The maintenance of chromosome integrity depends critically upon the accurate repair of double-stranded DNA breaks. Homologous recombination is a conserved DNA repair pathway, able to restore chromosome integrity without loss of genetic information. The human RAD51 protein is the core catalyst of homologous recombination, forming filaments on single-stranded DNA and performing the strand-exchange reaction.

Here we combined optical trapping and fluorescence microscopy to unveil the dynamic properties of individual RAD51-ssDNA filaments. We compared two naturally occurring isoforms of RAD51, K313 and Q313, and found that the alteration of a single amino acid results in dramatically different behavior of the RAD51 nuclei: RAD51-Q313 nuclei are static, and the time they remain bound to the ssDNA relates to their size. RAD51-K313 nuclei, on the other hand, can slide and hop along the ssDNA. In addition, we show that the diffusion of RAD51-K313 nuclei depends on their size: larger nuclei often bind without moving along the DNA, while RAD51 monomers predominantly display diffusive motion.

Next, we observed that RAD51-ssDNA filaments are profoundly susceptible to applied tension: filaments can occur in a compressed and an extended conformation. When ATP is bound, filaments are in the extended conformation, while in the ADP state the RAD51 conformation depends on tension: at forces above 9 pN filaments are extended and below they are compressed. Using Jarzynski’s equality we revealed the free energy difference between these two conformations is approximately 4 kB,T.

Revealing the impact of a single amino acid as well as the exquisite force balance of the filament conformations demonstrates the multi-faceted and finely balanced nature of RAD51 protein, the key player of human homologous recombination.
6.1 INTRODUCTION

Homologous recombination (HR) is a multi-step process able to repair endogenous and exogenous DNA double-stranded breaks (DSB). After occurrence and detection of a DSB, the broken DNA ends are processed by the end-resection machinery and 3’ single-stranded DNA (ssDNA) overhangs is created [142, 76]. Subsequently, RAD51, the central catalyst of HR, forms right-handed helical filaments around these overhangs, commonly referred to as the nucleoprotein filament (NPF). The NPF is able to search for and pair with the homologous sequence in the sister chromatid in order to exchange genetic information between the DNA molecules [14].

In vitro, the formation of the NPF can occur in presence of both ATP [174] and ADP [72]. The resulting filaments, however, display very different properties. Electron microscopy studies have revealed that human RAD51 bound to ssDNA in the presence of ATP adopts an extended conformation with a pitch of 9.8 nm [190]. The ADP-bound filament is more compact, with a pitch of approximately 8 nm and is often referred to as the compressed conformation [190]. These two states not only have distinct structural properties but also differ in terms of biochemical function: ATP-bound-extended RAD51 is able to perform strand exchange with high efficiency, while ADP-bound-compressed-RAD51 is less active, and might represent an intermediate state before disassembly [23]. X-ray crystallography identified the location of the ATP-binding pocket between adjacent monomers in the filament [38]. The monomer-monomer interface of the RAD51 filament is therefore crucial for the correct execution of homologous recombination.

Interestingly, two sequences of human RAD51 (D14134.1 and D13804.1) are deposited, differing in the amino acid at the position 313, which could be either a Lysine (K313) or a Glutamine (Q313). Presumably both variants occur among the human population. Within the RAD51 nucleoprotein filament, the residue 313 lies in the proximity of the monomer-monomer interface [81] and therefore could significantly affect the behaviour of RAD51 protein. Biochemical studies proposed that this mutation regulates RAD51 strand-exchange activity through a mechanism involving a differential filament-forming ability [81].
Structural studies provided hints that the recombinase filament is a dynamic entity, able to transition between these conformations in response to nucleotide hydrolysis. The interplay between ATP hydrolysis, structural transitions and disassembly of recombinase proteins was further verified at the single-molecule level. It was demonstrated that RAD51- filaments on dsDNA can undergo multiple transitions between the extended and the compressed state without dissociating, implying that this transition is dictated by nucleotide present at the monomer-monomer interface [136]. Also, it has been revealed that high levels of tension applied on the DNA can stall the filament disassembly process (F>50 pN), possibly by a mechanism in which the above mentioned conformational transition is halted [174]. In addition force has been shown to switch the conformation of bacterial RecA filaments on dsDNA from the compressed to the extended state [37]. So far, a characterization of these dynamic transitions of RAD51 nucleoprotein filaments on ssDNA is lacking.

