Non-Homologous End Joining (NHEJ) is an efficient mechanism to repair DNA double-strand breaks. XRCC4 and XLF are two structurally-similar core NHEJ proteins. They can directly interact at the protein-protein level and engage DNA by an unknown mechanism.

Here, we use optical tweezers and fluorescence microscopy to visualize XRCC4-XLF complexes on DNA in real time. We find that the behavior of XRCC4-XLF on DNA is multifaceted: complexes rearrange continuously and show periods of rapid diffusion along the DNA, interspersed by stationary binding. By manipulating two DNA molecules independently we study bridge formation and demonstrate that XRCC4-XLF complexes can directly bridge two independent DNA molecules.

Our approach reveals the transient and dynamic aspects of the interaction of XRCC4-XLF with DNA providing new insights on how they could function during DNA double-strand break repair by NHEJ.
7.1 INTRODUCTION

The maintenance of chromosome integrity depends critically upon the repair of double-stranded DNA breaks (DSB). Failure to efficiently execute DNA repair may result in chromosomal aberrations such as genomic translocations, a hallmark in human cancers [73, 65]. Two major DSB repair processes exist in humans: homologous recombination (HR) and non-homologous end-joining (NHEJ). While HR requires an homologous template to copy and restore the genetic information at the damaged site [142], NHEJ operates by direct religation of the two broken DNA ends [95]. Cells presenting malfunctions in genes associated with the NHEJ repair pathway display hypersensitivity to ionizing radiations [3] and severe immunodeficiency-syndromes [73].

The principal components of the NHEJ DNA repair consist of Ku70/80 [95], DNA-PKcs [152], XRCC4 [85], XLF [4, 94] and DNA Ligase IV (LigIV) [153]. Although how these proteins assemble and operate during NHEJ is still under research, two major steps have been identified: (i) Ku70/80 first recognizes the ends of the broken DNA molecules and recruits DNA-PKcs [95] and (ii) a complex of XRCC4-Lig IV ligates the broken ends together [95], stimulated by XLF [3]. XRCC4 and XLF display similar structural properties [4, 85], since both form homo-dimers characterized by N-terminal heads and alpha-helical tails. Recent X-ray crystallographic studies have indicated that XRCC4 and XLF are binding partners, forming long, helical filaments with a pitch of 72 to 84 nm and a large internal groove with a diameter of approximately 10 nm [64, 137, 182, 5]. In addition, the alpha-helical tails of XRCC4 show two intriguing features: they are involved in the formation of XRCC4 tetramers through a tail-to-tail interaction [85], and they serve as a binding platform for the C-terminal domain of LigIV (BRCT) [153].

Resolving how XRCC4 and XLF interact with DNA and what functions they execute during NHEJ are challenging research problems. Two contradicting DNA binding strategies have been proposed: in one, the XRCC4-XLF helical filament binds around the DNA [5], while in the other model is the DNA to wrap around the XRCC4-XLF protein complex [64]. Also, the exact role of XRCC4 and XLF in NHEJ is currently under debate. In the canonical view of NHEJ, XRCC4 and XLF intervene late during the repair process, stimulating the ligation step catalysed by LigIV [182]. In a recent
biochemical study it has been shown that XRCC4 and XLF are also present at the early stage of NHEJ, displaying a LigIV-independent function [141]. Also, recent bulk and AFM methods have shown hints that XRCC4 and XLF possess the capability of forming bridges between DNA molecules [5]. The possibility that XRCC4-XLF complexes are capable of holding together two DNA molecules is particularly intriguing, since it can provide a molecular explanation to the reported stimulatory role in the DNA ligation step [5] as well as their early function during NHEJ [141].

In order to provide a better understanding of the biochemical and biophysical properties of the XRCC4-XLF complex, we have generated a fluorescently labelled variant of XRCC4 with biochemical activity unaltered from wild-type. In experiments combining DNA manipulation via optical tweezers and protein visualization via single-molecule fluorescence microscopy we studied the DNA-binding properties of XRCC4 in the absence and presence of its binding partner XLF in real-time. In addition, we tested the DNA-bridging properties of XRCC4-XLF complexes in a novel assay involving the manipulation of two DNA molecules. Our results show that the DNA binding properties of XRCC4-XLF are complex, involving switching between fast diffusion along the DNA and static binding. Finally we demonstrate the ability of XRCC4-XLF to form bridges between DNA molecules.

