Methods

Preparation of expression vectors

Custom HELQ ORF was purchased from GeneArt and used as template during PCR to prepare plasmid (pFastbac1) compatible for bacmid preparation for the expression in insect cells. To prepare MBP-HELQ-FLAG construct, HELQ was amplified by PCR using primers HELQ_F and HELQ_FLAG_R. The amplified insert was digested with BamHI and XmaI and inserted into pFastbac1 vector containing MBP (maltose binding protein tag; previously inserted using EcoRV and HindIII restrictions sites). The resulting construct was pFB-MBP-HELQ-FLAG. To prepare helicase dead HELQ K365M, pFB-MBP-HELQ-FLAG was mutagenized with primers HELQ_K365M_F and HELQ_K365M_R using Q5 site-directed mutagenesis kit, according to manufacturer's instructions. The constructs for pET11c-RAD51 and, RPA-eGFP (pMM801) and RPA-mRFP1 (pMM802) were kind gifts from Lumir Krejci (MUNI, Masaryk University, Bruno) and Mauro Modesti (CRCM, Marseille) respectively.

Recombinant protein purification

To express proteins in insect cells, bacmid, primary and secondary baculovirus were prepared according to manufacturer's instructions (Bac-to-bac system, Life technologies). To express recombinant MBP-HELQ-FLAG, *Spodoptera frugiperda* (*Sf9*) insect cells were seeded at 500,000/ml and after ~ 24 hrs, cells were infected with MBP-HELQ-FLAG baculovirus. The infected cells were incubated at 27° C for 56 hrs with continuous agitation. Cells were harvested by centrifugation at 500 *g* for 10 min and washed once with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The harvested pellets were snap frozen in liquid nitrogen and stored at -80° C until further use. All subsequent steps were carried out either on ice or at 4° C. The cells pellets were resuspended in 3 volumes of lysis buffer containing 50 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), protease inhibitor cocktail tablets (Roche), 30 µg/ml leupeptin (Merck), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.1% NP-40 substitute (NP-40) and incubated for 15 min with continuous agitation. Next, 50% glycerol and 5 M NaCl were added sequentially to the final concentrations of 16.7% and 310 mM respectively, and suspension was further incubated for 30 min with continuous agitation. The suspension was centrifuged at ~ 48000 *g* for 30 min to obtain the soluble extract. The amylose resin

(NEB) was pre-equilibrated with amylose wash buffer I (50 mM Tris-HCl pH 7.5, 1 mM 2-Mercaptoethanol (β-ME), 1 M NaCl, 1 mM PMSF, 10% glycerol, and 0.1% NP-40) and added to 50 ml tubes containing soluble extract. These tubes were subsequently incubated for 1 h with continuous rotation. Post-incubation, resin was washed batchwise 4x by centrifugation at 2000 *g* for two min and 2x on column with amylose wash buffer I. Resin was washed two more times on column with amylose wash buffer II (same as wash buffer I but with 0.5 β-ME and 0.8 M NaCl). Protein was eluted from resin with amylose elution buffer (same as amylose wash buffer II supplemented with 10 mM Maltose) and total protein was estimated using Bradford assay. To remove the MBP tag, 1/8 (weight/weight) of PreScission protease (PP) to the total protein added to amylose eluate and incubated for 2 hrs without rotation but gentle agitation at regular intervals. The FLAG resin (anti-FLAG M2 resin, Sigma-Aldrich), pre-equilibrated with FLAG wash buffer (50 mM Tris-HCl pH 7.5, 0.8 M NaCl, 1 mM PMSF,10% glycerol), added to amylose eluate containing PP and incubated for 2 hrs with continuous rotation. FLAG resin was collected directly on column and washed 6x with FLAG wash buffer. Protein was eluted from FLAG resin with FLAG elution buffer (50 mM Tris-HCl pH 7.5, 0.5 mM β-ME, 150 mM NaCl, 1 mM PMSF,10% glycerol, 150 ng/µl 3XFLAG peptide (Sigma), aliquoted, frozen in liquid nitrogen and stored at -80° C. The same purification procedure was used to purify HELQ K365M.