Here, we have combined dual-optical trapping, single-molecule fluorescence microscopy, force-induced DNA melting and micro-fluidics [176, 174, 59, 25] to simultaneously control and manipulate a long ssDNA molecule while visualizing fluorescently labelled RAD51 bound to it. Our method allowed studying how the applied tension on the DNA affects the physical structure of the RAD51 nucleoprotein filament. We found that forces exceeding 9 pN are able to induce the elongation of ADP-bound RAD51 from the compressed the extended conformation. Furthermore we quantified the free-energy difference between these two structural forms, providing novel insights on the plasticity of the RAD51 recombinase filament.

Next, we visualized the interaction of two naturally occurring variants of RAD51, RAD51-K313 and RAD51-Q313 on ssDNA. Our results reveal that the interaction with ssDNA of K313-RAD51 and Q313-RAD51 are substantially different. K313-RAD51 interacts with ssDNA by both sliding along ssDNA and hopping between different sites. Q313-RAD51, on the other hand, forms filaments that are static.
6.2 MATERIALS & METHODS

6.2.1 RAD51 fluorescent labeling

RAD51 (C319S) fluorescent labelling was performed as previously described [114]. Further biochemical characterization showed that ATP hydrolysis, efficiency in joint molecule formation and DNA binding properties were indistinguishable from wtRAD51.

6.2.2 Preparation of DNA construct

To produce a 48.512 nt ssDNA melting Lambda DNA was biotinylated at the 3’ and 5’ end of the same strand using three oligonucleotides. First, the 5’-end of Lambda DNA and oligonucleotide 1 (5’- ggg cgg cga cct gga caa-3’) and 2 (5’- agg tbg ccc ccc ttt tTt TtT-3’) were phosphorylated for 30 min at 37°C in a reaction containing 14 nM Lambda DNA or 10 μM of the oligonucleotide and 0.25 U/μl of T4 Polynucleotide Kinase in 1x T4 Ligase buffer (Fermentas). Next, oligonucleotides 1 and 3 (5’- TtT tTt ttt aga gta ctg tac gat cta gca tca atc ttg tcc-3’) were annealed to the overhangs of Lambda DNA in a 10:1 oligonucleotide: DNA ratio (total volume 500 μl) by heating the reaction to 65°C and slowly cooling down to room temperature. The ligation reaction was then initiated by adding T4 DNA ligase (0.02 U/μl) and carried out for two hours. Next, oligonucleotide 2 was annealed in a 100:1 ratio to the Lambda DNA construct by incubation at 45°C for 30 minutes. Subsequently, the oligonucleotide was ligated to the DNA at room temperature. Finally, the DNA was purified by ethanol precipitation.

6.2.3 Experimental Conditions

Experiments were performed in 20 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM CaCl2, 10 mM DTT, unless otherwise specified.
6.3 RESULTS

6.3.1 Single-Molecule assay for studying RAD51 on DNA

Our approach, consisting of combining dual optical tweezers, single-molecule fluorescence microscopy and micro-fluidics has been described extensively in previous publications [176, 174, 59, 25].

![Diagram of the single-molecule experimental approach]

**Figure 45.** Single-molecule experimental approach for studying of the mechanical and dynamic properties of RAD51 nucleoprotein filaments. [A] Microfluidics is used for the in situ assembly of the single-molecule assay and the fluorescence visualization in the absence of fluorescent background. The experiment is composed of 5 steps: (i) capture two beads in the optical traps. (ii) Tethering of a single dsDNA molecule between the beads. (iii) force-induced melting and production of a long ssDNA tether. (iv) ssDNA is exposed to the buffer containing fluorescent RAD51 for a defined amount of time. (v) the ssDNA-RAD51 complex is imaged in the absence of fluorescence protein in solution. [B] Example of an individual RAD51 nucleoprotein filament resolved on a single ssDNA molecules held in an extended configuration by dual optical tweezers.

