Science
AAAS

Supplementary Materials for

RHINO directs MMEJ to repair DNA breaks in mitosis

Alessandra Brambati[1#*], Olivia Sacco[1#], Sarina Porcella[1#], Joshua Heyza[2,3], Mike Kareh[1], Jens C. Schmidt[2,3], and Agnel Sfeir[1*]

Corresponding author: Sfeira@mskcc.org and BrambatA@mskcc.org

The PDF file includes:

Other Supplementary Materials for this manuscript include the following:

Materials and Methods

Cell culture

UO2S and HEK293T cells were purchased from ATCC and grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco/Thermo Fisher) supplemented with 10% fetal bovine serum (FBS, Gibco/Thermo Fisher). *TRF1/2$^{\Delta/\Delta}$Ku80$^{-/-}$p53$^{-/-}$* and *TRF1/2$^{\Delta/\Delta}$Ku80$^{+/+}$p53$^{-/-}$* MEFs were previously established (*37*). Parental and *BRCA2$^{-/-}$* DLD-1 cells were purchased from ATCC (CCL-221) and Horizon, respectively (HD105-007) and maintained in RPMI medium (Gibco/Thermo Fisher) supplemented with 10% FBS. *MDC1$^{-/-}$* U2OS cells were previously established and validated (*69*). Cells expressing Halo-Polθ were grown in RPMI medium supplemented with 10% FBS. All cells were supplemented with 0.1 mM MEM non-essential amino acids (Gibco/Thermo Fisher), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Pen/Strep; Gibco/Thermo Fisher) and grown at 37 °C and 5% CO_2. To synchronize in G1, cells were treated for 18-24h with 2 mM of thymidine (MilliporeSigma, T1895-5G) followed by 16h of incubation with 9 μM of RO-3306 (SelleckChem, catalog no. S7747) to synchronize at the G2/M phase. To allow cells to enter and accumulate in mitosis, cells were released from G2/M blockade in 100 ng/ml of Nocodazole (MilliporeSigma, M1404). In experiments with PARP inhibitors, we used Olaparib at 1 μM or 10 μM as indicated in figure legend for DLD1 and U2OS (Selleckchem, S1060). For Zeocin-treated samples, cells were treated for one hour with 20 μg/mL (for imaging Polθ foci) or 100 μg/mL Zeocin (for live-cell single-molecule imaging in interphase) for 1 hour (ThermoFisher R25005). For experiments combining Zeocin and ATR inhibitor treatment, cells were simultaneously treated with 20 µg/mL Zeocin ± 1 μM VE-821 (Sigma, SML1415) for 1 hour before imaging. Cells were irradiated with X-RAD225 - Precision X-Ray – (Accela). AsiSI expression was induced with 2 µM 4-OHT (Sigma-Aldrich) and 3 µM Shield-1 (Takara Bio) for the indicated lengths of time. For the kinase inhibitor experiment in HEK293T, the following chemicals were used: 5 µM of VE-821 (ATR inhibitor; Sigma, SML1415); 10 µM of KU-55933 (ATM inhibitor; Tocris, catalog no. 3544); 10 µM of RO-3306 (CDK1 inhibitor; SelleckChem, catalog no. S7747); 0.1 µM of BI-2536 (PLK1 inhibitor; SelleckChem, catalog no. S1109); 1 µM of MLN8237 (Alisertib, AURK inhibitor; SelleckChem, catalog no. S1133). Treatments lasted 24 hours before harvesting lysate and performing Co-IP.

Plasmids

The sgRNAs used to knock-out *LIG4* were cloned in the pSpCas9(BB)-2A-GFP (pX458) (Addgene #48138). The sgRNA used for IncucyteS3 experiments were cloned into a pLenti-gRNA-GFP-2A-PURO gift from Jordan Young from Repare Therapeutics. The lentiviral Cas9-BLAST was purchased from Addgene (#52962). The shRNAs that knocked down *9-1-1/RHNO1*,