7.2 MATERIALS & METHODS

7.2.1 Protein expression, labeling and biochemical activity

Fluorescently labelled XRCC4 was prepared by the “Cys light” method [111]. Plasmid pBMM42 [112], a pET28a derivative expressing recombinant human XRCC4 tagged with polyhistidine at the C-terminus was modified by site directed mutagenesis to remove all Cys codons but one. Constructs XRCC4-41 (C93A C128A C130A C165A) and XRCC4-43 (C128A C130A C165A C218A) were generated and verified by DNA sequencing. Recombinant XRCC4 proteins were purified after expression in bacteria as described [112]. AlexaFluor 555-maleimide (Invitrogen) was used for specific labelling at position C218 (XRCC4-41) and C93 (XRCC4-43), respectively. Proteins were first buffer exchanged into 50 mM Tris pH 7.2,
300 mM KCl, 1 mM EDTA and 10% glycerol, adjusted to 2 mg/ml and labelled with AlexaFluor 555-maleimide (Invitrogen) at 5:1 dye-to-protein molar ratio for 2 hours at 4°C. Reactions were quenched by addition of fresh DTT (10 mM) and excess dye was removed by size exclusion chromatography (Econo-Pac 10DG columns, BIO-RAD) followed by Sepharose Q chromatography. Preparations were finally dialyzed against 20 mM HEPES pH 8, 150 mM KCl, 1 mM EDTA, 1 mM DTT and 10% glycerol and stored at -80°C. Functionality of the fluorescently labelled XRCC4 preparations were tested for DNA and LigIV tandem BRCT domains interaction by electromobility shift assays as described [5] and behaved as the unlabelled wild type protein. Recombinant wild-type human XLF was produced as described [4]. The XLF expression construct was modified by inserting an eGFP-His6 cassette at the C-term. The fusion protein was purified using the same protocol as for the wild-type protein. Wild-type XLF and XLF-eGFP were stored at -80°C in 20 mM HEPES pH 8, 150 mM KCl, 1 mM EDTA, 1 mM DTT and 10% glycerol. Functionality of the fluorescently labelled XLF preparations were tested for DNA and XRCC4 binding, for DNA bridging and for LigIV/XRCC4 stimulation as described [5] In these assays XLF-eGFP behaved like the wild-type unlabelled control except that it was deficient in the DNA bridging assay.

7.2.2 Single-molecule approach

The biophysical properties of XRCC4 and XRCC4-XLF complexes on DNA were studied at the single-molecule level using a combination of DNA manipulation and fluorescence visualization. Our technique permits the manipulation of a single Λ-DNA molecule between two optically trapped microspheres (4.5 µm in diameter) and the control over the applied tension with 0.1 pN accuracy [25, 59]. The single-molecule construct was assembled in situ using a microfluidic system and a computer-controlled movable microscope stage. Fluorescent XRCC4 (XRCC4-Alexa555) and wild-type XLF were introduced in the flow-cell in a separate channel using laminar-flow, in order to minimize mixing with the other components. The DNA molecule was allowed to interact with a high concentration (in the range of 50 to 400 nM) of the fluorescent proteins for a defined amount of time (between 30 seconds to 5 minutes). Following this incubation step, the DNA-protein complex was repositioned in the imaging channel, in the ab-
sence of fluorescent protein in solution. This step permits single-molecule fluorescence resolution. To study the DNA bridging properties of XRCC4-XLF complexes, a novel instrument was developed which combines dual DNA manipulation \cite{42} and fluorescence visualization. Using this instrument it was possible to manipulate independently two distinct DNA molecules and produce a crossed configuration where the two molecules are held in contact. DNAs kept in this configuration were incubated with XRCC4-Alexa555 and XLF. The specificity of the binding and the formation of inter-molecular bridges were tested using both direct imaging and the force response of the DNA-protein complex.

\subsection*{7.2.3 Buffer conditions}

Protein-DNA interaction and force-stretching experiments were conducted in 20 mM Tris-HCl pH 7.5 and variable concentrations of monovalent ions, in the range of 25 to 150 mM KCl and 1 mM DTT. Beads catching and DNA tethering were performed instead in phosphate buffered saline (PBS-Sigma).

\subsection*{7.2.4 Fluorescence tracking and diffusion analysis}

The fluorescent movies were analysed in the following way: first, the intensity in the direction longitudinal to the DNA was averaged over 5 pixels to produce a kymograph. The kymograph was further analysed using a custom written Labview routine. A one-dimensional Gaussian fitting was performed and the x-position of the fluorescent protein along the DNA was recorded. For a time trace of N point, the mean-squared-displacement at time lag n was calculated according to:

\[ MSD(n,N) = \frac{\sum_{i=1}^{N-n} (x_{i+n} - x_i)^2}{N-n} = 2Dn\Delta t + \sigma^2_{loc} \]  

(21)

The statistical variance of MSD(n,N), as demonstrated by \cite{129, 179, 88} was computed using the following expression:

\[ \sigma^2_{n,N} = \frac{(2Dn\Delta t)^2 (2n^2 + 1)^2}{[3n (N - n + 1)]} \]  

(22)
Where $\Delta t$ is the exposure time of the camera (typically from 50 to 100 ms). From each diffusion trajectory (with $N_{\text{frames}} > 50$) the diffusion constant $D$ was obtained using a weighted linear fit of $\text{MSD}(n,N)$ in the interval from $n=1$ to $n=5$.