In brief, dual-trap optical tweezers are used to trap streptavidin-coated beads Figure 45.A (4.26 µm diameter – Spherotech). Using a computer-controlled microscope stage and a micro-fluidics system, an individual
double-stranded DNA molecule (dsDNA) can be tethered between the beads. Applying tensions above 80 pN, force-melting can be triggered \cite{173, 60}. Following this method, an intact ssDNA molecule is produced. Next, the ssDNA molecule is exposed to a buffer containing a fluorescent variant of human RAD51 labelled at a specific cysteine residue (RAD51-Q313-C319S-Alexa555 which we refer to as RAD51-Q313 or RAD51-K313-C319S-Alexa555, which we refer to as RAD51-K313) in the presence of ATP and Mg\textsuperscript{2+} or Ca\textsuperscript{2+}. After incubation (for 1 to 5 minutes), the ssDNA molecule is removed from the RAD51-containing buffer and repositioned in the imaging channel. Successful formation of RAD51-nucleoprotein filaments is verified using fluorescence microscopy in absence of RAD51 in solution (Figure 45.B).

### 6.3.2 RAD51 residue 313 affects the dynamic properties of nucleoprotein filaments on ssDNA

To test whether RAD51-K313 and RAD51-Q313 differ in their interaction with ssDNA, we directly visualized individual nucleoprotein filaments on ssDNA in the presence of ATP-Ca\textsuperscript{2+} and ATP-Mg\textsuperscript{2+}. When ATP hydrolysis is prohibited \cite{23} (in the presence of Ca\textsuperscript{2+}), RAD51-Q313 forms static nucleoprotein filaments on ssDNA, as previously reported \cite{114, 174}.

Figure 46.A shows an example of a kymograph in which multiple static nucleoprotein filaments formed by RAD51-Q313 are detected on a single ssDNA molecule. RAD51-Q313 forms nucleoprotein filaments whose stability is dictated by their size (see Chapter 5). The decrease in fluorescence intensity in Figure 46.A follows a typical staircase pattern of single-molecule photobleaching traces, indicating that not the interaction time with the ssDNA but photobleaching limits the time RAD51-filaments can be observed under these conditions. In contrast to RAD51-Q313’s static binding to ssDNA, we found that RAD51-K313 is able to diffuse along the ssDNA showing signatures of both sliding and hopping. The kymograph in Figure 46.B indicates the presence of several RAD51-K313 filaments.

We notice that some filaments are able to display longitudinal motion (see patch “a” in Figure 46.B) while others appear static (see patch “b” in Figure 46.B). We therefore can exclude that the longitudinal displacement observed for these RAD51-K313 filaments are due to the ssDNA thermal fluctuations.
Figure 46: Dynamic properties of RAD51 filaments on ssDNA. [A] Kymograph showing static RAD51-Q313 filaments in the presence of ATP-Ca\(^{2+}\). [B] Kymograph showing RAD51-K313 filaments in the presence of ATP-Ca\(^{2+}\). Most of the nucleoprotein filaments diffuse along the ssDNA (see patch a). Some filaments show no sign of displacement (see patch b). [C] Time-trajectory of single RAD51-K313 nucleoprotein filaments “a” and “b”.

Remarkably, the fluorescent spots in in Figure 46.B disappear suddenly between consecutive frames, independently of their initial fluorescence intensity (see patch “a’ and “b” in 46.B). This indicates that also RAD51-K313 nuclei are able to spontaneously release into solution from the ssDNA, similarly to RAD51-Q313. Kymographs were analysed using single-particle tracking [25] (see Figure 46.C).
The diffusion coefficient of individual RAD51 filaments was determined by linear fitting of the mean-squared-displacements as a function of the time-lag. We restricted our analysis to RAD51 filaments that stayed bound on the DNA for at least 10 frames (N=270 in MgCl$_2$ and N=170 in CaCl$_2$) and obtained a diffusion coefficient of $(1.7\pm0.2)\times10^{-3}$ µm$^2$/s in the case of Mg$^{2+}$ and $(2.2\pm0.2)\times10^{-3}$ µm$^2$/s in the presence of Ca$^{2+}$ (mean±SE). In our experiment, we found that not all of the K313-RAD51 filaments show diffusive behaviour, but a fraction between 50 to 60%, according to the divalent cation present in the buffer (see Figure 47.A).