Recombinant human RAD51 was purified as described previously with few modifications[1]. The pET11c-RAD51 expression vector was transformed in *E. coli* BLR(DE3)pLysS cells and subsequent culture containing ampicillin (100 mg/l) and chloramphenicol (33. mg/l) was grown to ~0.7 O.D at $A_{600.}$ RAD51 expression was induced with 1 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) at 37° C for 3-4 hrs. All subsequent steps were carried out either on ice or at 4° C. Cells were harvested by centrifugation at 5000 *g*. Cell pellets were resuspended in cell breakage buffer (CBB) (50 mM Tris-HCl pH 7.5, 10% sucrose, 0.5 mM EDTA, 1 M KCl, 1 tablet/50 ml protease inhibitor cocktail tablets (Roche), 1 mM PMSF, 1 mM DTT and 0.01% NP-40), sonicated and centrifuged at 100,000 *g* for 1 h. To precipitate RAD51, 0.242 grams/ml ammonium sulphate was mixed with clarified supernatant and centrifuged for 20 min at 10,000 g. The pellet was resuspended with buffer K (20 mM K_2HPO_4 pH 7.5, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, 0.01% NP-40) and loaded on Q Sepharose Fast flow column (Cytiva), pre-equilibrated with K buffer-low (K buffer supplemented with 175 mM KCl). The column was extensively washed with K buffer-low

and protein was subsequently eluted with KCl gradient using K buffer-high (K buffer supplemented with 0.6 M KCl). The eluted fractions containing RAD51 were pooled and diluted with 6 volumes of dilution buffer (25 mM Tris HCl pH 7.5, 0.5 mM EDTA, 1 mM DTT, 0.01% NP-40). The diluted sample was loaded on HiTrap Heparin HP affinity column (Cytiva), which was pre-equilibrated with buffer H without glycerol (25 mM Tris HCl pH 7.5, 0.5 mM EDTA, 1 mM DTT, 0.01% NP-40, 150 mM KCl) and washed with buffer H containing 10% glycerol. Protein was eluted in buffer H with KCl gradient (0.1 M to 1 M KCl) and fractions containing RAD51 were pooled and dialyzed in buffer H without glycerol. The dialyzed sample was loaded on Mono Q 5/50 GL column (Cytiva), equilibrated with buffer Q (25 mM Tris HCl pH 7.5, 0.5 mM EDTA, 1 mM DTT, 0.01% NP-40, 100 mM KCl, 10% glycerol) and column was further washed with buffer Q containing 50 mM KCl but lacking glycerol. RAD51 was eluted from Mono Q column with KCl gradient (0.05 M to 1 M) in buffer Q lacking glycerol. The eluted fractions containing RAD51 were pooled and further concentrated with Vivaspin Centrifugal Concentrator (30 K MWCO). Glycerol was added to concentrated sample to reach final 10% concentration. Finally, samples were aliquoted, frozen in liquid nitrogen and stored at – 80° C. The RAD51 C319S mutant was purified using same procedure. RPA-mRFP1 and RPA-eGFP were purified as described previously[2]. Recombinant RecA (M0249) and ET SSB (M2401) were commercially purchased from NEB, England.

Preparation of labelled proteins

RAD51 C319S variant was expressed and purified as described earlier for wild type RAD51[1,3]. After purification, protein was fluorescently labelled with Alexa Fluor 488 C_5 maleimide (Thermo Fisher, cat. n. A10254) according to previously described protocol[2]. Labelled protein was purified away from the free dye using Zeba column gel filtration system (0.5 mL resin, 50.000 MWCO). Protein concentration was estimated by Coomasie staining and dye concentration was measured spectrophotometrically. Presence of minimum free dye concentration was assessed using SDS-PAGE on labelled proteins. Protein to dye concentration ratio was consistently 0.9-1.0. D-loop formation of labelled RAD51 was tested and gave yields comparable to unlabelled wild-type RAD51 protein, consistent with previous reports[3]. RPA-eGFP and RPA-mRFP1 were expressed and purified as described previously[2]. DNA binding of labelled RPA was tested. All RPA fusion proteins displayed similar ssDNA affinities within nanomolar K_d range.

DNA sequences of oligoes used in this study for *in vitro* analysis

The details of DNA substrate preparation and sequence of oligoes used is provided in table 1 and table 2 respectively.

