

Polyubiquitinated PCNA Recruits the ZRANB3 Translocase to Maintain Genomic Integrity after Replication Stress

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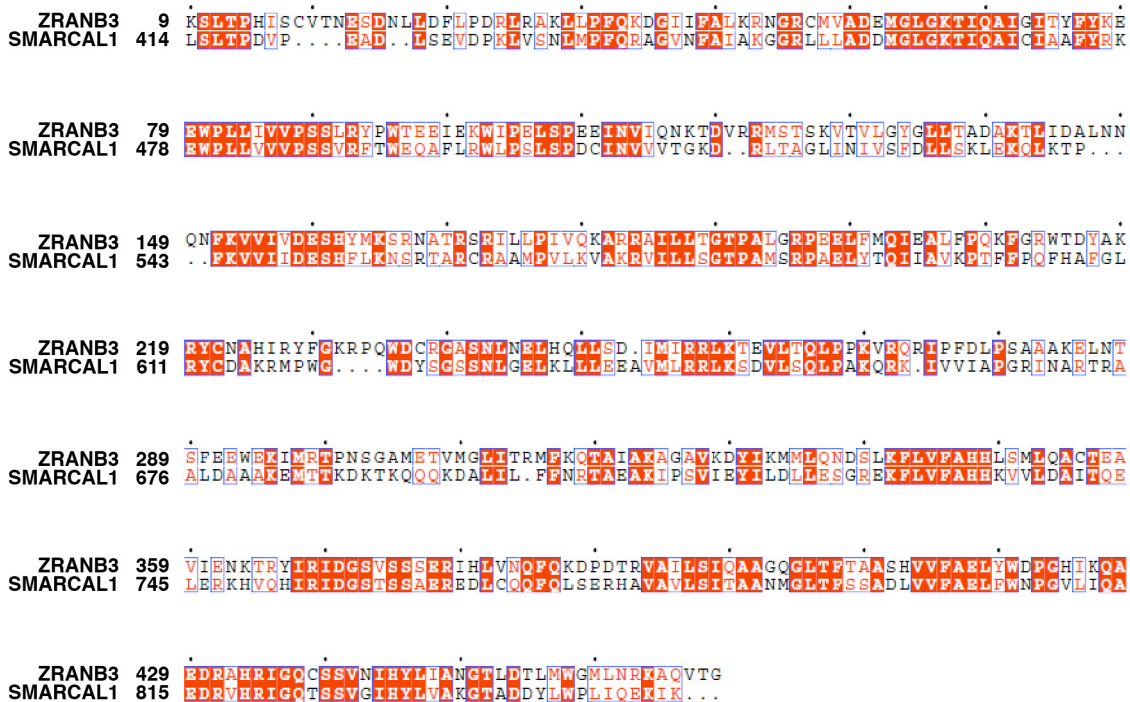


Figure S1 (related to Figure 1). Sequence Alignment of the Helicase Domains of ZRANB3 and SMARCAL1

Similar amino acid residues are shown in red. The alignment was performed using ClustalW.