Furthermore, we noticed that the dynamic properties of bright and dim RAD51 patches tend to differ: bright patches are mostly static while dimmer ones diffuse more frequently. To further quantify this, we determined the fraction of diffusing filaments as a function of fluorescence intensity. To this end, we estimated the lowest diffusion coefficient detectable under our experimental conditions to be $2\times10^{-5}$ µm$^2$/s (based on the localization accuracy (∼20 nm) and the average interaction time of the RAD51 filament in Mg$^{2+}$, approximately 10 to 20 seconds for small filaments). This lowest detectable diffusion constant was used as a threshold for classifying particles as diffusive or static. The histogram of the fraction of diffusive particles as a function of fluorescence intensity (Figure 47.B) indeed shows that brighter (larger) filaments have a higher probability to be statically bound to the ssDNA.

In addition to the reported diffusive behaviour, we found that in rare cases (approximately 5% of the diffusive filaments) RAD51-K313 nucleoprotein filaments exhibit displacements over micrometres within consecutive frames (see Figure 48.A and Figure 48.B). We attribute this behaviour to the release and the re-association of the filament to a different location (hopping). It is very unlikely that such large displacements are due to translocation while remaining bound to the ssDNA, considering the very low diffusivity of RAD51. In addition, Figure 48.C shows an example in which a static RAD51 filament (patch a) is located in between the unbinding and rebinding locations of another, hopping one (patch b). Considering the helical structure of RAD51 filaments, it is very unlikely that a purely sliding mechanism is responsible for the observed filament bypass. Therefore we conclude that RAD51-K313 diffusion occurs through a combination of both size-dependent sliding and hopping.
Figure 47: Diffusion of K$_{313}$-RAD51. [A] Distribution of diffusion coefficients for RAD51-K$_{313}$ in Ca$^{2+}$ (red columns, $N=170$) and Mg$^{2+}$ (blue columns, $N=270$). [B] Histogram showing the relationship between the fluorescence intensity (size) of RAD51-K$_{313}$ nucleoprotein filaments and the probability to display diffusive behaviour.

6.3.3 Disassembly of RAD51 nucleoprotein filaments shows pausing-burst behavior

Next, we studied ATP-hydrolysis-induced disassembly of individual RAD51 filaments from ssDNA. It has been shown before that RAD51 disassembly from dsDNA proceeds in bursts from filament ends, limited by ATP-hydrolysis of the terminal monomer [174]. To test whether the mechanism of disassembly from ssDNA is similar, fluorescent RAD51 (K$_{313}$ and Q$_{313}$) filaments were assembled on ssDNA in the presence of Mg$^{2+}$ and the disassembly was subsequently monitored in real time in the presence of Mg$^{2+}$, but absence of fluorescent RAD51 in solution (Figure 49.A). Measurement conditions were optimized to minimize photo bleaching, while retaining single-fluorophore sensitivity. To determine the disassembly kinetics, kymographs were analysed (Figure 49.B) and fluorescence intensity traces were obtained (Figure 49.C).

Similarly to disassembly from dsDNA, disassembly from ssDNA occurs in bursts involving multiple RAD51 monomers, separated by pauses (Figure 49.C). We found that pause durations are exponentially distributed,
Figure 48: Detection of hopping of K313-RAD51. [A] Example of kymograph of RAD51-K313 in the presence of ATP-Ca$^{2+}$ showing signature of both hopping (see patch “a”) and sliding (see patch “b”). [B] Single-particle tracking of the kymograph in panel A. Patch (a), changes position of approximately 1 µm within two consecutive frames (3.5s-4.0s). Patch (b) shows a purely diffusive behaviour. [C] Hopping of a RAD51 filament (patch b) allows bypassing a second filament bound to the ssDNA (patch a).

on average lasting 55±5 seconds (Figure 49.D). This value is substantially shorter than the value determined for RAD51-filament disassembly from dsDNA (150±10 seconds) [174], which is in accordance with the three-fold higher ATP-hydrolysis rate observed for RAD51 in the presence of ssDNA compared to dsDNA [166].