7.3 RESULTS

7.3.1 XLF stimulates the binding of XRCC4 to DNA

We first addressed the DNA binding properties of XRCC4 and XLF using a combination of optical tweezers and fluorescence microscopy. In order to study the interaction of XRCC4 and XLF with DNA, it is important to understand how their DNA binding affinity is affected by their relative stoichiometry.

![Figure 54: XLF stimulates binding of XRCC4 on DNA.](image)

[A] Single DNA molecule observed after an incubation step of three minutes in a buffer containing: (i) 200 nM of XRCC4-Alexa555; (ii) a premixed solution with XRCC4-Alexa555 and XLF (200 nM:200 nM); (iii) 200 nM XRCC4-Alexa555 and 2 $\mu$M XLF. All measurements are performed in a buffer solution containing 20 mM Tris-HCl pH 7.5, 25 mM KCl and 1 mM DTT.

[B] DNA coverage (measured as the fraction of stained and unstained DNA) as a function of XLF concentration (XRCC4-Alexa555 concentration was 200 nM).

To this end, we investigated how increasing concentrations of XLF modulate the formation of XRCC4-XLF complexes on DNA. For this experiment we used a single cysteine mutant of XRCC4 labelled with the fluorophore Alexa555 (XRCC4-Alexa555) and wild-type unlabelled XLF. The
DNA molecule was held at a defined tension (in the range from 1 to 30 pN) using dual optical tweezers and subsequently incubated in the channel containing the mixture of XRCC4 and XLF (for an incubation time of 3 minutes) and visualized using fluorescence in the imaging channel devoid of fluorescent proteins. After incubation with XRCC4-Alexa555 alone, many fluorescent spots of differing intensity were bound on the DNA molecule (Figure 54.A). Incubation with XRCC4 at the same concentration in the presence of an equal concentration of XLF resulted in enhanced protein binding and DNA coverage. Adding XLF in large excess (10:1, XLF:XRCC4) resulted in a complete and homogeneous coverage of the DNA, almost instantaneous on the time-scale of our experiments. Our results thus indicate that XLF stimulates the binding of XRCC4 to DNA in a concentration-dependent manner (Figure 54.B). The head-to-head interaction between XRCC4 and XLF involves both hydrophobic contacts and hydrogen bonds and is crucial for filament formation, as highlighted by mutational analysis [64]. Therefore we tested the effect of increasing salt concentration on the DNA binding properties of XRCC4 and XLF. Increasing the KCl concentration from 25 mM to 150 mM resulted in a drastic inhibition of filament formation on DNA (Figure 55). Paradoxically, at salt concentrations close to the physiological range (approximately 150 mM) the formation of filament on DNA is practically entirely suppressed. This indicates the fundamental role of electrostatic interactions in the formation of a XRCC4-XLF-DNA complex.

XRCC4 is present in solution as a homo-dimer but can from higher-order structures [112, 113]. The sparse and bright fluorescent spots observed after incubation with XRCC4-Alexa555 alone indicates that binding to DNA of XRCC4 oligomers [85] is slow and inefficient. When XLF is added to the XRCC4-Alexa555 reservoir, protein complexes containing both XRCC4 and XLF are formed in solution [182]. Because large excess of XLF strongly stimulates XRCC4-Alexa555 binding to DNA (Figure 54.C), we presume that the interaction between the DNA with a solution containing XRCC4 and XLF in equal amount will result predominantly in the formation of XRCC4-XLF complexes and not in XRCC4-alone binding to the DNA. In most of the experiments that are going to be discussed below, we used a 1:1 ratio of XRCC4-Alexa555 and XLF. We therefore refer to the fluorescent patches obtained following this protocol as XRCC4-XLF complexes. Furthermore, we verify that using equal amount of XRCC4 and XLF results
in XRCC4-XLF complex formation on DNA using multi-color fluorescence imaging (Supplementary Figure S71).