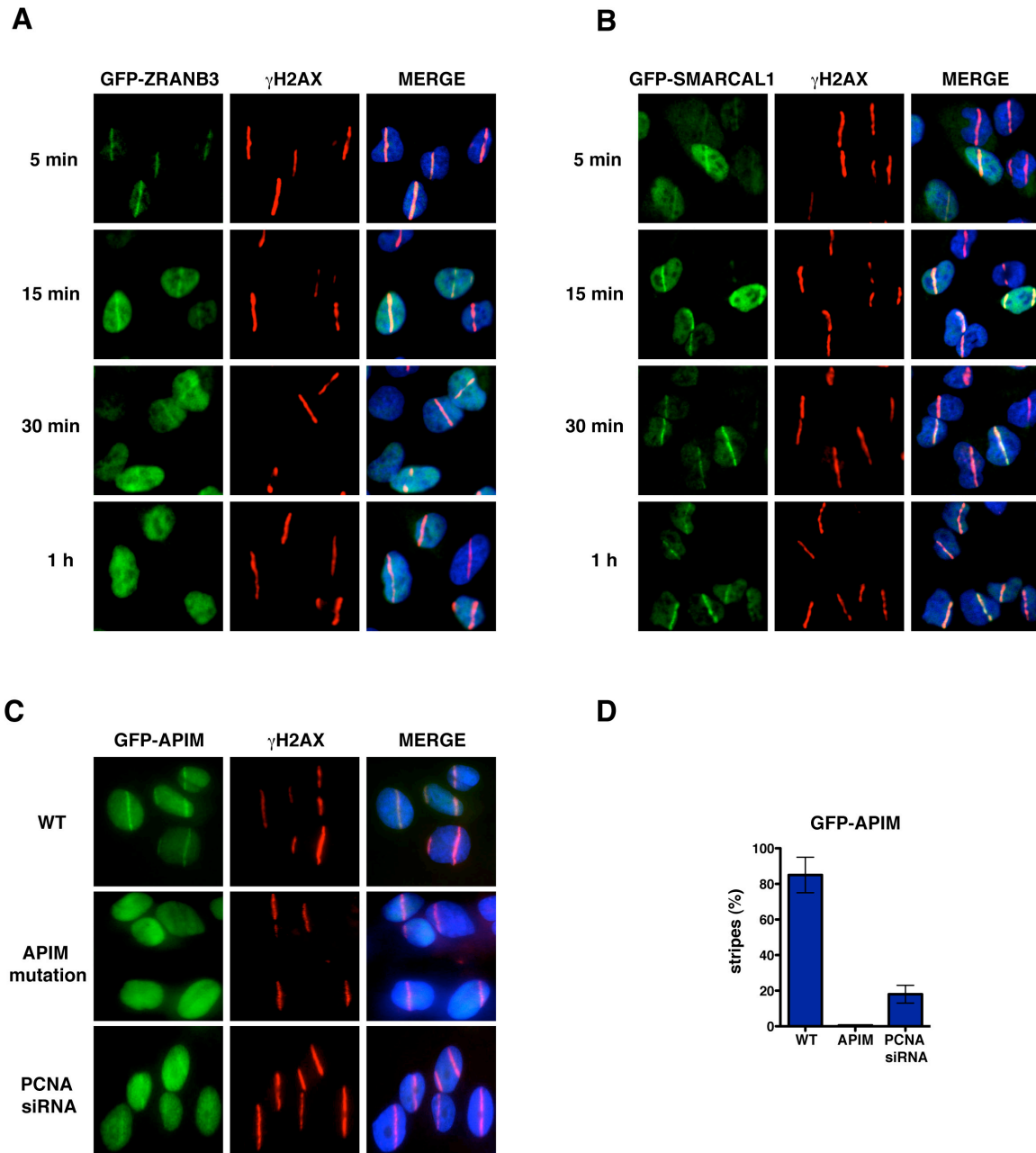


Figure S2 (related to Figure 1). Recruitment of ZRANB3 and SMARCAL1 to DNA Damage Sites

(A) Time course experiment monitoring the recruitment of GFP-ZRANB3 to DNA damage sites. U2OS cells expressing GFP-ZRANB3 were fixed at different time points following UV laser microirradiation. Cells were then stained with anti-GFP (green) and anti- γ H2AX (red) antibodies and with DAPI (blue).

(B) Time course of the recruitment of GFP-SMARCAL1 to laser-generated stripes. The experiment was performed as described in (A).

(C) Localization of the APIM motif of ZRANB3 to damage sites. U2OS cells expressing a WT or mutant APIM motif of ZRANB3 fused to a GFP tag and a nuclear localization sequence were UV microirradiated and stained with anti-GFP (green) and anti- γ H2AX (red) antibodies and with DAPI (blue). Cells expressing WT GFP-APIM are shown with or without PCNA siRNA treatment before microirradiation.

(D) Graphical representation of the percentage of U2OS cells that display co-localization of GFP-APIM with γ H2AX at laser-generated stripes. The data represent the average and standard deviation of three independent experiments.

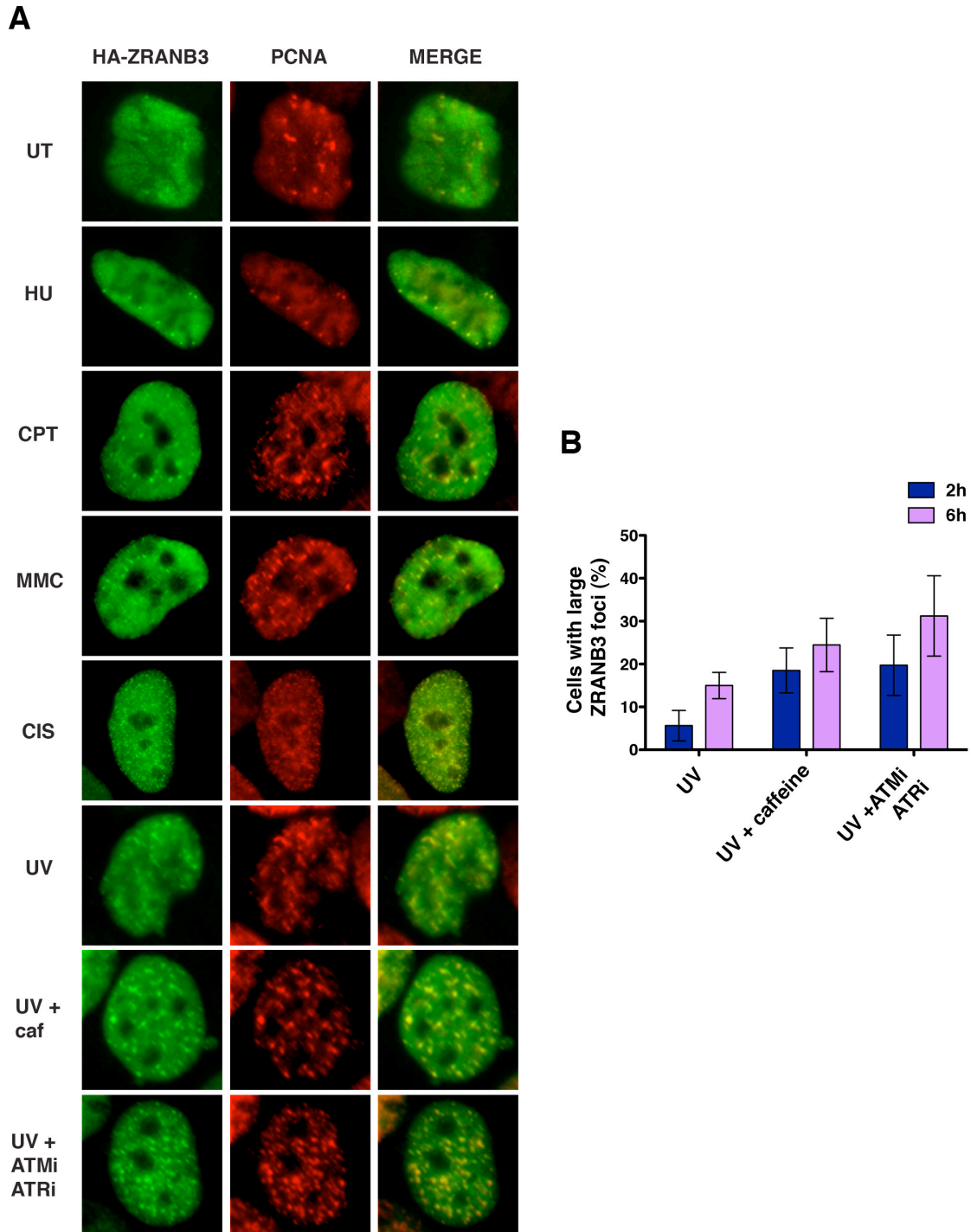


Figure S3 (related to Figure 1). Co-localization of ZRANB3 with PCNA Following Replication Stress

(A) U2OS cells expressing HA-ZRANB3 were left untreated (UT) or subjected to hydroxyurea (HU, 2 mM), camptothecin (CPT, 50 nM), mitomycin C (MMC, 300

nM), cisplatin (CIS, 5 μ M) or UV radiation (25 J/m²) treatment. Cells subjected to UV radiation were also treated with caffeine (2 mM) or ATM and ATR inhibitors (10 μ M and 2 μ M, respectively). Cells were stained with anti-HA (green) and anti-PCNA (red) antibodies.