DNA unwinding assay

The unwinding assays were carried out in 15 µl helicase buffer containing 25 mM Tris-HCl pH 8.0, 2 mM ATP, 2 mM $MgCl^2$, 1 mM DTT, 50 mM NaCl, 0.1 mg/ml bovine serum albumin (BSA, New England Biolabs), 1 mM PEP (Phosphoenolpyruvate, Sigma-Aldrich), 10 U/ml pyruvate kinase (Sigma-Aldrich) and 5' end FITC (Fluorescein isothiocyanate) labelled 25 nM DNA substrate (in molecules). All steps except assembling reactions, and protein addition were performed in dark. The reactions were assembled on ice and recombinant proteins were added, mixed and incubated at 37° C for 30 mins. The reactions were stopped with 5 µl of 2% stop solution (0.2% SDS, 30% Glycerol, 150 mM EDTA, bromophenol blue) and 1 µl Proteinase K (Roche, 18.4 mg/ml) and incubated further for 10 min at 37° C. To prevent reannealing, 2% stop solution was supplemented with 10-fold excess of unlabelled oligo with the same sequence as FITC-labelled one. The products were resolved by 10% native polyacrylamide gel (19:1 acrylamide-bisacrylamide, Biorad) using Mini-Protean Tetra Cell electrophoresis system (Biorad) at 100 V for 1 h. The gels were directly imaged in ChemiDoc MP imaging system.

Quenching-based kinetic assay for DNA unwinding

These assays were carried out in 60 µl helicase buffer with 20 nM DNA substrate (in molecules). The oligo F (49-mer) in DNA substrate was labelled with 6-flouroscein amidite(6-FAM) at 5' end whereas oligo R (22-mer) was labelled at 3' end with rhodamine (ROX). The reactions were assembled on ice in 96-microwell plate and recombinant proteins were directly added to their respective wells. Microplate was transferred to microplate reader (CLARIOstar, BMG Labtech) at 37° C and 6-FAM intensity was continuously monitored at every 30 seconds for 60 min. The final values were plotted in graph forms using PRISM graphpad.

Electrophoretic mobility shift assay

EMSA reactions (15 µl) were carried out in binding buffer containing 25 mM Tris-HCl pH 8.0, 2 mM ATP, 2 mM $MgCl^2$, 1 mM DTT, 50 mM NaCl, 0.1 mg/ml bovine serum albumin (BSA, New England Biolabs) and 5' end FITC (Fluorescein isothiocyanate) labelled 25 nM DNA substrate (in molecules). All steps except assembling reactions, and protein addition were performed in dark. Reactions were assembled on ice and recombinant proteins were added to reactions, mixed and incubated for 10 min at 37° C in dark. Reactions were supplemented with 5 µl of EMSA loading buffer (50% glycerol, bromophenol blue) and resolved with 6% native TBE polyacrylamide gel (19:1 acrylamide-bisacrylamide, Biorad) using Mini-Protean Tetra Cell electrophoresis system (Biorad) at 80 V for 45 min on ice. Finally, gels were imaged using ChemiDoc MP imaging system.

RPA stripping gel-based assay

Stripping assay was performed as described for EMSA except products were resolved at room temperature and longer 6% TBE native gel was used.

DNA annealing assay

DNA annealing assays were carried out in 15 µl annealing buffer containing 25 mM Tris-HCl pH 8.0, 2 mM ATP, 2 mM $MgCl^2$, 1 mM DTT, 50 mM NaCl and 0.1 mg/ml bovine serum albumin (BSA, New England Biolabs). All steps except assembling reactions, and protein addition were performed in dark. For DNA substrate, 10 nM (in molecules) complementary oligos (5' FITC-Oligo 1 and oligo 2) were separately incubated in 7.5 µl annealing buffer and as indicated, with or without RPA on ice for 2 min. Recombinant proteins were added to FITC-oligo 1 reactions (7.5 µl) on ice, immediately followed by the addition of oligo 2 reactions (7.5 µl). Reactions were incubated for 10 min at 37° C. The final concentration of both individual oligo and annealed dsDNA product was 5 nM. The reactions were stopped with 5 µl of 2% stop solution (0.2% SDS, 30% Glycerol, 150 mM EDTA, bromophenol blue) and 1 µl Proteinase K (Roche, 18.4 mg/ml) and incubated further for 10 min at 37° C. To prevent the detection of spontaneous annealing during deproteination, 25-fold excess of unlabelled oligo 1 to FITC-oligo 1 was included in 2% stop solution. The products were resolved and imaged identically as described for unwinding assays.