Figure 55.: The binding affinity of XRCC4 and XLF complexes is influenced by the salt concentration. [A] Fluorescent images obtained after three minutes of incubation in 200 nM XRCC4-Alexa555, 200 nM XLF and 20 mM Tris (pH=7.5) in (I) 150 mM KCl, (II) 75 mM KCl, (III) 50 mM KCl, (IV) 25 mM KCl. [B] The average protein coverages on the DNA (%) as a function of salt concentration, we found 41±6% (N=16), 22±9% (N=7), 19±6 (N=10) and 0% (N=5) for 25, 50, 75 and 150 mM, respectively. DNA-protein complex formation clearly decreases at higher ionic strengths.

7.3.2 Mechanical properties of DNA in complex with XRCC4-XLF

It is not known how XRCC4-XLF complexes interact with DNA. In general, it is well known that protein binding can affect the mechanical properties of the DNA, depending on the nature of the DNA-protein interactions [32]. For XRCC4-XLF, two contradicting views on the interaction of these proteins with DNA have been proposed: in one the DNA and proteins interact in a histone-like fashion [64], while in the second the proteins form helical filaments around the DNA [5, 182]. If the first view were correct, one would expect a substantial shortening of the DNA contour length. In addition, depending on the DNA-binding strategy employed by the XRCC4 and XLF, the assembly kinetic can be influenced by the stretching force applied on the DNA.

In order to gain insights in the DNA binding mechanism of XRCC4-XLF binding to DNA we recorded force-extension curves. To this end,
DNA subjected to tensions ranging from 1 to 30 pN was incubated with XRCC4 and XLF at high concentrations (20 mM Tris pH 7.5, 25 mM KCl, 200 nM XRCC4-Alexa555 and 2 µM XLF). Fluorescence images confirmed that DNA molecules were fully covered with XRCC4-XLF, independent of tension (see Figure 56). Next, force-distance curves were recorded and compared to curves obtained before incubation with XRCC4 and XLF. An example, in Figure 57, shows that the force-extension curve of DNA is not altered by the presence of XRCC4-XLF (as confirmed by the fluorescence image, inset), indicating that the binding of XRCC4-XLF does not influence the elastic properties of DNA in a detectable way.

We also studied whether the nature of the DNA, single-stranded versus double-stranded, affects binding of XRCC4-XLF. To this end, DNA was overstretched, beyond its crystallographic contour length to a length of 26 µm. In this way, at low salt concentrations (25 mM KCl), a spatially segregated ssDNA-dsDNA hybrid structure was created due to force-induced melting [60]. Next, this hybrid was incubated with XRCC4-XLF while the tension was fixed at 65 pN and fluorescence images were recorded. Figure 58.A shows that part of the hybrid-DNA molecule was not fluorescent, while the rest was fluorescent, in the same way as a completely double-stranded DNA (Figure 58.A). This result indicates that XRCC4-XLF binds specifically to dsDNA and not to ssDNA under tension.
XRCC4-XLF does not alter the mechanical properties of dsDNA. Force-distance curve of a DNA molecule before (black curve) and after (red) incubation in 200 nM of XRCC4-Alexa555 and 2 µM of XLF. In the inset, a fluorescent snapshot of the DNA-protein complex after the incubation step confirming the full fluorescent coverage.

7.3.3 Dynamic properties of XRCC4-XLF filaments on DNA

A more detailed inspection of sequences of fluorescence images of XRCC4-Alexa555 bound to dsDNA indicated that some of the XRCC4 complexes move along the DNA while remaining bound. Other complexes appear instead to be static. XRCC4, therefore, displays two distinct binding modes on the DNA: a dynamic and static one (Figure 59.A). Before further analysis of the motility parameters, we tested whether XRCC4-XLF complexes show the same behaviour. Using large excess of XLF (1:25), image sequences show that also XRCC4-XLF complexes display both DNA-binding modes (see Figure 59.B). This suggests that the dual DNA binding mode is a feature of both XRCC4 and XRCC4-XLF complexes.

In addition, we also observed individual XRCC4-XLF complexes switching from static to dynamic behaviour and vice versa (see Figure 60.A, B and C). In some cases, we observed that the motion of dynamic complexes appeared to be confined (yellow line Figure 60.D). The molecular causes of both switching and confinement are unknown. We speculate that both effects are triggered by recognition of altered DNA structures, for example nick or gaps [112] or of specific sequences on the DNA. Further
Figure 58: XRCC4-XLF binding is specific for dsDNA. [A] A dsDNA molecule stretched at 50 pN shows full coverage after incubation with 200 nM of Alexa555-XRCC4 and 2 µM of XLF (incubation time=1min). [B] Fluorescent image showing a spatially separated fluorescent coating. The DNA molecule was first overstretched (L\text{contour}=26 µm and F=65 pN) and after incubated with 200 nM of XRCC4-Alexa555 and 2 µM of XLF (Incubation time=1min). The proteins bind specifically to the regions corresponding to the intact segments of dsDNA.