(B) Graphical representation of the percentage of U2OS cells displaying large HA-ZRANB3 foci as shown in (A) following UV treatment with or without caffeine or ATM and ATR inhibitors. The time in which the cells were fixed after UV radiation is indicated. The data represent the average and standard deviation of three independent experiments in which more than 200 cells were counted.

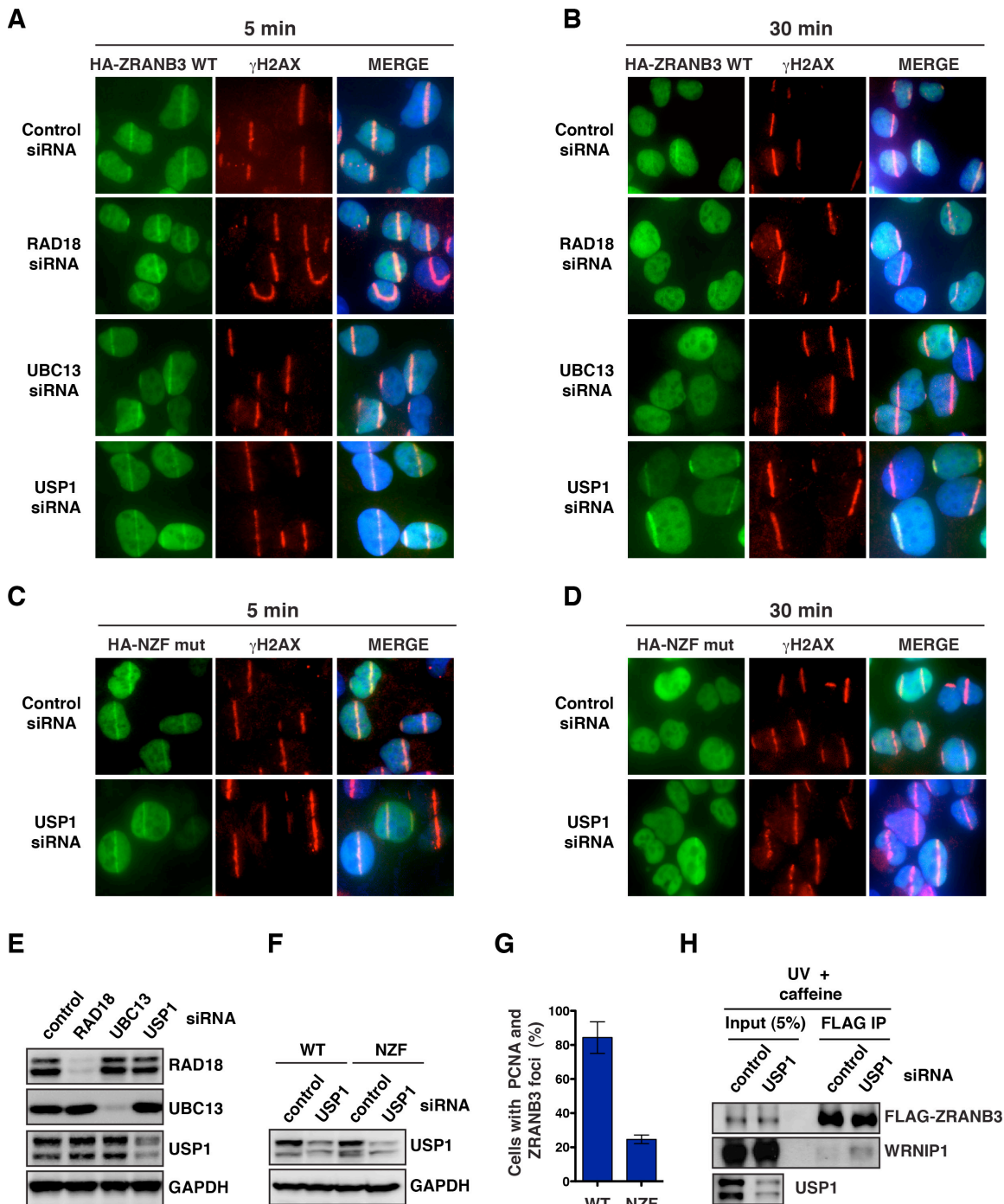


Figure S4 (related to Figure 3). Ubiquitination-Dependent Association of ZRANB3 to DNA Damage Sites.

(A) Localization of WT HA-ZRANB3 to laser-induced stripes following treatment with control, RAD18, UBC13 or USP1 siRNAs. Cells were fixed 5 min after

microirradiation and then stained with anti-HA (green) and anti- γ H2AX (red) antibodies and with DAPI (blue).

(B) U2OS cells expressing WT HA-ZRANB3 treated as in (A) were fixed 30 min after microirradiation and then stained with anti-HA (green) and anti- γ H2AX (red) antibodies and with DAPI (blue).

(C) Localization of NZF mutant HA-ZRANB3 to laser-induced stripes following treatment with control or USP1 siRNAs. Cells were fixed 5 min after microirradiation and then stained as in (A).

(D) U2OS cells expressing NZF mutant HA-ZRANB3 treated as in (C) were fixed 30 min after microirradiation and then stained as in (A).

(E) Western blot showing the efficiency of knockdown of RAD18, UBC13 and USP1 in the experiments represented in (A) and (B).

(F) Western blot for efficiency of knockdown of USP1 in the experiments represented in (C) and (D).