Interaction assay

To study the interaction in between HELQ and RAD51, MBP-HELQ-FLAG baculovirus was expressed in 300 ml insect cells and soluble extract from collected pellet was prepared as described for protein purification procedure. Reagent volumes for soluble extract preparation were scaled down accordingly. Soluble extract was divided equally into two parts and incubated with amylose (E8021, NEB), and anti-FLAG M2 resin (A2220, Sigma-Aldrich) for 1 h at 4 C. Next, amylose resin and anti-FLAG M2 resin were washed with wash buffer I (50 mM Tris-HCl pH 8.0, 1 mM DTT, 310 mM NaCl, 10% glycerol, 1 mM PMSF). Both resins were divided into 50 µl volumes in separates microtubes. 4 µg recombinant RAD51 was added to all except one tube for each resin and incubated for 1 h at 4° C. Resins were washed with wash buffer II (same as wash buffer I but containing 100 mM NaCl). Proteins were eluted from resin in 1X SDS buffer by boiling at 95°C for 4 min. The eluate was separated by 4-12% native SDS-PAGE gel (NuPAGE Bis-Tris, Invitrogen) and stained with instant blue Coomassie protein stain (Abcam).

DNA capture assay

The capture assays were carried out in 20 µl DNA annealing buffer supplemented with 0.05% Tween-20. Reactions were assembled on ice and where indicated, 82 nM RPA, 10 nM biotinylated dT43 (bio-dT43) and 10 nM 3'Cy3-dT79 were added to reactions. Next, HELQ was added to reactions as indicated. Reactions were mixed and incubated at 37° C for 8 mins in dark. To pull down bio-dT43, magnetic streptavidin beads were washed 2X with PBS-0.1% Tween 20 (Dynabeads M-280, ThermoFisher) and 5 µl bread were added to each reaction. Reactions were further incubated for 4 min in dark at room temperature and then washed 2X with 80 µl washing buffer (25 mM Tris-HCl pH 8.0, 2 mM ATP, 2 mM $MgCl^2$, 1 mM DTT, 100 mM NaCl, 0.5 mg/ml bovine serum albumin, NP-40) on magnetic rack. Finally, beads were resuspended in 30 µl loading buffer (7.5 µl 2% STOP solution and 22.5 µl washing buffer) and boiled at 95° C for 4 min. Sample were centrifuged at high speed for a min and 25 µl volume sample was loaded immediately on 10% native polyacrylamide gel and ran as described for unwinding assay. The gels were directly imaged in ChemiDoc MP imaging system (Biorad).

Substrate and flow cell preparation for single-molecule imaging

Experiments were performed using commercially available C-trap (LUMICKS) setup. Protein channels of the microfluidics chip were first passivated with BSA (0.1% w/v in PBS) and Pluronics F128 (0.5% w/v in

PBS), minimum 500 µl of both flowed through prior to use. Biotinylated ssDNA precursor was prepared as described previously[4]. To generate gapped λ DNA, a protocol described previously was employed[5]. Briefly, biotinylated hairpin oligonucleotides were annealed to ds-λDNA ends and ligated[6]. S. p. Cas9 D10A nickase (IDT) bound to previously described[7] guide RNAs were subsequently used to generate targeted DNA nicks. The reaction was then stored at 4 °C and directly diluted in PBS on the day of the experiment. DNA was captured between 4.5 -µm SPHERO Streptavidin Coated polystyrene beads at 0.005% w/v using the laminar flow cell, stretched and held at forces of 100 pN (for ssDNA) or 65 pN (λ gDNA 4/5) until the strands were fully melted. The presence of ssDNA and/or ssDNA gap was verified by comparison to built-in freely joined chain model. For confocal imaging, three excitation wavelengths were used, 488 nm for eGFP and Alexa Fluor 488, 532 nm for Cy3 and 638 nm for Cy5, with emission detected in three channels with blue filter 512/25 nm, green filter 585/75 nm and red filter 640 LP.