Our data further support the notion that RAD51 disassembles from filament ends. In addition, we observed that under conditions permitting ATP hydrolysis, Q313-RAD51 is also able to diffuse along the ssDNA (Figure 49.D). This occurs only in a small fraction of the filaments (∼6.5%) and always in the last phase of its interaction with ssDNA, just prior to release into solution.
Figure 49: RAD51-Q313 disassembly kinetic. [A] Fluorescence image showing an ssDNA construct immediately after incubation with RAD51-Q313 in solution, in the presence of ATP and Mg$^{2+}$, conditions that allowed ATP hydrolysis. [B] Kymograph showing ATP-hydrolysis-induced disassembly. Asterisk denotes a RAD51 nucleus that moves, prior to disassembly. We found that approximately 6.5% of RAD51-Q313 moves during disassembly. [C] Fluorescence intensity time traces of RAD51 filaments 1, 2 and 3 of kymograph B show that disassembly occurred in bursts separated by pauses. [D] Red columns represent the pause duration histogram (pauses below 20s cannot be detected experimentally). The blue dotted line is the exponential fit to the distribution indicating a mean pause duration of 55±5 seconds.

6.3.4 Nucleoprotein-filament elasticity during ATP hydrolysis

Next, we try to resolve the mechanical response of RAD51-filaments on ssDNA to tension in the presence of ATP hydrolysis and absence of free RAD51 in solution.
To this end, we densely coated ssDNA with fluorescent RAD51 (RAD51-K313) using a buffer (20 mM Tris pH 7.5, 10 mM Mg(OAc)$_2$, 2 mM CaCl$_2$, 2 mM ATP, 1 mM DTT) in which RAD51 is active for strand exchange and ATP hydrolysis and retains its interactions with recombinase mediators [27]. After incubation, the extent of RAD51 coverage of the ssDNA was checked using fluorescence visualization (see Figure 51.A). Subsequently, force-distance curves were recorded by consecutive stretching and relaxing the ssDNA molecule at a constant rate of 670 nm/s (see Figure 50).

The first remarkable observation is that both stretch and relax curves of RAD51-coated ssDNA are different from the force-curves of naked ssDNA. This indicates that the stretching (relaxation) of RAD51-ssDNA filament is more complex than the entropic extension (relaxation) of a polymer chain. A second, even more striking observation, is that (in contrast to naked ssDNA) stretching and relaxation curves do not overlay in the regime of 15 to 28 µm extension. In other words, the RAD51-coated ssDNA molecule shows hysteretic force-extension behaviour. Such hysteretic behaviour has been reported before for RecA [69]. However, two interpretations were put forward to interpret that observation: (i) the recombinase filaments bound to ssDNA are able to switch between a compact and extended conformation in response to the applied tension or (ii) extension of the ssDNA can stimulate RecA binding to the interstitial sites between filaments.

Hysteretic force-extension behaviour is due to a conformational transition

To confirm that the hysteresis is caused by a force-induced conformational transition, we recorded fluorescence images before and after the extension and relaxation of the ssDNA (see Figure 51.A and Figure 51.B). The images show no appreciable difference in fluorescence intensity, indicating that the difference between the two curves is not due to force-induced detachment of RAD51. Also, our microfluidic approach permits to exclude the presence of residual recombinase proteins in solution during the force-extension cycle. Therefore we can exclude both RAD51 binding and unbinding as a possible reason of the observed hysteresis.

We assume that at low force, all ADP-bound recombinase proteins are in their natural compact form. Consistently with previously published data, we assume also that ATP-bound RAD51 retains the extended conformation independently of the force applied [175]. A possible explanation of the observed lengthening is that during DNA stretching, the ADP-bound RAD51 is able to undergo a force-induced structural transition and switch to a new state, which resembles the extended conformation. In the force-
extension experiments, the conformational switching of the recombinase proteins occurs in non-equilibrium, resulting in the observed hysteresis. By decreasing the extension rate, one would expect the system to stay closer to equilibrium, resulting in a lower degree of hysteresis. Indeed, upon decreasing the extension rate from 670 nm/s to 100 nm/s (see Figure 50.B), the hysteresis in the force-extension curves of RAD51-coated ssDNA is significantly lower. By independently monitoring the fluorescence intensity, we found that it decreases as a function of time (see Figure 51.C), due to ATP-hydrolysis induced detachment of RAD51 from the ssDNA.