ETAA1, and *ATRIP* in MEFs were cloned into the pLKO.1 vector from the RNAi consortium (Broad Institute). The 9-1-1 plasmids were a gift of Robert Weiss (pCMV-RAD1-HA, pCMV14-HUS1-3XFLAG, pCMV-RAD9A-MYC). The HA-CDC20 and HA-CDH1 plasmids were purchased from Addgene (#11594 and #11596, respectively). A lentiviral RHINO-MYC-FLAG plasmid (pCMV6-RHNO1-Myc-FLAG) was purchased from Origene (RC203020). Full-length human *POLQ* cloned into pLPC-Flag vector was previously described (*1*). Potential regulatory motifs within RHINO's sequence were found using The Eukaryotic Linear Motif resource (http://elm.eu.org). To generate RHINOΔDK, residues 125-132 (D-box) and residues 174-178 (Ken-box) within RHINO in the pCMV6-RHNO1-Myc-FLAG were deleted using New England BioLabs Q5® Site-Directed Mutagenesis Kit (NEB #E0554). To generate PLK1-7A, PLK1-A-G, CDK1-7A, and ΔTOPBP1, gene blocks were ordered through Integrated DNA Technologies (IDT). For PLK1-7A, Ser51, Thr52, Ser141, Ser162, Ser163, Ser164, Ser178 were mutated to Alanine. For each PLK1-A through G mutants, gene blocks were ordered through IDT with mutations as follows: S51A (A), T52A (B), S141A (C), S162A (D), S163A (E), S164A (F), S178A (G). The gene block for CDK1-7a contained the mutations S58A, S98A, S133A, T159A, T198A, S191A, and T202A. RHINO ΔTOPBP1 contained a single mutation: T202A. The original pCMV6-RHNO1-Myc-FLAG backbone was digested via restriction enzymes to remove the WT RHINO sequence. Gene blocks were PCR amplified using primers with overhangs containing restriction enzyme sites complementary to the digested backbone. PCR-amplified and digested gene blocks were cloned into the digested pCMV6-Myc-FLAG backbone, and cloned products were confirmed via restriction enzyme digest, Sanger sequencing, and functionally validated via western. Cell lines overexpressing these RHINO mutants and WT were made via lentiviral transduction into WT DLD1 cells. Cells were selected using 3 µg/mL puromycin and maintained in media containing 1.5 µg/mL. To tag endogenous *POLQ* at the N-terminus with a 3xFLAG-LoxP-SV40-Puro-Lox-HaloTag (Addgene #86843), ~ 5 x 10^5 U2OS cells were transfected using FuGene6 (Promega) with 1 µg of gRNA/Cas9 (px330) and HDR plasmid in 6 well plates. Edited cells were selected with 1 µg/mL of puromycin. Single-cell clones were sorted, and N-terminally edited cells were transfected with a plasmid encoding CRE to recombine the PuroR cassette generating a 3xFLAG-HaloTagged-*POLQ* protein. Cells were labeled with 250 nM JFX650-HaloTag ligand sorted based on the JFX650 signal (*70*). Homozygous clones were identified by genomic PCR using primers orientated outside each homology arm. To visualize RPA in live imaging experiments, ~ 6 x 10^5 U2OS cells were transfected with 1.8 µg of a mNEON-RPA32 plasmid. To generate endogenously tagged RHINO, we employed CRISPR/Cas9-mediated gene targeting using a guide targeting its C-terminus. Cas9 RNP and a ssDNA donor containing a mutated PAM site, linker, and 3xFLAG sequence were nucleofected into 1 x 10^6 DLD1 cells. 72 hours later, cells were harvested, and

genomic DNA was subjected to PCR using primers outside of the homology arms to assess donor incorporation efficiency and cutting efficiency via TIDE. Cells were plated at clonal density, and single clones were picked, subjected to PCR flanking the edited regions. Clones with PCRs showing homozygous alleles at the expected edited size were chosen for further evaluation, including PCR, Sanger sequencing, and western blot analysis. A list of primers used to tag RHINO or POLQ is provided in Supplementary Table 2-5. To generate the DLD1 AsiSI-expressing cell line, cells were infected with a lentiviral construct, a gift from Roger Greenberg.

List of antibodies

Primary antibodies used for Western blot included FLAG (Clone M2, Sigma; 1: 10000 dilution), MYC (9B11; Cell Signaling; 1: 1000 dilution), LIG4 (HPA001334; Sigma; 1: 500 dilution), p53 (sc126; Santa Cruz; 1: 1000 dilution), RAD1 (NB100-346; Novus Biologicals; 1: 1000 dilution), RAD9 (611324, BD Transduction Laboratories, 1: 500 dilution), HUS1 (D4J9H, Cell Signaling Technology; 1: 1000 dilution), Polθ (gift from Repare Therapeutics; 1: 1000 dilution), phospho-H2AX (S139, 9718 S; Cell Signaling Technology; 1: 1000 dilution), phospho-H3 (S10, 9718 S; Cell Signaling Technology; 1: 10000 dilution), phospho-Chk1 (Ser345, 2348 S; Cell Signaling Technology; 1: 1000 dilution), phospho-RPA32 (S4/S8, A300-245A, Bethyl, 1: 1000 dilution) γ-tubulin (GTU-88; Sigma Aldrich; 1: 5000 dilution), GAPDH (0411, Santa Cruz; 1: 10000 dilution), LaminB1 (ab16048; Abcam; 1: 10000 dilution), A custom RHINO polyclonal antibody was outsourced from GenScript using the recombinant protein described in the “Protein purification section” with 6xHIS tag retained and produced in rabbit (GenScript; 1:1000 dilution). The secondary antibodies were mouse IgG HRP-linked (NA931, GE Healthcare; 1:5000) or rabbit IgG HRP-linked (NA934, GE Healthcare; 1:5000).

Antibodies used for immunofluorescence included phospho-γH2AX (S139, 05-636 l; Millipore; 1: 5000 dilution), MYC (9B11; Cell Signaling; 1: 500 dilution), 53BP1 (100-304, Novus Biologicals; 1: 1000 dilution) and FLAG (14793; Cell Signaling; 1: 5000 dilution). Secondary antibodies were conjugated with Alexa Fluor 488 (Life Technologies), Alexa Fluor 568 (Life Technologies), or Cy5 (Life Technologies) and diluted 1:500.

siRNA transfection

For DLD1 experiments, 125,000 cells were reverse transfected with 30 pmol of a pool of siRNAs (Dharmacon, SMARTpool, L-003462-00-0005) targeting *BRCA2* or a pool of scrambled sequences (siNT, Dharmacon, SMARTpool, D-001810-10-05 or esiBRCA2 from Sigma, EHU031451) with RNAimax kit (Invitrogen), according to the manufacturer’s instructions. Halo-Polθ U2OS cells were nucleofected for live-cell mitotic imaging using a Lonza nucleofector with

the indicated siRNAs targeting *BRCA2* and *RHNO1* using ~200 ng siRNA/100,000 cells. To knock down *RHNO1*, U2OS cells were nucleofected with two separate siRNA (hs.Ri.RHNO1.13.1 and hs.Ri.RHNO1.13.2 from IDT).