(G) Quantification of the percentage of cells expressing either WT or NZF mutant HA-ZRANB3 that display co-localization of PCNA and HA-ZRANB3 after USP1 depletion. The data represent the average and standard deviation of three independent experiments in which more than 50 cells were counted.

(H) Interaction of ZRANB3 with WRNIP1 after USP1 depletion. 293T-Rex cells expressing FLAG-ZRANB3 were treated with control or USP1 siRNAs prior to UV radiation (30 J/m^2) and caffeine (2 mM) treatment. Protein complexes were crosslinked, subjected to anti-FLAG immunoprecipitation and then detected by western blotting.

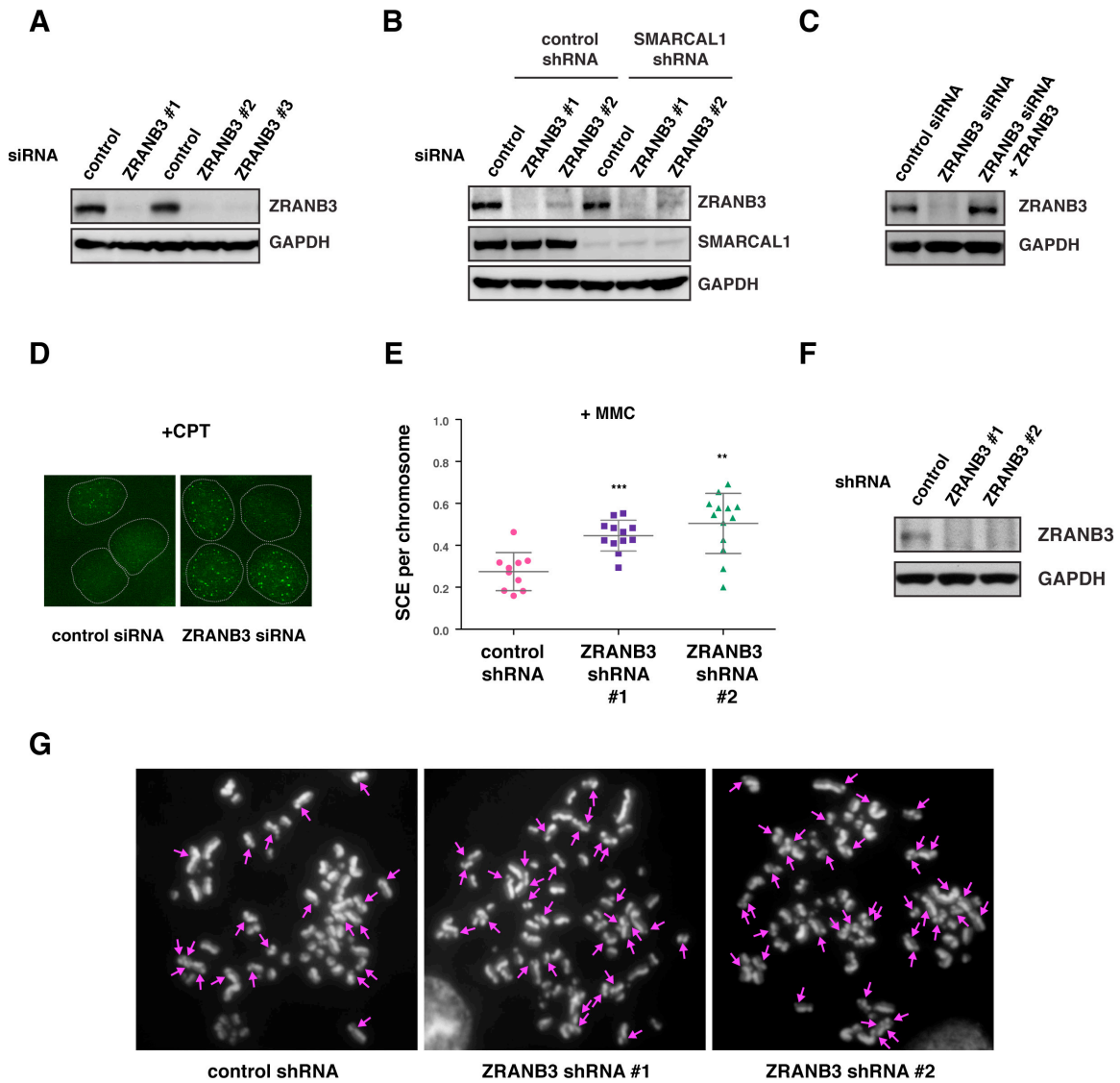


Figure S5 (related to Figure 4). Cellular Effects of ZRANB3 Depletion

(A) Western blot for the knockdown of ZRANB3 in the cells subjected to the competition assay represented in Figure 4A.

(B) Western blot showing the protein levels of ZRANB3 and SMARCAL1 in the cellular samples of competition assay represented in Figure 4B.

(C) Detection by western blotting of the protein levels of ZRANB3 in the cells subjected to the sister chromatid exchange assay represented in Figure 4C.

(D) Formation of RAD51 foci (green) in U2OS cells treated with control or ZRANB3 siRNA and fixed 12h after 1h camptothecin (CPT, 10 nM) treatment.

(E) Graphical representation of the number of sister chromatid exchanges (SCEs) of mitotic chromosomes isolated from U2OS cells transfected with control or ZRANB3 shRNAs after mitomycin C (MMC) treatment. The average frequencies of SCEs and the standard deviation are indicated. Statistically significant p-values calculated using the Mann-Whitney test are indicated by asterisks (**p<0.01, ***p<0.001).

(F) Detection by western blotting of ZRANB3 protein levels after treatment with control or ZRANB3 shRNAs shown in (E).

(G) Chromosome spreads from U2OS cells expressing control shRNA or ZRANB3 shRNAs shown in (E). SCEs are indicated by pink arrows.