Single-molecule DNA unwinding assay

For all the unwinding assays λ gDNA 4/5 construct was held at constant force of 50 pN. Beads and DNA were kept in PBS during the experiment, while DNA was melted in 0.5xNTM buffer (25 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM $MgCl_2$) supplemented with 0.2 mg/ml BSA and 1 mM DTT. 50 nM HELQ and/or 25 nM RAD51(A488) were flowed into the system in 1xHELQ buffer (25 mM Tris-HCl pH 8.0, 2 mM $MgCl^2$, 50 mM NaCl) supplemented with 2 mM ATP, 0.2 mg/ml BSA and 1 mM DTT. Unwinding was monitored by change in distance between the beads over time. To directly image fluorescent RAD51, following image acquisition setup was employed: 5% blue laser power, 0.1 ms/pixel dwell-time, 100 nm pixel size, 1 s inter-frame wait time.

Single-molecule DNA oligonucleotide capture assay

For all the unwinding assays λ ssDNA was held at constant force of 10 pN. Beads and DNA were kept in PBS during the experiment, while DNA was melted in 0.5xNTM buffer (25 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM $MgCl_2$) supplemented with 0.2 mg/ml BSA and 1 mM DTT in the presence of 5 nM RPA-eGFP. 5 nM HELQ, 5 nM RPA-eGFP and 2 nM 5'Cy3-(λ4)80 oligonucleotide (5'-Cy3-CCTGAACGACCAGGCGTCTTCGTTCATCTATCGGATCGCCACACTCACAACAATGAGTGGCAGAT ATAGCCTGGTGGTTC-3') or 2 nM 5'Cy3-(dT)79 oligonucleotide (5'-Cy3-

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTT-3’) were flowed into the system in 1xHELQ buffer (25 mM Tris-HCl pH 8.0, 2 mM $MgCl^2$, 50 mM NaCl) supplemented with 2 mM ATP, 0.2 mg/ml BSA and 1 mM DTT. To directly image fluorescent oligonucleotide capture, following image acquisition setup was employed: 15% green laser power, 0.1 ms/pixel dwell-time, 100 nm pixel size, 0.5 s inter-frame wait time.

Image processing, and data analysis

Real-time force, distance and fluorescence data were exported from Bluelake HDF5 files and analysed using custom-written scripts in Pylake Python package. Force was down sampled to 3 Hz for plotting. Worm-like chain (WLC) model for λ dsDNA was used as a reference for force-extension curve comparison. Bead distance-time traces were processed in GraphPad Prism 7. First derivative and smoothing of the traces was performed to extract individual unwinding stroke rates. Unwinding stroke rate distribution was analyzed in GraphPad Prism 7 by fitting single or double-gaussian curve. Dwell-times and binding frequencies were estimated in Fiji. Dwell-time frequency distribution was analysed in GraphPad Prism 7. Mann-Whitney test was used to assess statistical significance of the data where appropriate.

For the Mean-Square-Displacement and velocity analysis, we used custom-made single-particle tracking algorithm. The sub-pixel position of the molecule in each frame of the kymograph (line time = 0.997 s) was calculated by fitting the signal intensity of a 3-frame moving window with 1D Gaussian function. For the obtained trajectories, MSD was calculated using the following equation:

$$MSD(n, N) = \sum_{i=1}^{N-n} \frac{(X_{i+n} - X_i)^2}{N - n}$$

where: N is the total number of time frames in the kymograph, n is the number of time frames within a moving window (interval time) from which the square displacement was calculated (ranging from 1 to N-1). X_i is the molecule position along the DNA in time. The position was translated from pixels to microns knowing that 1 pixel = 0.1 μm.

The resulting function MSD(*interval time*) generated a plot which can be linear in the case of unconstrained 1D diffusion, partially linear (at short interval times) in the case of constrained diffusion, quadratic for unidirectional movement, and flat in the case of a stationary molecule.

To estimate the average velocity of the translocating molecule, the total route of the molecule (a sum of frame-to-frame displacements) was divided by the total trajectory time. Here, every trajectory was smoothed using Savitzky-Golay filter (smoothing factor = 51) to eliminate tracking inaccuracies and to minimize the effect of thermal fluctuations of the DNA tether on the frame-to-frame displacements.

To estimate the loop size formed by the HELQ-RPA-DNA complex, the contour length after each unfolding event in the force-distance curve was fitted by the Worm-Like-Chain model. The difference between contour lengths of the neighbouring events corresponds with the loop size.