shRNA interference

shRNAs were cloned into a pLKO.1-Puro backbone as AgeI–EcoRI dsDNA oligomers (IDT) and introduced by four lentiviral transduction at 4 h intervals in the presence of 8 µg/ml polybrene (MilliporeSigma) using supernatant from transfected HEK293T cells. MEFs cells were selected with 3 µg/ml puromycin for 3 days and recovered for an additional day before evaluating the silencing efficiency. As a control, MEFs cells were infected with a pLKO.1-Puro plasmid encoding a scrambled shRNA sequence (Addgene plasmid #1864). A list of the shRNA sequences is provided in Supplementary Table 2-2.

Lentiviral transduction

Lentiviral particles were produced in HEK293T cells in 10 cm plates by co-transfection of packaging vectors VSV-G, pMDLg/RRE, and pRSV-REV along with 10 µg of the desired plasmid using polyethylenimine (PEI). Virus-containing supernatant was collected ~48-72 h post-transfection, cleared through a 0.2-µm filter, supplemented with 8 µg/mL polybrene, and used for transduction. The medium was refreshed 6 to 12 h later.

RT–qPCR

Total RNA was purified with the NucleoSpin RNA Clean-up (Macherey-Nagel) following the manufacturer's instructions. Genomic DNA was eliminated by on-column digestion with DNase I. A total of 1 µg of RNA was reverse transcribed using iScript Reverse Transcription Supermix (Biorad), and cDNA was diluted 1:5 or 1:10. Reactions were run with ssoAdvanced SYBR Green Supermix (BioRad) with standard cycling conditions. Relative gene expression was normalized using *ACT1* or *GAPDH* as a housekeeping gene, and all calculations were performed using the ΔΔCt method. A list of the primers sequences is provided in Supplementary Table 2-3.

TLR assay

Lentiviral constructs coding for TLR (#31482) and I-SceI with donor e-GFP (#31476) were purchased from Addgene. To avoid the confounding effect of NHEJ on the repair of I-SceI-induced DNA breaks, we stably integrated the TLR construct into *LIG4*$^{-/-}$ HEK293T. Cells were transduced with Cas9-RNP with sgRNAs against *TP53* and *9-1-1/RHNO1*, followed by virus particles of the

I-SceI with donor e-GFP. Cells were collected 72 h later and analyzed on a BD LSRFortessa™ Cell Analyzer (BD Biosciences). Data were analyzed using FloJo software v.10 (TreeStar).

CRISPR/Cas9-mediated gene knockout

To generate *LIG4* knockout, 10^6 cells were transfected with 2.5 µg of two plasmids containing Cas9 and the gRNA against exon4 in LIG4. Single clones were genotyped by PCR with primers amplifying a 600-bp sequence around the cutting sites to ascertain the presence of insertions or deletions. Clones with possible homozygote status were amplified and subjected to confirmation by western blotting. *TP53*$^{-/-}$ cells were generated by delivering the ribonucleoprotein complex of a single gRNA targeting exon 4 and Cas9 protein (IDT). Cells that survived a 7-day treatment of 10 µM Nutlin-3 were used for the Western blot for p53 expression. *RHINO1*$^{-/-}$ cells were generated by delivering the ribonucleoprotein complex of a single gRNA targeting exon 3 and Cas9 protein (IDT). PCR genotyped single clones with primers amplifying a 600-bp around the cutting sites to ascertain the presence of insertions or deletions. A list of the gRNA sequences is provided in Supplementary Table 2-1 and the primers used for TIDE (*74*) in Supplementary Table 2-4.

Genome-wide CRISPR/Cas9 screens

CRISPR screens were performed as described (*71*). Briefly the DLD1 cells were transduced with the lentiviral TKOv3 library at a low MOI (~0.2–0.3) and selected with 4 µg/ml of puromycin for 48 h post-transduction. This was considered the initial time point (day 0). Cells with different genetic backgrounds were grown for additional 14 doublings. Cell pellets were frozen at each time point for genomic DNA (gDNA) isolation. A library coverage of ≥500 cells per sgRNA was maintained at every step. gDNA from cell pellets was isolated using the Quick-DNA Midiprep Plus Kit (ZymoResearch, D4075) and genome-integrated sgRNA sequences were amplified by PCR using the Q5 Mastermix (New England Biolabs Next UltraII). i5 and i7 multiplexing barcodes were added in a second round of PCR, and final gel-purified products were sequenced on Illumina HiSeq2500 or NextSeq500 systems to determine sgRNA representation in each sample. BAGEL2 was used to identify essential genes (*72*).

Clonogenic and proliferation assays

500-1000 cells were seeded in 6-well plates for clonogenic assays. For IR sensitivity assays, cells were irradiated with 2 Gy, collected after 30 min, counted, and plated. After 10 days medium was removed, cells were rinsed with PBS and fixed with 4% paraformaldehyde for 10 minutes. Colonies were then rinsed with PBS and stained with 0.4% (w/v) crystal violet in 20% (v/v) methanol for 5 minutes. The stain was aspirated, and plates were rinsed in water three times and

air-dried. Colonies were counted with the Fiji ImageJ plugin “Colony Area”. Data were plotted as surviving fractions relative to untreated cells. For the clonogenic assay on mitotic cells, cells were synchronized as described in Fig. 4A, and after irradiation, mitotic cells were recovered by mitotic shake-off. For proliferation assay, an IncuCyte Live-Cell Analysis Imager (Essen/Sartorius) was employed to monitor confluency over time. Cell confluency was monitored every 24 h.