Experiments involving sequence-specific nicks and, better defined DNA-orientation might shed further light on these intriguing observations.

7.3.4 Diffusion of XRCC4-XLF on DNA

Next, we quantified the motility parameters of the dynamic XRCC4 and XRCC4-XLF complexes, by tracking the fluorescent spots with an accuracy of less than 10 nm using two-dimensional Gaussian fitting [25] (Figure 61.A). From trajectories obtained in this way, we determined the mean squared displacements (MSD) as a function of lag time (see Figure 61.B). We found that MSD increases with time in a linear way, a hallmark of free diffusion (Figure 61.C). By fitting a line to the MSD as a function of lag time we determined the one-dimensional diffusion coefficient (Figure 61.C). For XRCC4-Alexa555 (20 mM Tris; 25 mM KCl) we found a diffusion coefficient of $1.07\pm0.02 \mu m^2/s$ (mean$\pm$SE, N=10). For XRCC4-XLF complexes (DNA molecules incubated in a 1:1 solution), a similar value was obtained ($0.97\pm0.02 \mu m^2/s$, mean$\pm$SE, N=40). Increasing the KCl concentration to 75 mM resulted in a slightly slower diffusion of the XRCC4-XLF complex ($0.59\pm0.02 \mu m^2/s$, mean$\pm$SE, N=30).
Figure 59. XRCC4 and XRCC4-XLF show both diffusive and static behaviors. [A] Kymograph showing a DNA segments after incubation with XRCC4 (100 nM). We observe both static and diffusive fluorescent complexes. [B] Kymograph showing a DNA segments after incubation with XRCC4-XLF ([XRCC4-Alexa555]=100 nM and [XLF]=2.5 µM). Also in this case, the two binding modes are retained.

In the cell, XRCC4-XLF complexes bind and move along DNA with a free (broken) end, unlike the experiments described above, where DNA ends are both engaged with the microspheres required for optical trapping. To test whether end-specific effects occur, we attached the DNA with only one of its ends to an optically trapped microsphere and extended it in a constant buffer flow, leaving the other end free. In these experiments, the molecule was incubated in 200 nM XRCC4-Alexa555 and 200 nM XLF in presence of 20 mM Tris pH 7.5 and 50 mM KCl. We observed that in most of these flow-stretching experiments, XRCC4-XLF was found bound only at the free DNA end (Figure 62.A). We wondered whether this observation is the result of specific end-binding or an artifact due to the hydrodynamic drag pushing diffusing XRCC4-XLF complexes towards the DNA end. To answer this, we returned to the dual beads configuration and investigated the effect of buffer flow on diffusing XRCC4-XLF complexes. We found that complexes are moved in the direction of the buffer flow, as shown in Figure 62.B. We therefore conclude that buffer flow can bias the diffusion of DNA-bound XRCC4-XLF complexes, resulting in the DNA-end-bound complexes observed in the flow-stretching experiments.
Figure 60. XRCC4-XLF complexes switch dynamically from diffusive to static states. [A] Kymograph shows a fluorescent XRCC4-XLF complex undergoing a transition from static to a diffusive state (yellow arrow). [B] Kymograph shows the diffusive to static switching (red arrow). [C] Kymograph shows the diffusive to static switching (yellow arrow) and a consecutive static to diffusive switching (red arrow). [D] Kymograph shows a fluorescent XRCC4-XLF complexes not able to bypass the region on the DNA marked with the dotted yellow line (confined diffusion). Experiments were performed using 200 nM XRCC4-Alexa555 and 200 nM XLF in 20 mM Tris-HCl pH 7.5 and 25 mM KCl.

7.3.5 Formation of XRCC4-XLF filaments

We also investigated the mechanism of formation of XRCC4-XLF complexes on DNA. Two distinct strategies have been described for protein complexes forming on DNA. Recombinase proteins such as RAD51 and RecA bind to DNA from solution in a highly cooperative fashion, involving a rate-limiting nucleation step followed by polymerization [72, 55]. For
Proteins characterized by a low cooperativity in DNA binding, such as mitochondrial transcription factor A, an alternative mechanism has been proposed, with individual proteins or protein-complexes randomly moving along the DNA colliding and coalescing into larger structures [51].
Figure 62: Single particle tracking of XRCC4-XLF complexes diffusing along DNA. [A] Kymograph shows the diffusion of a single XRCC4-XLF complex along a single DNA molecule after incubation with 150 nM XRCC4-Alexa555 and 150 nM XLF. [B] Single-particle tracking allows determining the time-trajectory of the fluorescent spot. [C] Mean squared displacement (MSD) analysis of the diffusive particle. The diffusion coefficient is extracted from the linear fit (dashed black line) to the MSD plot, in this case $D = 0.66 \pm 0.01 \mu m^2/s$.