RPA stripping via single Molecule FRET

Flow chambers were prepared as described previously[1,2] . Quartz slides and coverslips were passivated with polyethylene glycol (5% biotinylated) and flow chambers constructed using double-sided sticky tape and sealed with epoxy. 5′-biotin and internal amino linker modified DNA oligonucleotides were labelled with Cy3-NHS ester and HPLC purified as previously described [3]. DNA (6 pM) was immobilised via biotin–streptavidin interactions. All experiments we performed in the standard HELQ buffer with addition of the PCA/PCD oxygen scavenger system with 5 mM PCA, 100 nM PCD and saturating Trolox. The flow chambers were imaged on a home-built, prism-based total internal reflection microscope with a 532nm excitation laser (~3.8 mW), and images acquired on an EM-CCD camera (Andor) with a 30 ms exposure time. FRET efficiencies were calculated from integrated donor (I_D) and acceptor (I_A) intensities as FRET= $I_A/(I_D + I_A)$[1,3] Images and data were analysed by custom IDL, MATLAB and R scripts, available upon request. FRET efficiency histograms were constructed by averaging the first 10 frames of each trajectory, with bins of 0.1. The dwell times of the free (high FRET) and bound (low FRET) states were measured, and dwell-time histograms plotted. These were fit with single exponential fits to obtain average dwell times.

Cell culture

U2OS, human osteosarcoma cell line, was grown in DMEM supplemented with 10% bovine growth serum (BGS), 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 units/mL penicillin. U2OS-EJDR cells were cultured in DMEM supplemented with 10% tetracycline-free fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 units/mL penicillin. U2OS-DR cells contain a stably integrated DR-GFP reporter to measure DSB repair by HR as previously described[8]. U2OS-SA cells contain a stably integrated SA-GFP reporter to measure DSB repair by SSA as previously described[9]. U2OS-DR cells containing a stably integrated EJ-RFP reporter to measure DSB repair by mutagenic end-joining, constitute the U2OS-EJDR cell line as previously described[10]. U2OS-RFP-SCR cells contain a stably integrated RFP-SCR reporter for quantifying short and long tract gene conversion in HR as previously described[11].

U2OS-DR HELQ-/- and U2OS-SA HELQ-/- cells were generated using the CRISPR-Cas9 system. Knockouts verified by Sanger sequencing and immunoprecipitation/western blot.

siRNA

siRNA oligonucleotides used to transiently deplete HELQ: HelQsp (Horizon siGENOME SMARTpool, M-015379-01-0005), HelQ_M[4] (CAAAGGAAGATTTCCTCCAACTAAA), HelQ_4 (Qiagen FlexiTube siRNA, TTGGGTGATCATTCTATGAAA). RAD52 was depleted with On-Target plus SMART pool siRNA L-011760-00-0005 (Horizon). BRCA2 was depleted with On-Target plus SMART pool siRNA L-003462-00-0005 (Horizon). On-Target plus Non-targeting siRNA pool used for NT controls (D-001810-10-05, Horizon).

DSB repair assays

$0.25x10^6$ cells were reverse transfected with 30 pmol siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to manufacturer instructions. After 48 hours, cells were transefected with 2 µg of pCMV-ISceI-3xNLS or pCMV 3xNLS empty vector and 30 pmol siRNA using Lipofectamine 2000 (Invitrogen). Cells were harvested for analysis by flow cytometry at 72 hours using the LSR Fortessa instrument (BD Biosciences). For each experiment, % GFP or RFP positive cells in the empty vector control was subtracted from the I-SceI transfected cells. Data from each reporter assay represents the

mean and standard error of at least 3 independent experiments, and statistical analysis was performed using a two-tailed paired t-test.