Micronuclei scoring

Cells were synchronized in mitosis by double thymidine block (two treatments of 18h with 2 mM of thymidine) followed by 16h of incubation with CDKi to synchronize at the G2/M phase. Cells were released in mitosis by washing out CDKi. After 30 minutes, most of the cells were in mitosis and subjected to 2 Gy irradiation. 24h after irradiation, cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS, and permeabilized with 0.5% Triton X-100 buffer for 10 minutes. Cells were washed with PBS 3 times for 5 min each, counterstained with DAPI for 1h, and coverslips were mounted on slides with anti-fade reagent (Prolong Gold, Invitrogen). Images were captured using a Nikon Eclipse 55i upright fluorescence microscope at 40× magnification and analyzed with Nikon software.

IF-FISH

IF-FISH was performed as previously described (*37*). Briefly, MEFs grown on coverslips were fixed with 4% paraformaldehyde for 10 min, washed with PBS, and treated with permeabilization buffer (0.5% Triton X-100, 20 mM HEPES pH 7.9, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) for 10 min. Cells were then incubated in blocking solution (1 mg/mL BSA, 3% goat serum, 0.1% Triton X-100, 1 mM EDTA in PBS) at room temperature for 30 min, followed by incubation with primary antibody diluted in blocking solution for 2 hours at room temperature. After washing with PBST (0.1% Tween 20 in PBS) 3 times for 5 minutes each, cells were incubated with Alexa Fluor labeled secondary antibody (Thermo Fisher Scientific) for 45 minutes at room temperature. After washing with PBST 3 times for 5 minutes each, cells were dehydrated with ethanol series (70%, 95%, then 100%) and hybridized with TAMRA-OO-(TTAGGG)3 PNA probe (Applied Biosystems) in formamide hybridization solution (70% formamide, 0.5% blocking reagent (Roche), 10 mM Tris-HCl, pH 7.2) at 80°C for 5 min. Cells were allowed to cool at room temperature for 2 hours, then washed 4 times for 10 minutes each with formamide washing solution (70% formamide, 10 mM Tris-HCl pH 7.2). Cells were washed with PBS 3 times for 5 min each, counterstained with DAPI, and coverslips were mounted on slides with anti-fade reagent (Prolong Gold, Invitrogen). Images were captured using a Nikon Eclipse 55i upright fluorescence microscope at 60–100× magnification and analyzed with Nikon software.

Immunofluorescence

For the indicated treatments, cells were plated on 12-mm circular glass coverslips (Fisher Scientific), and immunofluorescence was performed using standard techniques. For POLθ and RHINO immunofluorescence cells were pretreated with cold CSK buffer (0.5% Triton, 1 mM EDTA pH 8, 10 mM PIPES pH 7, 100 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, 1 mM EGTA pH 8) for 5 min on ice before fixation. In brief, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were washed with PBS, permeabilized with 0.5% (v/v) Triton X-100 for 10 min and blocked for 30 min with a blocking solution (1 mg/mL BSA, 3% goat serum, 0.1% Triton X-100, 1 mM EDTA in PBS), 0.1% Triton X-100 and 1 mM EDTA. Cells were incubated with the same buffer containing primary antibodies for 1 hour at room temperature, followed by incubation with secondary antibodies overnight at 4C. DNA was counterstained with 5 µg/ml DAPI. Cells were mounted with ProLong Gold Antifade (Thermo Fisher Scientific), imaged on a Nikon Ti2 Eclipse upright fluorescence microscope at 40× magnification, and analyzed using Fiji ImageJ software. We employed a Nikon CSU-W1 Spinning Disk Confocal microscope with a 100x oil lens to image RHINO foci. Quantification of γ-H2AX in irradiation experiments was done using a script written in Fiji, briefly defining the ROI with the pH3S10 staining and quantifying the total integrated density of γ-H2AX staining (the sum of the values of the pixels in the image). The quantification of γ-H2AX foci in Olaparib-treated cells or RHINO foci in mitosis was done manually with blinded investigators.

Western blot analysis

Cells were collected by trypsinization and lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate). After two cycles of water-bath sonication at medium settings, lysates were incubated at 4°C on a rotator for an additional 30 min. Lysates were clarified by centrifugation for 30 min at 14,800 rpm at 4°C, and the supernatant was quantified using the enhanced BCA protocol (Thermo Fisher Scientific, Pierce). Equivalent amounts of proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk in TBST or 5% BSA in TBST in the case of phosphorylated proteins for at least one hour at room temperature. Incubation with primary antibodies was performed overnight at 4°C. Membranes were washed and incubated with HRP-conjugated secondary antibodies at 1:5000 dilution, developed with Clarity ECL (Biorad), and acquired with a ChemiDoc MP apparatus (Biorad) and ImageLab v.5.2. Antibodies against LaminB1, GAPDH, or γ-tubulin were used as loading controls. We prepared SDS-PAGE gel using

25 μM of Phosbind Acrylamide (APExBIO) to resolve phosphorylated protein on a Western blot and 100 μM of $MnCl_2$. To detect Halo-Polθ, cells were labeled with 150 nM JFX650-HaloTag ligand for 30 minutes, harvested, lysed in 2x Laemmli buffer containing β-mercaptoethanol, and loaded onto a 4-15% Mini-PROTEAN TGX Stain-Free polyacrylamide gel. Fluorescently labeled protein was detected on a BioRad Chemidoc using the Cy5.5 filter. Protein loading was detected using the Stain-Free filter on a BioRad Chemidoc after 45-second UV activation with 5-second exposure.