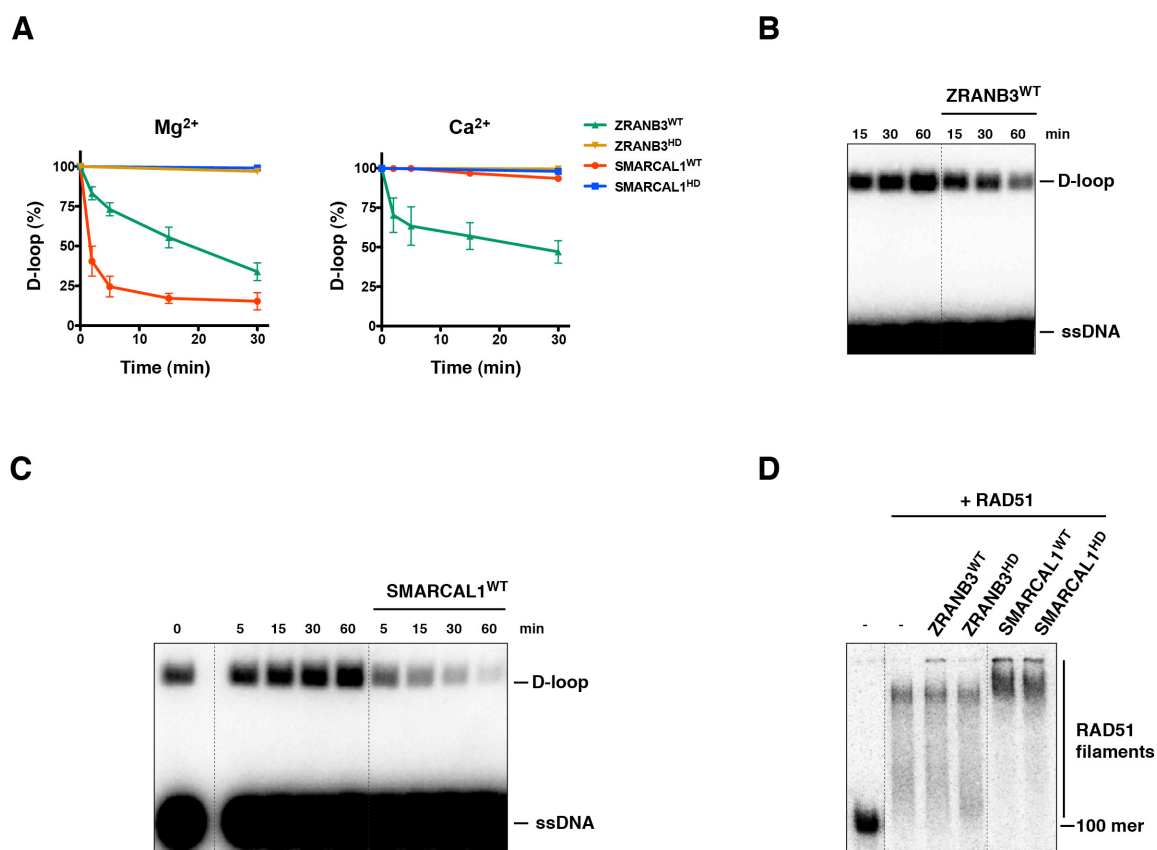


Figure S6 (related to Figure 7). In Vitro Activities of ZRANB3 and SMARCAL1 on D loop Structures and RAD51 Filaments

(A) Quantification of the dissociation of D loop structures by ZRANB3 and SMARCAL1 proteins in a time course reaction in either magnesium or calcium containing buffer as represented in Figure 7A-B. Points with error bars represent the average and standard deviation of three or more independent experiments.

(B) Disruption of preformed RAD51-containing D loop structures by ZRANB3 (100 nM) in a time course reaction as in Figure 7E.

(C) Disruption of preformed RAD51-containing D loop structures by SMARCAL1 (25 nM) in a time course reaction as in Figure 7E.

(D) Stability of RAD51-ssDNA nucleofilaments in the presence of WT or mutant ZRANB3 and SMARCAL1 proteins (100 nM).

Supplemental Experimental Procedures

DNA Clones

The Gateway entry vector pDONR201-ZRANB3 was purchased from Open Biosystems (clone # OHS5894-99169786). The Gateway entry vectors pDONR201-ZRANB3-PIP and pDONR201-ZRANB3-APIM were obtained from pDONR201-ZRANB3 by site directed mutagenesis of the amino acid sequence QHDIRSFF to AHDARSAA (amino acids 519-526 of ZRANB3) or by deletion of the last 6 amino acids of ZRANB3, respectively. The Gateway entry vector pDONR201-ZRANB3-PIP+APIM contains all the changes in the PIP box and APIM motifs described above. The Gateway entry vector pDONR201-ZRANB3-NZF was obtained from pDONR201-ZRANB3 by site directed mutagenesis of the amino acid sequence TY to LV (amino acid 631-632 of ZRANB3). The Gateway entry vector pDONR223-APIM was generated by PCR amplification of the region between amino acid 1049 and 1077 of ZRANB3. The Gateway entry vector pDONR223-APIM-mutant was generated by introducing the mutation F1073A in pDONR223-APIM. The Gateway entry vector pDONR223-NZF was obtained by PCR amplification of the region between amino acid 500 and 800 of ZRANB3. The Gateway entry vector pDONR223-NZF-mutant was generated from pDONR223-NZF by introducing the NZF mutation described above. The mammalian expression vectors pMSCV-GFP-ZRANB3, pMSCV-GFP-ZRANB3-PIP, pMSCV-GFP-ZRANB3-APIM, pMSCV-GFP-ZRANB3-PIP+APIM, pMSCV-FLAG-HA-ZRANB3, pMSCV-FLAG-HA-ZRANB3-PIP+APIM, pMSCV-FLAG-HA-ZRANB3-NZF were obtained by Gateway recombination between pDONR201-ZRANB3, pDONR201-ZRANB3-PIP, pDONR201-ZRANB3-APIM, pDONR201-ZRANB3-PIP+APIM, pDONR201-ZRANB3-NZF and pMSCV-GFP or pMSCV-FLAG-HA. pMSCV-FLAG-HA-ZRANB3, pMSCV-FLAG-HA-ZRANB3-PIP+APIM and pMSCV-FLAG-HA-ZRANB3-NZF contain a doxycycline inducible promoter. The mammalian expression vectors pMSCV-GFP-APIM and pMSCV-GFP-APIM-mutant were obtained by Gateway recombination between pDONR223-APIM, pDONR223-APIM-mutant and pMSCV-GFP. The Gateway entry vector