Immunoprecipitation/Western blot

Cells were lysed in RIPA buffer (Teknova, cat#R3792) with HALT protease inhibitor cocktail (Thermo Scientific). As HELQ is expressed at low levels in human cell lines and commercially available antibodies tested did not dependably detect endogenous HELQ by western blot, we validated siRNA mediated HELQ knockdown by HELQ immunoprecipitation. 10^6 cells were transfected with 2µg siRNA by electroporation using the Amaxa nucleofector system, and plated into 150mm dishes. After 72 hours, cells were harvested and whole cell extracts were used for immunoprecipitation. 2mg protein was incubated with 1ug HEL308 antibody (Novus Biologicals NBP1-91842) at 4°C overnight with rotation. After washing 0.25mg Pierce Protein A/G Magnetic Beads (Thermo Scientific cat #88802), the antigen sample/antibody mix was added to the beads and incubated for 1 hour at room temperature with rotation. The beads were washed four times and eluted in SDS-PAGE reducing sample buffer (Invitrogen) for ten minutes at 96°C. Samples were loaded onto 4-12% Bis-Tris precast gels (Invitrogen) for SDS-gel electrophoresis and transferred onto Immobilon-P PVDF membrane (Millipore). Membranes were blocked for one hour at room temperature with Pierce clear milk blocking buffer. For Western blot analysis of RAD52 and BRCA2, 50 µg of protein was loaded onto 10% Bis-Tris or 3-8% Tris-Acetate precast gels (Invitrogen) for SDS-gel electrophoresis. Proteins were transferred onto a PVDF (polyvinylidene difluoride) membrane (BioRad) for BRCA2 detection.The iBlot Gel Transfer System (Invitrogen) was used to perform dry blotting of proteins onto nitrocellulose membranes for RAD52 detection. Membranes were blocked for one hour at room temperature with Pierce clear milk blocking buffer. Antibodies for Western blot: anti-HEL308 (Santa Cruz Biotechnology, sc-81095), anti-RAD52 (Santa Cruz Biotechnology, sc-365341), anti-Actin (Millipore Sigma, MAD1501), anti-BRCA2 Ab-1 (Millipore Sigma, OP95), anti-SMC1 (Bethyl laboratories, A300-055A).

Software

Statistical analysis was carried out using Graphpad Prism (version 8.4).

Flow cytometry data was analyzed using BD FlowJo (version 10.6).

316 Schematics for reporter assays designed using Biorender.com.

317

318 **Table. 1 Detail of preparation of *in vitro* substrates**

DNA substrates	**Annealed oligos**
ssDNA	Oligo 1
3’ overhang	Oligo 1 + Oligo 4
5’ overhang	Oligo 1 + Oligo 5
dsDNA	Oligo 1 + Oligo 2
Y structure	Oligo 1 + Oligo 6
Lagging-strand fork	Oligo 1 + Oligo 4 + Oligo 6
D-loop	Oligo 23 + Oligo 22 + Oligo 21 + Oligo 20
3’ overhang (used for Quenching kinetic assay)	Oligo F + Oligo R

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320 **Table. 2 DNA sequence of oligos used in *in vitro* analysis**

Oligos	**DNA sequence (5’ to 3’)**
Oligo 1	5’ **FITC**-AGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT (49-mer)
Oligo 2	5’AGCTATGACCATGATTACGAATTGCTTAATTCGTGCAGGCATGGTAGCT 3’ (49-mer)
Oligo 4	5’ AATTCGTGCAGGCATGGTAGCT 3’ (22-mer)
Oligo 5	5’ AGCTATGACCATGATTACGAATTGCTT 3’ (27-mer)
Oligo 6	5’ AGCTATGACCATGATTACGAATTGCTTGGAATCCTGACGAACTGTAG 3’ (47-mer)
Oligo 23	5’ **FITC**-GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC 3’ (61-mer)
Oligo 22	5’ GGGTGAACCTGCAGGTGGGCGGCTGCTCATCGTAGGTTAGTTGGTAGAATTCGGCAGCGTC 3’ (61-mer)
Oligo 21	5’ TAAGAGCAAGATGTTCTATAAAAGATGTCCTAGCAAGGCAC 3’ (41-mer)
Oligo 20	5’ TATAGAACATCTTGCTCTTA 3’ (20-mer)
Oligo F	5’ 6-**FAM**-AGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT (49-mer)
Oligo R	5’ AATTCGTGCAGGCATGGTAGCT 3’ **ROX** (22-mer)

λ4	5'**Cy3-** CCTGAACGACCAGGCGTCTTCGTTCATCTATCGGATCGCCACACTCACAACAAT GAGTGGCAGATATAGCCTGGTGGTTC-3' (80-mer)
dT 79	5'**Cy3**TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTT 3' (79-mer)
B-dT43	5' TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT 3' **Biotin** (43-mer)

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Author contributions

R.A, E.B, S.P and S.B conceived, planned and performed majority of experiments and wrote the paper. O.B and M.N performed and analysed SMI and FRET-based experiments. A.K analysed part of SMI-based DNA capture experiments with D.R. All authors contributed to assemble and finalize the manuscript.

Conflict of interest

S.J.B. is also cofounder and VP Science Strategy at Artios Pharma.