Protein expression and purification

To test whether RHINO and Polθ directly interact, we outsourced purified recombinant human RHINO protein from GenScript. RHNO1 cDNA with N-terminal 6xHIS tag and TEV cleavage sequence was cloned into a pET30a vector and expressed in *E. coli* Artic Express (DE3). The cell lysate supernatant was isolated by ultracentrifugation and then applied to a Ni(II) chelate column. The target protein was further purified using a Superdex 200 column. The resulting purified protein was subjected to TEV protease cleavage to remove the 6xHIS tag and cleared via Ni(II) chelate column for *in vitro* assays. Purified Polθ was outsourced from Repare Therapeutics and purified as previously described (*31*). Purified 9-1-1 was kindly provided by the laboratory of Dr. Dirk Remus and purified as previously described (*73*).

In vitro immunoprecipitation

Dynabeads Protein A was resuspended in a solution of 5 μg RHINO antibody:200μl PBS 0.1% tween (PBST) per 50 μl of beads and incubated with rotation at room temperature for 1 hour. The supernatant was removed, and conjugated beads were washed with PBST with gentle pipetting. Beads were resuspended with PBST to make a 50% slurry. Before immunoprecipitation, 10 μl of packed beads were washed once with 1X Master Mix (25 mM HEPES KOH pH 7.6, 150 mM NaCl, 0.02% NP40, 1 mM EDTA, 5% glycerol, 0.1 mg/mL BSA, 1 mM DTT). 1 μM purified RHINO, 1 μM purified 9-1-1, and 0.311 μM purified Polθ were used for *in vitro* immunoprecipitation experiments. 1X Master Mix was added to the conjugated beads and the appropriate amount of purified protein to a total volume of 100 μl. The immunoprecipitation reaction was carried out at 37C for 30 minutes with gentle agitation. The supernatant was cleared, and the beads were washed twice with 1X Master Mix. Immunoprecipitated proteins were eluted from the beads using 50 mM glycine pH 2.8 for 20 minutes at room temperature with gentle agitation and immediately neutralized with Tris pH 8.0. Laemmli buffer was added to 25% of the eluant, boiled at 95C for 5 minutes, loaded into SDS-PAGE gels, and subjected to western blot.

Coomassie stain

Purified Polθ, 9-1-1, and RHINO proteins were diluted to the concentrations reflecting those in the in vitro immunoprecipitation assay and boiled with Laemmli buffer for 5 minutes at 95C. Proteins were loaded in a 4-20% gradient SDS-PAGE gel and subjected to Coomassie staining. The SDS-PAGE gel was fixed for 1 hour in 50% ethanol:10% acetic acid solution and then washed overnight in 50% methanol:10% acetic acid. After, the gel was stained in Coomassie stain (0.1% w/v Coomassie blue R250, 50% methanol:10% acetic acid) for 4 hours at room temperature. The gel was washed until the stain was removed using 50% methanol:10% acetic acid. Then, the gel was incubated with a 5% acetic acid solution for 1 hour before imaging. All were performed at room temperature with gentle agitation.

Coimmunoprecipitation

HEK293T cells were co-transfected with the indicated plasmid constructs using polyethyleneimine (PEI). 48 hr post-transfection cells were harvested in cold PBS, washed once with PBS, and lysed for 15 min on ice in 500 µL Lysis Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.05% SDS, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). Salt boost was performed by adding 25 µL 5M NaCl and incubating on ice for an additional 5 min. Then, 500 µL cold H_2O was added, and lysates were spun at max speed for 10 min to pellet debris. 60 µl of protein G Sepharose beads conjugated to M2 Flag antibody (Millipore Sigma Cat. M8823-5ML) was added to each sample. Samples were incubated for three hours, rotating at 4°C. Beads were washed 4 times in Wash Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), and proteins were eluted in 2X Laemmli buffer by boiling at 95°C for 5 min for Western blot.

Immunoprecipitation

DLD1 RHINO-MYC-FLAG cells were harvested by either trypsinization or mitotic shake-off, washed once with PBS, and resuspended in 1mL of IP lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.1% sodium deoxycholate, 5% glycerol, 1 mM EDTA), supplemented with 1X complete protease inhibitor cocktail, 1 mM DTT, and 375 U/mL Benzonase nuclease (Sigma, E1014). Cells were incubated on ice for 1 hour, and lysates were cleared by centrifugation at max speed at 4C for 15 min. 3 mg of cell extract was incubated with 60 µl/sample of Anti-FLAG® M2 Magnetic Beads (Millipore Sigma, M8823-1ML) overnight on a rotating wheel at 4C. Immunoglobulin-antigen complexes were washed three times for 15 min in cold TBS before elution (1X TE, 1% SDS).