test whether XRCC4-XLF follows one of these strategies, we monitored complex formation in real-time (see Figure 62). We observed that XRCC4-XLF complexes appear randomly and abruptly along the DNA and remain bound for a long time. The fluorescence intensity of the fluorescent complexes remains constant for the duration of the measurement indicating that growth of the complexes is a rare event. In several cases, we observed the collision of distinct, diffusing particles. Upon collision, particles either merged, forming larger complexes (see Figure 63.A), or "bounced" (see Figure 63.B), resulting in rapid dissociation of collision complexes. We also observed complexes bound to the DNA to break into fragments (Figure 63.C), further highlighting that XRCC4-XLF complexes on DNA are highly dynamic, undergoing continuous rearrangement.

7.3.6 DNA bridging at the single-molecule level

Finally, we tested whether XRCC4-XLF complexes possess the ability of bridging two DNA molecules. In a first attempt to study bridging, we trapped a single DNA molecule between two microspheres. While incubating for three to five minutes in 200 nM XRCC4-Alexa555 and 200 nM XLF (in 20 mM Tris (pH 7.5), 25 mM KCl), the DNA was kept in an extended configuration (tension in the range of 1-50 pN).

Subsequently the DNA was relaxed to an end-to-end distance of 7 \mu m for two minutes allowing distinct XRCC4-XLF-coated segments to come...
Figure 63: XRCC4-XLF assembly on DNA is visualized in real-time. The appearance of fluorescent patches on DNA occurs randomly at different positions. Once bound, the fluorescence intensity stays constant, indicating that filament growth is not taking place during the timescale of the experiment.

Figure 64: Assembly and fragmentation of XRCC4-XLF complexes. [A] Kymograph showing multiple XRCC4-XLF complexes colliding (orange arrows) and forming a larger protein structure on the DNA. [B] Collision between XRCC4-XLF filaments resulting in transient complex formation (orange arrows). [C] Individual XRCC4-XLF complex fragmenting (orange arrow) into smaller filaments.
Figure 65: XRCC4-XLF catalyzes the formation of intra-molecular bridges. [A] The force distance curve of naked DNA molecule is shown in black. The force-extension after incubation with XRCC4-XLF is shown in red. [B] Kymograph recorded during bead separation shows the rupture of an intra-molecular bridge mediated by XRCC4-XLF.

into contact due to DNA thermal motion. Afterwards, force-extension curves were measured (see Figure 65.A). These force-extension curves showed complex behavior: extension involved many force ramps and sudden force drops, most likely representing the rupture of intra-molecular bridges formed by XRCC4-XLF. At forces above 50 pN the XRCC4-XLF-coated DNA is extended comparably to naked DNA. Notably, force-extension curves recorded upon relaxing the XRCC4-XLF-coated DNA are indistinguishable from naked DNA.

A kymograph obtained from fluorescence images recorded during extension of a XRCC4-XLF coated DNA molecule, Figure 65.B, clearly shows the rupture (arrow) of a DNA bridge formed between two XRCC4-XLF complexes bound on different parts of the DNA. This observation confirms that XRCC4-XLF bound to DNA has DNA-bridging activity. Note that the use of fluorescence visualization is essential to discriminate between intra-molecular bridges formed by XRCC4 and XLF and DNA-protein adsorption to the optically trapped microspheres (Supplementary Figure S72).

In order to obtain further insight in the DNA-bridging activity of XRCC4-XLF and to avoid artefacts due to DNA binding to the optically trapped microspheres, we developed a novel assay for studying the DNA-end bridging catalysed by XRCC4 and XLF. The experiment was performed us-
Figure 66.: Intra-molecular bridging catalyzed by XRCC4-XLF complex and creation of three-way junction. [A] Fluorescent visualization of two crossed DNA molecules with three distinct XRCC4-XLF complexes bound. [B] The top-right bead is moved towards the right, stretching the black DNA molecule into the overstretching transition. [C] After rupture of the black DNA strand due to force-induced unpeeling across a nick, a three way junction is formed. We noticed an increased fluorescence intensity at the DNA crossing position. This indicates that the segments of the black DNA molecule recoiled at the junction, probably extruding a DNA loop.

In this preliminary experiment, we trapped four individual microspheres in each of the four controllable optical traps. After a dual DNA tether system was produced, the DNA molecules were subsequently crossed. Next, the DNA molecules in the crossed configuration were incubated in 300 nM XRCC4-Alexa555 and 800 nM XLF (20 mM Tris, 25 mM KCl) for 30 seconds. Subsequently the DNA molecules were transferred to the observation channel for further inspection and manipulation. We observed a static XRCC4-XLF complex located at the location where the two DNA molecules
were expected to cross (Figure 66.A). To verify that this indeed represented an actual inter-DNA bridge, we stretched one of the two DNAs by moving the top-right bead to the right (Figure 66.B).

