8A. The I345T structure did not contain ATP γ S and ssDNA, while the H352Y structure does not contain nucleotide and ssDNA. The inward orientation of F187, R188, S192, and Q326 in I345T and H352Y in ‘no nucleotide’ conformation may reflect the transitional state of Rad51 between ADP-release and new ATP-binding (Fig. 8B). This configuration might partly constitute the structural deviation between the wild-type Rad51 and I345T/H352Y, particularly in helical pitch (Supplementary Fig. S6A–C).

For the first time, we detected the local dynamics of amino acids of yeast Rad51 involved in binding ATP, DNA, and proteins. We used this information to analyze the structural changes of yeast Rad51–I345T and Rad51–K342E, and provided satisfying explanations of their biochemical defects. We also created a structure of Rad51–dsDNA with modeled-in dsDNA. We envision that the Rad51–dsDNA filament would be useful to analyze certain Rad51 mutant filaments formed on dsDNA, considering the highly conserved positions of DNA in Rad51–ssDNA and RAD51–dsDNA filaments (Fig. 7A, B). In summary, our study has provided the first high-resolution structure of the yeast wild-type Rad51 filament with detailed information on Rad51–ATP and Rad51–DNA interaction. Additionally, we captured the local dynamics of Rad51 amino acids, which are expected to contribute to the understanding of RAD51 filament formation in HR repair.

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Author contributions: J.L. and S.G. conducted cryo-EM sample preparation and data collection; J.L. performed cryo-EM image analysis and model building; J.L., S.G., and W.D.H. designed experiments and interpreted results; J.L. and W.D.H. designed the study; J.L., S.G., and W.D.H. wrote the manuscript.

Supplementary data

Supplementary data is available at NAR online.

Conflict of interest

None declared.

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Data availability

The density maps and data have been deposited in the Electron Microscopy Data Bank under accession codes EMD-44104 and EMD-44105. The atomic coordinates and data have been deposited in the Protein Data Bank under accession code 9B2D (2.35 Å) and 9ED3 (2.52 Å).

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