We quantified the disassembly kinetics and found a rate constant of $0.047 \pm 0.01 \text{ min}^{-1}$, negligible during a single stretch-relax cycle ($\sim 1 \text{ minute}$). This indicates that the hysteresis between subsequent stretching and relaxation curves is not due to ATP-hydrolysis induced detachment of RAD51 from ssDNA, but intrinsic to the RAD51-coated DNA, confirming the existence of a force-induced molecular transition of the ADP-bound RAD51 from a compressed to an extended structure.
Figure 51.: RAD51 filament disassembly kinetic. Characterization of RAD51 filament disassembly kinetic using single-molecule fluorescence microscopy. [A] Image an ssDNA molecule covered with fluorescent RAD51 before the stretching cycle. [B] Image of single ssDNA molecule covered with fluorescent RAD51 after the stretching cycle. [C] Fluorescence intensity as a function of time shows that disassembly of RAD51 can be characterized by an exponential decay using $0.047 \pm 0.01 \text{ min}^{-1}$ as RAD51 disassembly rate.

To test how ATP hydrolysis and RAD51 detachment affects these hysteretic force-extension curves of RAD51-coated ssDNA, we repeatedly recorded cycles of stretch and relax force-extension curves, over a time scale of an hour. We noticed that over the course of several minutes, both stretch and relax force-extension curves dramatically changed (see Figure 52). The contour length of the DNA-protein complex, indicated approximately by the end-to-end distance at which the sudden force increase occur during the stretching (indicated by the circles in Figure 52), shifts, over time, to lower extension. We hypothesized that this shift is caused by the hydrolysis of ATP to ADP. We therefore quantified the hysteresis, calculating the area between subsequent stretches and relax curves as a function of time (see Figure 52). Note that in a force-extension curve, the area corresponds to work. The hysteresis area as a function of time shows biphasic behaviour: first the hysteresis area increases with time, until it reaches a maximum and decreases to zero.

We modelled this behaviour using the following model: (i) ATP-RAD51 bound to ssDNA can occur in only one form, the extended conformation; (ii) ADP-RAD51 bound to ssDNA, can be either in this extended or in a compact form, depending on the tension applied; (iii) only ADP-bound RAD51 is able to detach from ssDNA. Our model consists of two coupled differential equations describing the time evolution of the system, involv-
Figure 52: Effect of ATP hydrolysis on force-distance curves of RAD51-ssDNA complexes.Inset of consecutive forward (blue) and backward stretching cycles (red). During the forward stretching we observe the presence of both an entropic and enthalpic behaviour up until the force of 9 pN. Above the force-threshold of 9 pN (blue dotted line) a saw-tooth behaviour is observed, suggesting the presence of a molecular transition. During the backward stretching cycle we observed that the reverse transition occur below the force of 6 pN (red dotted line). The cross-point between forward and backward stretching for consecutive cycles moves progressively towards shorter extensions.

ing the hydrolysis of ATP by ssDNA-bound RAD51 with rate \( k_{hyd} \) and subsequent detachment of RAD51 from the ssDNA with rate \( k_{off} \):

\[
\frac{dN_{ATP} (t)}{dt} = -N_{ATP} (t) k_{hyd} \tag{18}
\]

\[
\frac{dN_{ADP} (t)}{dt} = N_{ATP} (t) k_{hyd} - N_{ADP} (t) k_{off} \tag{19}
\]