Cell cycle analysis by FACS

0.5 × 10^6 cells were collected by trypsinization, washed with cold PBS, and fixed with 70% ice-cold ethanol at 4°C for at least 24h. After fixation, cells were washed once with PBS, and DNA was labeled with propidium iodide (50 µg/ml) in the presence of RNase A (0.2 mg/ml, Qiagen) and Triton X-100 (0.01%) for 30 minutes at room temperature. Data on DNA content were acquired on a BD LSRFortessa™ Cell Analyzer (BD Biosciences) using BD FACSDiva™ Software (BD Biosciences) and analyzed using FlowJo v.10 (TreeStar).

Live cell imaging microscopy and single-particle tracking (SPT)

Cells were plated onto glass-bottom 24-well plates for live-cell imaging experiments and imaged with an Olympus IX83 inverted microscope. Imaging was performed at 37C and 5% CO2 with the 100x objective and the 640 nm laser with highly inclined laminated optical sheet illumination (lightly angled 0.5). Thymidine block was performed using 2 mM Thymidine and 9 µM RO-3306 to synchronize cells at the G2/M boundary. Cells were treated with 1 µM Olaparib for ~16 hours or 20 µg/mL Zeocin (in the presence or absence of 1 µM ATR inhibitor (VE-821) for one hour. For mitotic imaging, cells were released in 100 ng/mL nocodazole media. Halo-Polθ cells were labeled with JFX650 at 150 nM for 30 – 60 minutes. After labeling, cells were washed three times with complete medium and allowed to rest for 10 minutes before adding full fresh medium.

Z-stack images were acquired at 150 nm intervals for live-cell mitotic and interphase Polθ imaging. Mitotic Polθ foci were counted by visual inspection in ImageJ. They were classified as foci if a particle remained static and could be visualized through at least four consecutive Z-frames. For live-cell single-molecule imaging of Polθ in interphase, cells were seeded onto glass-bottom 24-well plates on the day before imaging. The following day, cells were treated with 100 µg/mL Zeocin for one hour, labeled with 50 nM JFX650 and 1 µg/mL Hoechst for 10 minutes, washed three times with complete media, and allowed to rest for 10 minutes before adding fresh complete medium. Imaging was performed at 37°C and 5% CO_2 with a 60x objective and a 640 nm laser on an Olympus IX83 inverted microscope with highly inclined laminated optical sheet illumination (light angled at 2.22). Images were acquired at 202 frames per second for 3000 frames followed by an image of Hoechst which was used to generate a nuclear mask to separate cytoplasmic and nuclear Polθ particles. Single-particle tracking was performed in MATLAB 2019a as previously described (*69, 74*). Settings for SPT used in the analysis: Exposure Time: 4.95 ms; NA: 1.49; Pixel Size: 0.1083 µM; Emission Wavelength: 664 nm; *D*max = 5 µm2/s; Number of gaps allowed = 2; Localization Error = -5; Deflation Loops = 0. To separate cytoplasmic and nuclear Polθ tracks, the Hoechst image was used to generate a nuclear mask in Fiji. Nuclear

tracks from SPT were subsequently analyzed in SpotOn (*74*) using the following settings: Time Gap: 4.95 ms; dZ: 0.700 μm; Gaps Allowed = 2; Time Points = 8; Jumps to Consider = 4; Bin Width: 0.01 μm; PDF-fitting; D_free_2State = [0.5 25]; D_bound_2State = [0.0001 0.5]. At least 20 cells were imaged and analyzed for live-cell single-molecule imaging experiments.

Statistical analysis

All statistical analysis was performed with GraphPad Prism 9. Sample sizes and the statistical tests used are specified in the figure legends.