pDONR223-SMARCAL1 and the expression vector pMSCV-GFP-SMARCAL1 have been previously described (Ciccia et al., 2009). The bacterial expression vectors pET60-NZF and pET60-NZF-mutant were obtained by Gateway recombination between pDONR223-NZF, pDONR223-NZF-mutant and pET60-DEST (Novagen). The baculoviral vectors pFASTBAC-FLAG-ZRANB3 and pFASTBAC-FLAG-SMARCAL1 were generated by cloning ZRANB3 or SMARCAL1, which were PCR amplified with a forward primer containing the FLAG tag sequence, into the EcoRI and XhoI sites of pFAST-BAC1 (Invitrogen). The baculoviral vectors pFASTBAC-FLAG-ZRANB3-PIP+APIM and pFASTBAC-FLAG-ZRANB3-NZF were generated by site directed mutagenesis of pFASTBAC-FLAG-ZRANB3 by introducing the mutations in the PIP box and APIM motif or in the NZF motif described above. The baculoviral vectors pFASTBAC-FLAG-ZRANB3-HD and pFASTBAC-FLAG-SMARCAL1-HD were generated from pFASTBAC-FLAG-ZRANB3 or pFASTBAC-FLAG-SMARCAL1 by site directed mutagenesis of the amino acid sequence DE to AA (amino acids 157-158 or 549-550 of ZRANB3 or SMARCAL1, respectively).

Protein Purification

Sf9 insect cells (1×10^8 cells) were harvested 3 days after infection with baculoviruses expressing FLAG-ZRANB3, FLAG-ZRANB3-HD, FLAG-SMARCAL1 and FLAG-SMARCAL1-HD. Cell pellets were resuspended in 2 ml of buffer A (50mM Tris pH 7.6, 0.01% NP40, 1 mM EDTA, 10% glycerol) supplemented with 150 mM KCl, Cytobuster (1:5 dilution, EMD4Bioscience), phosphatase inhibitors cocktail set I and II (Bd Pharmigen) and protease inhibitors (Roche). Cell lysates were gently rocked for 30 min at 4°C and then centrifuged at 14,000 rpm for 15 min. Cell pellets were then resuspended in 1 ml of buffer A supplemented with 500 mM KCl, sonicated (2 x 20 sec) and rocked for 1 h at 4°C. After centrifugation, the low and high salt extracts were mixed and the salt concentration was adjusted to 100 mM KCl. The cell lysate was loaded onto a 0.5 ml CM sepharose (Pharmacia) column, which was then washed with 3 x 10 ml of buffer A containing 100 mM KCl. Proteins were eluted in buffer A

supplemented with 500 mM KCl (6 x 0.5 ml elutions), the fractions containing the proteins of interest were combined and the salt concentration was adjusted to 200 mM KCl. FLAG-tagged proteins were then immunoprecipitated overnight using 20 μ l of EZview red anti-FLAG M2 beads (Sigma). Immunoprecipitated proteins were then washed 4 times with buffer A containing 200 mM KCl and then eluted with 3 x 50 μ l of buffer B (50 mM Tris pH 7.5, 100 mM KCl, 10% glycerol) supplemented 3X FLAG peptide (500 μ g/ml, Sigma) by gently rocking for 10 min at room temperature. Eluted proteins were stored at -80°C. Purification of PCNA and its in vitro poly-ubiquitination reaction were performed as previously described (Unk et al., 2008). RecA and human RAD51 were purified according to published protocols (Mirshad and Kowalczykowski, 2003; Sigurdsson et al., 2001).

In Vitro Pulldowns

E. coli BL21 (DE3) bacteria (100 ml) carrying pET60-NZF or pET60-NZF-mutant were grown at 30°C to OD₆₀₀ = 0.3 and induced for 5 h with 0.1 mM IPTG. Cell pellets were resuspended in 1.5 ml of GST buffer (50 mM Tris pH 7.5, 500 mM NaCl, 1% Triton, 1 mM DTT, 10% glycerol) supplemented with 10 mg/ml lysozyme, Bugbuster (10X, EMD4Bioscience) and protease inhibitors (Roche). Following sonication (2 x 30 sec), cell lysates were centrifuged at 14,000 rpm for 20 min. Supernatants were then incubated with 50 μ l of glutathione beads (GE Healthcare) for 2 h at 4°C and the beads were then washed 5 times with GST buffer. Glutathione beads bound to GST-NZF or GST-NZF-mutant (approx. 5 μ g) were incubated with mono-ubiquitin, K48- or K63-linked poly-ubiquitin chains (1 μ g, Boston Biochem) in 100 μ l of binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton, 10% glycerol). Following gentle rocking for 3 h at 4°C, beads were washed 4 times with binding buffer and the proteins bound to GST-NZF or GST-NZF-mutant were analyzed by gel electrophoresis and western blotting.

Sf9 insect cells (1 x 10⁸ cells) were harvested 3 days after infection with baculoviruses expressing FLAG-ZRANB3, FLAG-ZRANB3-PIP+APIM or FLAG-ZRANB3-NZF. Cell pellets were lysed and FLAG-tagged proteins were pulled

down with EZview red anti-FLAG M2 beads (Sigma) as described in the protein purification section. Anti-FLAG beads bound to FLAG-ZRANB3, FLAG-ZRANB3-PIP+APIM or FLAG-ZRANB3-NZF were incubated with purified unmodified PCNA (0.6 μ g) or with a mixture of unmodified PCNA (0.6 μ g) and poly-ubiquitinated PCNA (1.2 μ g) in 100 μ l of FLAG binding buffer (50 mM Tris pH 8, 400 mM NaCl, 10% glycerol, 0.1% triton). After 4 h of gentle rocking at 4°C, beads were washed 4 times with FLAG binding buffer and then proteins bound to FLAG-ZRANB3, FLAG-ZRANB3-PIP+APIM or FLAG-ZRANB3-NZF were analyzed by gel electrophoresis and western blotting.