Upon applying tensions exceeding the overstretching force (F>65 pN), DNA starts unpeeling. In cases where the force-induced unpeeling runs across a nick, the DNA molecule breaks, as is observed in Figure 66.C. Upon DNA rupture, part of the broken strand recoiled to the initial bridge point, resulting in an increase of the fluorescence intensity. This observation suggests that part of the broken DNA strand formed a loop as depicted in Figure 66.C. In this way, a three-way junction was formed as can be confirmed by DNA manipulation and, more directly, by fluorescence visualization, since one of the fluorescent spots diffuses between the bottom-left microsphere and the crossing point. The three-way junction held with three optical traps was further manipulated by moving the top-left microsphere further to the left, resulting in tensions above 65 pN on
Moving optically trapped microspheres confirms the stability of the bridged DNA molecules. [A] Single DNA tether consisting of two DNA molecules bridged by XRCC<sub>4</sub>-XLF (same as Figure 66.C). [B] Moving the top-left microsphere (red) confirms the existence of a stable bridge between both DNA molecules. [C] Release of the untethered microspheres and positioning of the DNA molecule in a horizontal configuration.

Upon rupture, one of the DNAs (red in Figure 67.A). The high tension applied, again, resulted in the rupture of one of the DNA molecules. Upon rupture, one of the fluorescent spots moves to the junction point (yellow arrow in Figure 66.B and Figure 66.C). We hypothesized that the resulting DNA tether between two beads consisted of two DNA molecules bridged by XRCC<sub>4</sub>-XLF. To test this, we kept on moving the top-left bead until the tether was horizontal (see Figure 68). Finally, we stretched the bridged DNA molecules taut, until the intra-molecular bridge formed by XRCC<sub>4</sub>-XLF is broken and both DNA molecules recoiled on the trapped microspheres (see Figure 69). Obviously, these experiments provide the first hints of the power of our experimental strategy and the first direct proof of the bridging capability of XRCC<sub>4</sub> and XLF.
In summary, we have studied the mechanisms of DNA binding and bridging by XRCC4 and XRCC4-XLF complexes. We have shown that both XRCC4 and XRCC4-XLF complexes are able to bind to DNA under tension, without affecting its mechanical properties. A DNA-binding mechanism by XRCC4 and XRCC4-XLF complexes resembling histones is therefore highly unlikely, since this would result in shortening of the DNA [64]. Our observations are consistent instead with XRCC4-XLF forming a filament around the DNA.

This hypothesis agrees very well with x-ray structural data, reporting an internal diameter of the XRCC4-XLF helix of approximately 10 nm, enough to accommodate dsDNA [5, 64, 137, 182]. The inhibitory effect on the DNA binding kinetic exerted by physiological salt concentrations suggest that in vivo, accessory factors might be required for the efficient formation of a DNA-protein complex. Cellular studies demonstrated that recruiting of XRCC4 at the site of damage depends on the presence of Ku70/80 [102]. Future experiment will clarify the role of Ku70/80 at the single-molecule level, in order to obtain a full picture of the regulation of XRCC4-XLF filament formation. Once bound, XRCC4-XLF filaments interact with the DNA for long periods, and can be observed for up to 20 minutes. This sug-
gests that the DNA binding is a near-irreversible process, and that XRCC4-XLF filaments are highly stable on the DNA. This also might imply that the disassembly of the XRCC4-XLF filaments could be a regulatory-point during NHEJ in vivo. A possible actor in the disassembly of the filaments is DNA-PKcs, which has been shown to phosphorylate XRCC4 and ablate its bridging function [141].

Nevertheless, these long binding times allowed us to unravel the dynamics of XRCC4-XLF bound to DNA. While a fraction of the XRCC4-XLF complexes remained statically bound to the same spot on the DNA, many complexes moved along the DNA, showing random diffusive behaviour. We have observed transitions between both XRCC4-XLF binding modes. Interestingly, bulk biochemical approaches have shown before that two binding modes exist and that only one of them is able to bridge DNA [5], although further characterization of the two modes was not possible.