Figure S1

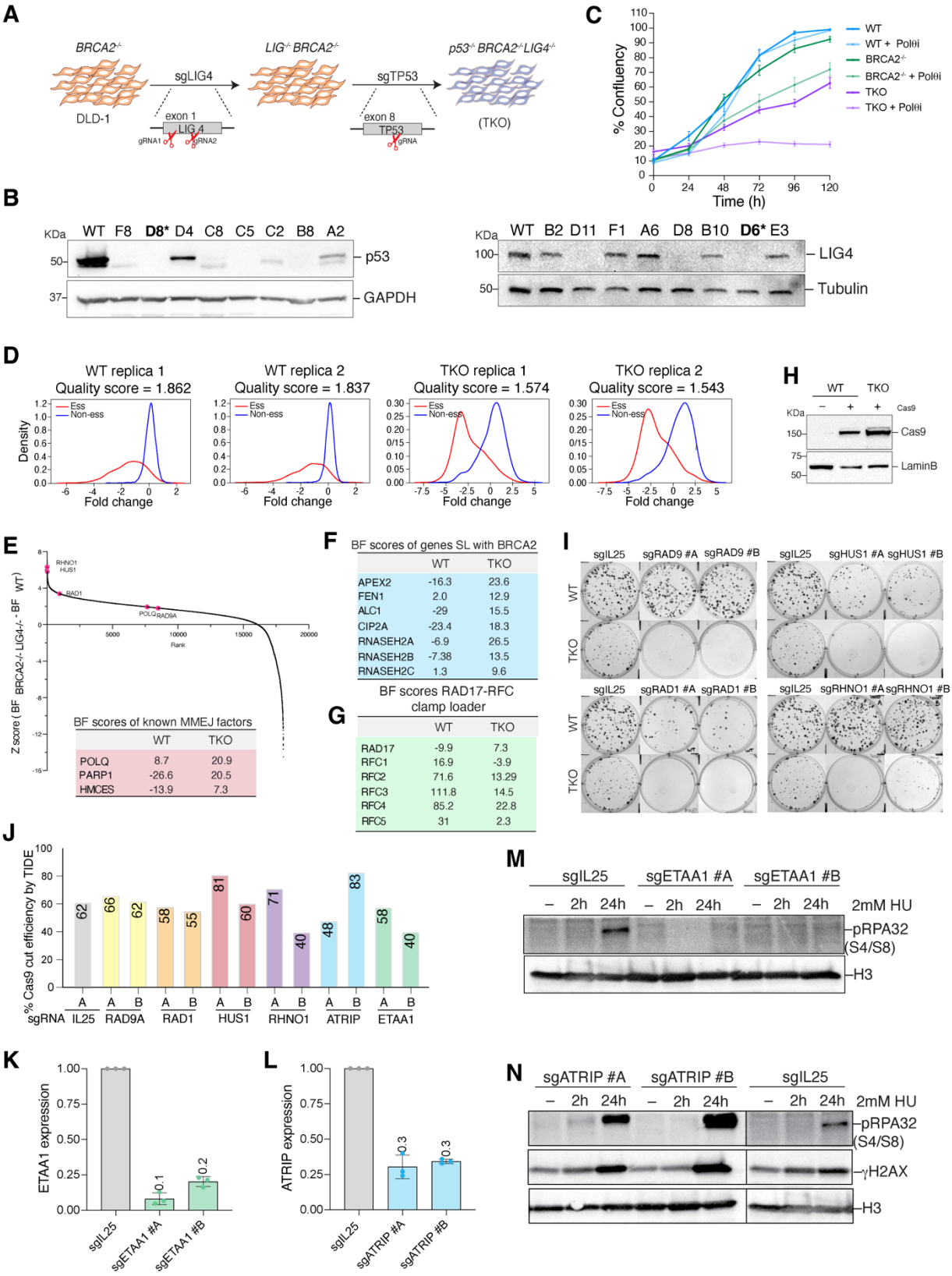

Fig. S1. The 9-1-1/RHINO complex is a novel MMEJ factor.

(**A**) Schematic of CRISPR/Cas9 strategy for sequential knock out of *TP53* and *LIG4* from DLD1 cells. (**B)** Western blot of cells transduced with sgRNA against *TP53* or *LIG4*. Lysates were probed with antibodies detecting p53 and GAPDH (loading control) or against Lig4 and Tubulin (loading control). (**C**) TKO cells were functionally validated by treating them with a small molecule inhibitor of Polθ, RP6685 (10uM) (*31*). (**D**) Kernel density plots that estimates reference core-essential (red) and non-essential (blue) genes in each CRISPR/Cas9 screen replicate (*71*, *72*). (**E**) Results from the CRISPR/Cas9 dropout screen in TKO cells as ranked z-scores of the difference in Bayes factor (BF) for essential genes in TKO *vs. TP53*$^{-/-}$. BF scores for known MMEJ genes are included below the graph. (**F**) BF scores for genes known to be essential for the survival of *BRCA2*$^{-/-}$ cells. SL = synthetic lethal. (**G**) BF scores for the RAD17-RFC clamp loader. (**H**) Western blot analysis showing Cas9 levels in WT and TKO cells. Lamin B1 antibody is used as a loading control. (**I**) Representative images of clonogenic survival of *TP53*$^{-/-}$ and TKO cells targeted with the indicated sgRNAs. (**J**) Editing efficiency of the indicated sgRNAs. Genomic DNA was isolated from WT cells expressing FLAG-Cas9 and the indicated sgRNAs. The region flanking the sgRNA cut site was amplified by PCR and sequenced. Results from the TIDE analysis (*75*) are shown. (**K-L**) qPCR analysis of *ETAA1* and *ATRIP* mRNA expression after targeting with sgRNAs in cells used for IncucyteS3 growth curve in Fig 1F. Relative gene expression was normalized to *ACT1*, a housekeeping gene. (**M-N**) Functional validation of *ETAA1* and *ATRIP* depletion. Cells expressing FLAG-Cas9 and the indicated sgRNAs were treated for 2 and 24 hours with 2 mM hydroxyurea (HU). Western blot analysis was performed with phospho-RPA (S4/S8) and γ-H2AX antibodies. H3 was used as a loading control.