Cell Culture

The human osteosarcoma cell line U2OS and human embryonic kidney fibroblast cell line 293T-Rex were maintained in McCoy's or DMEM medium, respectively, supplemented with 10% fetal bovine serum. Stable U2OS cell lines expressing GFP-ZRANB3, GFP-ZRANB3-PIP, GFP-ZRANB3-APIM, GFP-ZRANB3-PIP+APIM, GFP-APIM, GFP-APIM-mutant, HA-ZRANB3, HA-ZRANB3-PIP+APIM and HA-ZRANB3-NZF were obtained after puromycin selection of cells infected with retroviruses generated from the vectors pMSCV-GFP-ZRANB3, pMSCV-GFP-ZRANB3-PIP, pMSCV-GFP-ZRANB3-APIM, pMSCV-GFP-ZRANB3-PIP+APIM, pMSCV-GFP-APIM, pMSCV-GFP-APIM-mutant, pMSCV-FLAG-HA-ZRANB3, pMSCV-FLAG-HA-ZRANB3-PIP+APIM and pMSCV-FLAG-HA-ZRANB3-NZF. Stable 293T-Rex cells expressing FLAG-ZRANB3 were obtained after infection with retroviruses generated from the pMSCV-FLAG-HA-ZRANB3 vector.

Cell Competition Assay

Cell competition assay was performed essentially as previously described (Smogorzewska et al., 2007). In particular, parental U2OS cells or U2OS cells expressing FF or SMARCAL1 shRNAs, were transfected with control or ZRANB3 siRNAs. After 3 days, the siRNA transfected cells were mixed in a 1:1 ratio with GFP⁺ U2OS cells and plated in triplicate (10,000 cells/well of 6 well plate). The

following day, cells were treated with camptothecin (5 nM), hydroxyurea (2 mM) or cisplatin (0.5 μ M) for 16 h. After 7 days the ratio of uncolored to GFP⁺ U2OS cells was determined by flow cytometric analysis.

Immunofluorescence

U2OS cells expressing GFP-ZRANB3 or HA-ZRANB3 wild-type and mutant proteins were stained with rabbit polyclonal anti-PCNA (1:1000, Abcam, ab18197), anti-WRNIP1 (1:200, Genetex, GTX24731), mouse monoclonal anti-RAD18 (1:100, Novus Biologicals, H00056852-M01), anti-HA (Covance, HA.11), anti-ubiquitinated proteins (1:500, Millipore, clone FK2), anti- γ H2AX (1:500, Millipore, JBW301), and chicken anti-GFP (1:1000, Abcam, ab13970) antibodies. Microirradiation experiments were performed as previously described (Ciccia et al., 2009). For foci experiments, U2OS cells expressing GFP-ZRANB3 were treated with 2 mM hydroxyurea, 50 nM camptothecin, 5 μ M cisplatin, 300 nM mitomycin C for 12 h or UV irradiated (25 J/m²) and cells were fixed and stained 6-8 h after washout of the drug or irradiation. UV irradiated cells were treated with 2mM caffeine or with ATM inhibitor (10 μ M, Calbiochem, KU55933) and ATR inhibitor (2 μ M, VE-821) (Reaper et al., 2011) 8 h before and 2-6 h after irradiation. Cells treated with USP1 siRNA (50 nM) were fixed and stained 3 days after siRNA transfection. For RAD51 foci staining, cells were treated for 1 h with camptothecin (10 nM), fixed 6-12 h after washout of the drug and stained with mouse monoclonal anti-RAD51 antibody (Abcam, ab213).

Immunoprecipitation of Protein Complexes

Stable 293T-Rex (1 x 10⁷ cells) with inducible expression of FLAG-ZRANB3 or U2OS cells (5 x 10⁶ cells) stably expressing FLAG-ZRANB3 were transfected with control or USP1 siRNA (20 nM). After 72 h, 293T-Rex cells were treated for 24 h with 2 μ g/ml doxycycline to induce the expression of FLAG-ZRANB3. Caffeine (2 mM) was added 12 h before UV irradiation (30 J/m²) and cells were harvested 4 h after irradiation. Cells were then fixed in 10 ml of 0.25% or 1% (for 293T-Rex or U2OS cells, respectively) formaldehyde (diluted in PBS) for 10 min

at room temperature and the reaction was stopped by addition of glycine (0.125 M final concentration). After 2 washes of the cell pellet with PBS, cells were resuspended in 1 ml of TE buffer (10 mM Tris pH 8, 1 mM EDTA) with 1% SDS. Following centrifugation at 3000 rpm for 5 min, pellets were washed twice with 2 ml of TE buffer. Pellets were then resuspended in 1 ml of TE buffer containing 0.1% SDS and protease inhibitors and were sonicated with the Bioruptor Sonicator (10 x 30 sec). Lysates were then centrifuged at 14,000 rpm for 10 min and the supernatant was supplemented with 150 mM NaCl and 0.1 % NP-40. Protein complexes were then immunoprecipitated with EZview red anti-FLAG beads (5 μ l) overnight at 4°C. Anti-FLAG beads were then washed 5 times with wash buffer (10 mM Tris pH 8, 1 mM EDTA, 0.1% NP40, 150 mM NaCl) and protein crosslinks were reversed by incubating the beads in gel loading buffer at 95°C for 30 min. Proteins were then analyzed by gel electrophoresis and western blotting. For immunoprecipitation of PCNA complexes, cell lysates after sonication were diluted 1:10 in dilution buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% NP40) with protease inhibitors. Protein complexes were then immunoprecipitated with anti-PCNA antibody (Santa Cruz, PC10). Following 4 washes with dilution buffer, protein crosslinks were reversed and proteins analyzed as described above.