These coupled equations can be solved and compared to the time-dependence of the area of hysteresis as:

\[
\Delta W (t) = Ye^{-tk_{off}} \left( \frac{\chi_{ATP} k_{hyd}}{k_{off} - k_{hyd}} \right) \left( -1 + e^{-t(k_{off}-k_{hyd})} \right) \tag{20}
\]
where \( \Upsilon \) represents the conversion factor between the number of ADP-bound monomers and the hysteresis area, \( \chi_{ADP/ATP} \) the fraction of ATP/ADP-bound RAD51 molecules in the beginning of the experiment. Our experimental results can be well fitted by this equation, resulting in an initial increase of the hysteresis due to the ATP-to-ADP conversion, a maximum value of the hysteresis and a subsequent exponential decay due to detachment of RAD51 from the ssDNA after ATP hydrolysis. Using a fixed value of \( k_{off} \) of 0.05 min\(^{-1}\) (see Figure 51.C) we obtain fitting values of \( \Gamma \) of \((60\pm1) \times 10^3 \) k\(_B\)T/molecule, and \( X_{ATP} \) of 0.68±0.03 and \( k_{hyd} \) of 0.12±0.01 min\(^{-1}\). The quality of the fits indicates that the hysteretic force-extension behaviour is caused by force-induced switching of ADP-RAD51 from a compact to an extended form upon increasing tension on the ssDNA and vice versa (see Figure 53).

**Figure 53.:** 
Hysteresis in successive F-D cycles computed from experimental data. [A] The computed difference in the work performed during consecutive forward and backward stretching cycles \( \Delta W = W_{forward} - W_{backward} \) is plotted as a function of time. The time dependence is due to the ATP hydrolysis and disassembly of RAD51. Under the assumption that only the ADP-bound RAD51 undergoes a non-equilibrium molecular transition we fitted the experimental curve with equation xyz. [B] Schematic representation of the force-induced conformational change. Upon application of force above 9 pN the natural compressed ADP form (white circles) undergoes a molecular transition to a new state (red circles) whose physical structure resembles the extended-ATP bound conformation (black circle).
6.4 Discussion

6.4.1 RAD51 filament dynamics

The interface between RAD51 monomers within the nucleoprotein filament, of which RAD51 residue 313 is part, is crucial for homologous recombination, since it regulates both the structure and the biochemical function of the filament. It has been shown that two naturally occurring variants of RAD51, differing only with respect to the nature of residue 313 have distinct filament-formation and strand-exchange efficiencies [81].

Here, we report another difference between these variants using a combination of single-DNA molecule manipulation and single-protein visualization experiments. Q313-RAD51 shows a size-dependent lifetime on the DNA, as previously described (see Chapter 5). Only under conditions permitting ATP hydrolysis, Q313-RAD51 diffuses along the ssDNA, in a small fraction of the filaments (6.5%) and always in the last phase of its interaction with ssDNA, just prior to release into solution. K313-RAD51 behaves quite differently: monomers and dimers interact with ssDNA through a combination of sliding and hopping, showing considerable motility also under conditions inhibiting ATP-hydrolysis (Ca^{2+}-ATP). Filaments composed of more than three K313-RAD51 monomers are significantly more stable and form ssDNA-protein complexes that do not move along the DNA. A recent linear dichroism study has shown that DNA bases in RAD51-ssDNA filaments are less ordered when ATP hydrolysis can take place [54], indicating that the ADP-bound RAD51 interacts less strongly with ssDNA. Based on these observations, we speculate that this weakly bound-state is also able to slide along the ssDNA.

Recombinase proteins such as RecA [172] and RAD51 (see Chapter 5) form filaments on ssDNA with high cooperativity and starting from multimeric units. Such an assembly mechanism results in incomplete coverage of the ssDNA substrate, as demonstrated using Monte-Carlo modeling and magnetic tweeze measurements [172]. A possible strategy allowing the formation of continuous filaments over μm-long segments of ssDNA would involve the redistribution of pre-bound proteins [90, 172]. Proposed possible mechanisms included that individual monomers could translocate between filament ends either via unidirectional motion, diffusion or hop-
ping [172]. Here we have shown that small nucleoprotein structures can translocate along ssDNA over significant distances (>100 nts).

Next, we investigated the structural properties of RAD51-ssDNA filaments under ATP-hydrolysis conditions. Electron micrographs studies have proposed that the RAD51-ssDNA filaments are characterized by a high degree of conformational freedom. The variety of structural forms reported for human RAD51 are likely due to a rotational movement of the N-terminal domain which can locally modify the contact between the filament and the ssDNA [190]. Multiple conformations therefore are possible, reflecting the intrinsic flexibility of the RAD51 nucleoprotein filament.