Quantitative analysis of the diffusion of DNA-bound XRCC4-XLF revealed an unusually high diffusion coefficient, in the order of 1 \( \mu m^2/s \), comparable to hOgg1, a globular protein with a hydrodynamic radius of only 3.2 nm [19], which spins around the DNA double helix [18]. XRCC4-XLF forms extended helical filaments consisting of many tens of monomers, much larger than hOgg1. Consequently, it is very surprising that the diffusion coefficients of these proteins are similar while their size is so different. Most likely, the mechanism with which XRCC4-XLF complexes diffuse along DNA is substantially different. A possible mechanism might be that the helical XRCC4-XLF complex forms a filament around the DNA that only weakly interacts with it, but can hardly detach. This is confirmed by the observation that forces as weak as the hydrodynamic drag (\( \sim 10 fN \)) caused by the buffer flow, alter substantially the diffusion process, causing drift. Interestingly, the flow was able to accumulate fluorescent XRCC4-XLF protein on the free-end of the DNA. It is difficult to reconcile the filamentous structure of XRCC4-XLF shown by X-ray crystallography [5] with this end-accumulation without invoking a binding mechanism specific for DNA-ends.

Another interesting aspect of XRCC4-XLF interaction with DNA is that increasing the salt concentration slows down diffusion, indicating that better screened charges increase the interaction between DNA and XRCC4-XLF. By visualizing the formation of XRCC4-XLF complexes in real-time we also found that complexes do not form via a canonical nucleation-
Figure 70.: Schematic representation of the role of XRCC4 and XLF role in NHEJ. [A] XRCC4-XLF forms alternating helical filaments on the broken DNA molecule. The filaments are highly mobile on the DNA through a 1-dimensional diffusion mechanism. [B] One-dimensional diffusion permits the XRCC4-XLF complex to reach the broken DNA ends through a specific recognition mechanism. [C] The tail domains of XRCC4 interact, creating an intermolecular bridge between the broken DNAs.

growth mechanism. Instead, we propose a different filament assembly scheme. XRCC4-XLF complexes bind to and diffuse along the DNA, occasionally colliding with each other. Upon collision, complexes can coalesce and form larger DNA-protein structures, similarly to the assembly of TFAM on mitochondrial DNA [51] and Ase1p on microtubules [87]. XRCC4 is able to self-assemble in solution into long homopolymers [85]. XLF can remodel these structures, forming short hetero-complexes of XRCC4 and XLF [182]. These XRCC4-XLF complexes show, in turn, fast binding kinetic to DNA and are able to form regular helical filaments on the DNA, as indicated by the homogeneous fluorescent signal noticed in our measurements. Finally we demonstrated a novel assay, allowing the direct visualization and verification of the bridging properties of XRCC4-XLF complexes.
In conclusion, based on our results we propose that diffusion (sliding), switching from dynamic to static states (pausing) and bridging are the key ingredients for in the function of XRCC4-XLF in NHEJ (see Figure 70). Fast diffusion and dynamic switching state might represent a strategy to localize DNA breaks and bind to them in an effective way. The thus formed DNA-bound XRCC4-XLF complexes are able to form stable bridges between DNA molecules, without the need for other proteins. We argue that DNA bridging by XRCC4-XLF is vital for end ligation for two reasons: (i) physically linking the two broken DNA molecules may prevent the broken DNA ends to move apart and (ii) LigIV has been shown to interact with the C-terminal tail of XRCC4, the putative bridging domain of XRCC4 [153, 5, 113]. The fact that both DNA-bridging activity and LigIV binding involves the C-terminal domain of XRCC4 could indicate that XRCC4-XLF bridging DNA localizes Lig IV to the broken DNA ends, where it can perform the final ligation step.
Figure S71. Dual color excitation and emission of XLF-eGFP and XRCC4-Alexa555. [A] XLF-eGFP is excited with a 491 nm laser and the emitted light is filtered using a dichroic mirror (552 nm) and a single-band band-pass filter (550/88 nm). The EMCCD region for eGFP detection is colored in green. [i] eGFP-XLF complexes (1 and 2, orange arrows) are detected in the eGFP channel of the EMCCD. [B] Excitation with a 532 nm laser predominantly excites Alexa555 fluorophores. The emitted light that is led through the dichroic mirror (552 nm) is further filtered using a single-band band-pass filter (580/60 nm). The EMCCD region for Alexa555 detection is colored in red. [ii] Kymograph showing the Alexa555 channel when excitation is performed using a 532 nm laser. Complexes 3 and 4 consist only of XRCC4-Alexa555. Complexes 1 and 2 are present in both kymographs and represent XRCC4-XLF complexes.

Figure S72. Force-induced melting of dsDNA was employed to produce ssDNA templates. Force-stretching curve of a 38412 bp dsDNA(Black line). The molecule was pulled beyond the overstretching plateau (F>85 pN). The force-stretching curve (red line) confirmed the generation of a single ssDNA molecule tethered between the beads.