In Vitro Assays

Fork Regression Assay. Fork regression assays were performed in reaction buffer (20 mM Tris pH 7.5, 0.1 mg/ml BSA, 1 mM DTT, 10% glycerol, 5 mM ATP, 5 mM MgCl₂) supplemented with 100 mM or 50 mM NaCl in the presence of ZRANB3 or SMARCAL1 (50 nM), respectively, together with ³²P-labeled synthetic fork (0.5 nM) or plasmid based fork (5 nM) substrates. Description of the fork substrates and the experimental details of the assays have been previously provided (Blastyak et al., 2007).

Preparation of DNA for D loop Assays. A 100-mer oligodeoxynucleotide, complementary to nucleotides 2451-2550 of the minus strand of pUC19, was purchased from Sigma Genosys, and gel purified on a 12%

denaturing polyacrylamide gel. The 100-mer was 5'-³²P-labeled using T4 polynucleotide kinase and [γ -³²P]ATP, and purified using MicroSpin G-25 columns (GE Healthcare). Supercoiled pUC19 DNA was purified by non-alkaline lysis followed by cesium chloride density gradient centrifugation (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Concentrations of the 100-mer and pUC19 (2,686 bp) were determined using molar (nucleotide) extinction coefficients at 260 nm of 9.98×10^3 and $6.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. DNA concentrations are expressed in moles of nucleotides (nt) or molecules, as indicated.

Generation of 3-stranded DNA Structures (D loops). D loops were formed using RecA as follows: 5'-³²P 100-mer (0.9 μM nt; 9 nM molecules) was preincubated with RecA (0.3 μM) in a buffer containing 25 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT, 2.5 mM ATP γ S and 100 $\mu\text{g/ml}$ BSA for 5 min at at 37°C. The reaction was supplemented with pUC19 Form I DNA (48 μM . nt; 9 nM molecules) and further incubated for 5 min at 37°C. The reaction was deproteinized using the Qiagen PCR cleanup system followed by removal of excess un-annealed oligonucleotide by gel filtration through Chroma Spin + TE-1000 columns (BD Biosciences). To determine the yield of D loop formation, a fraction of the RecA catalyzed D loop reaction was analyzed by electrophoresis in 0.8% agarose gel (4.5 V/cm for ~1h). Following electrophoresis, the gels were dried on DE81 paper (Whatman), analyzed and quantified using a Molecular Dynamics Storm 860 PhosphorImager (GE); the yield of D loops was 35-40%. The DNA concentration was ~ 15 μM nt (~3 nM molecules).

Dissociation of D loops. Dissociation reactions with D loops (final concentration ~400 nM nts; 75 nM molecules) and ZRANB3 or SMARCAL1 (concentrations indicated in figure legends) were performed in a buffer containing 20 mM Tris acetate, 5 mM magnesium acetate (or calcium acetate), 1 mM DTT, 5 mM ATP and 100 $\mu\text{g/ml}$ BSA. Reactions were incubated at 37°C and terminated by the addition of termination buffer (final concentration: 2% SDS, 3 $\mu\text{g}/\mu\text{L}$ proteinase K, 50 mM EDTA, incubation time 20 min). Reaction products

were analyzed by electrophoresis in 0.8% agarose gel (4.5 V/cm for ~1h). Following electrophoresis, the gels were dried on DE81 paper (Whatman), analyzed, and quantified using a Molecular Dynamics Storm 860 PhosphorImager (GE). The amount of D loop in the reactions was calculated as percentage of total signal (D loop + 100 mer) relative the value obtained from reactions without proteins.

D loop Formation by Human RAD51 and Disruption Assay. D loops were formed as follows: nucleoprotein filaments were first formed by preincubating a 5'-³²P 100-mer (0.9 μM nt, 9 nM molecules) with RAD51 (0.3 μM) in a buffer containing 25 mM Tris-acetate (pH 7.5), 2.5 mM magnesium acetate, 2.5 mM calcium acetate, 1 mM ATP and 100 μg/ml BSA for 5 min at 37°C. Following filament formation, pUC19 supercoiled DNA (scDNA) (16 μM; 3 nM molecules) was added and the reactions were incubated for an addition 15 min at 37 °C. The reactions were stopped by the addition of termination buffer (final concentration: 2% SDS, 3 μg/μL proteinase K, 50 mM EDTA, incubation time 30 min). When indicated, ZRANB3 (WT or HD) or SMARCAL1 (WT or HD) were added either after filament formation or after formation of D loops and incubated at the indicated times. Reaction products were analyzed by electrophoresis in 1% agarose gel (4.5 V/cm for ~1.5 h). Following electrophoresis, the gels were dried on DE81 paper (Whatman) and exposed to a StoragePhosphor screen. The reaction products were visualized and quantified with a Molecular Dynamics Storm 860 using ImageQuant version 5.2. Lines on the gels may indicate samples of the same experiment that were resolved separately. The amount of D loop in the reactions was calculated as percentage of total signal (D loop + 100 mer) relative the value obtained from reactions containing RAD51 alone.

Disruption of RAD51-ssDNA Nucleoprotein Filaments. RAD51-ssDNA filaments were formed by incubating RAD51 (0.15 μM) and 100-mer ssDNA (0.45 μM) for 15 min in the same buffer used for D loop assays. When indicated, ZRANB3 or SMARCAL1 proteins (100 nM) were included in the reaction. The reaction products were analyzed by 6% PAGE in Tris-Borate-EDTA buffer (12.5 V/cm, 2 h) at 4°C. Following electrophoresis, the gels were dried on DE81 paper

(Whatman) and exposed to a StoragePhosphor screen. The reaction products were visualized and quantified with a Molecular Dynamics Storm 860 using ImageQuant version 5.2.

Supplemental References

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