To what extent do the ssDNA conformation and the thermal fluctuations influence the geometry of the human RAD51 nucleoprotein filament? Using force spectroscopy measurements we observed that the ssDNA conformation and the structure of the nucleoprotein filament are intimately related. Applying forces above 9 pN induces a structural transition in the RAD51-ADP-bound filament between a compressed to an extended conformation, resulting in an elongation of the filament. We used two different extension velocities (670 and 100 nm/s) and found that in both cases force-extension curves recorded in the stretching and relaxation directions are not equal: the system shows hysteresis. This indicates that the system is not in equilibrium during the force-extension experiments. Thus, the work performed on the system does not provide a good estimate of the free energy difference between the two RAD51 conformations. The equilibrium free energy difference between these conformation can, nevertheless, be recovered using the Jarzynski’s equality [82]: \( e^{-\frac{W}{k_B T}} = e^{-\Delta G/k_B T} \). Since the number of ADP-bound molecules changes during our measurement due to ATP hydrolysis and RAD51 dissociation, we used a moving average obtained from 5 consecutive force-distance curves and compare the computed free energy with the expected coverage of the ssDNA molecule. We found, in the limit of initial coverage higher that 60% of the ssDNA molecule, a weak dependence of the expected value of \( \Delta G \), which is estimated in the range of 4.7 \( k_B T \) (for an estimated initial coverage of 70%) to 3.3 \( k_B T \) (for the completely saturated case). i.e. the energy required to extend ADP-bound RAD51 on ssDNA is approximately 4 \( k_B T \) of energy.
6.4.2 Implications for strand exchange

What is the structure of the RAD51 recombinase filament in vivo? RAD51 binds on ssDNA in the presence of ATP. While dissociation of ADP-bound RAD51 monomers can occur only from filament ends [84, 174], ATP hydrolysis can occur anywhere within the RAD51 filament. Therefore, when ATP-hydrolysis is permitted (when Mg$^{2+}$ is present), the structure of the recombinase filament is likely heterogeneous, alternating segments in the ATP-bound and ADP-bound states, as confirmed by the force distance curves reported in Figure 49. In the canonical view of homologous recombination, only the ATP-bound extended conformation is active for strand exchange. Recent structural data on the bacterial homolog of RAD51 (RecA) have shown that ATP hydrolysis induces a dramatic conformational change which affects the interaction network between the recombinase protein and the ssDNA [33]. This conformational change is responsible for the inactivation of the filament for strand exchange. The question how the strand exchange reaction proceeds in the presence of a mixture of ADP and ATP nucleotides remain, therefore, unchallenged. A possibility is that such hybrid structures never occur in vivo, either because ATP molecules from solution can replace the ADP bound at the monomer-monomer interface [136], or because the conformational transition is blocked by the action of recombinase mediators, such as the tumour suppressor protein BRCA2 [49].

Filament plasticity is conserved through evolution and we argue that constitutes a key ingredient for understanding strand exchange. Our work demonstrated that a work input of approximately 3 to 4 k$_B$T is sufficient to induce a conformational transition from the compressed to the extended state of RAD51. This amount is considerably lower than the energy required for the chemical synthesis of an ATP molecule (∼20 k$_B$T).

In the presence of thermal fluctuations, therefore, we expect that a small fraction (approximately 3% when ΔG=3.5 k$_B$T) of RAD51 proteins are in the active state for initiating the strand exchange reaction, irrespective if ATP or ADP is bound at the monomer-monomer interface. Also, if strand-exchange is initiated, the energetic penalty required to convert a RAD51 protein from the inactive to the active state may be supplied by the base-pairing energy and the interaction between the secondary binding site of the filament and the displaced ssDNA strand [103, 104]. This ADP-specific
energetic penalty therefore leads to more stringent homology requirements for stable pairing compared to the ATP-bound RAD51 case. Our results indicate a possible strategy for RAD51 to execute strand exchange even in the presence of locally ADP-bound RAD51 and explain the role of RAD51 filament’s plasticity in determining the selectivity of the homology recognition process. Future single-molecule experiments will aim at elucidating how the applied tension modulates not only the physical properties but also the biochemical function of individual RAD51 filaments.