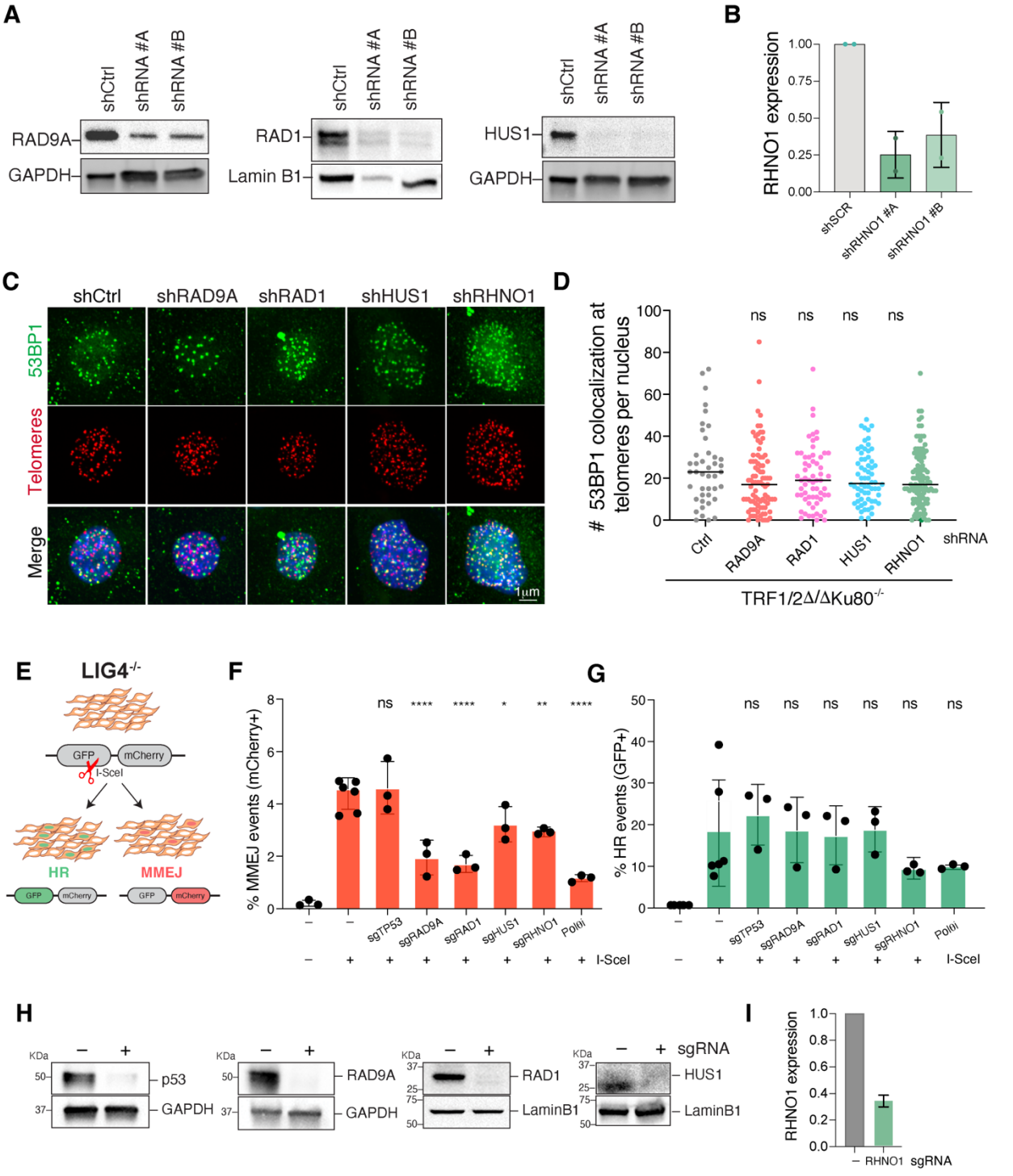

Fig. S2. Investigating the function of 9-1-1/RHINO in MMEJ.

(**A**) Western blot analysis of 9-1-1 complex members in *TRF1/2*$^{\Delta/\Delta}$*Ku80*$^{-/-}$ MEFs following shRNA knockdown of the indicates genes. GAPDH and Lamin B1 antibodies were used as loading

controls. (**B**) qPCR analysis of *RHNO1* mRNA expression after shRNA knockdown. Relative gene expression was normalized using *TBP* as a housekeeping gene. Data represent the mean of two independent experiments. (**C**) Representative images of telomere dysfunction-induced foci (TIFs) analyzed by IF-FISH in *TRF1/2$^{\Delta/\Delta}$Ku80$^{-/-}$* cells depleted for 9-1-1 subunits and *RHNO1*. Telomeres are stained with a FISH probe (red), and 53BP1 is stained using an antibody (green). DNA is stained with DAPI (blue). (**D**) Quantification of colocalization of 53BP1 with telomeres (TTAGGG) in *TRF1/2$^{\Delta/\Delta}$Ku80$^{-/-}$* cells upon shRNA knockdown of 9-1-1/*RHNO1* complex members. Each dot on the graph represents a single cell. The graph includes at least 40 nuclei from two independent experiments, with black lines indicating the mean. Statistical analysis was performed using one-way ANOVA. (**E**) Diagram illustrating the Traffic Light Reporter (TLR) in NHEJ-deficient cells (*LIG4$^{-/-}$*) (*39*). To limit repair to MMEJ and HR, we stably expressed the TLR reporter in *LIG4$^{-/-}$* cells where NHEJ is blocked. We first treated these cells with sgRNA targeting 9-1-1 and *RHNO1*, then introduced I-SceI to trigger break formation. The schematic depicts the potential repair outcomes after an I-SceI-induced DSB: HR-mediated repair (GFP+) and MMEJ-mediated repair (mCherry+). (**F-G**) Quantification of MMEJ (F) or HR (G) events assessed by TLR after 9-1-1 and *RHNO1* depletion via Cas9-RNP. Statistical analysis was performed for three separate experiments using one-way ANOVA (****p<0.0001, **p<0.01, *p<0.05). Depletion of subunits of the 9-1-1 complex and RHINO led to a significant reduction in the percentage of mCherry by TLR in cells, indicative of reduced MMEJ activity. (**H**) Western blot of 9-1-1 complex subunits in HEK293T following their knockdown. GAPDH and Lamin B1 antibodies served as loading controls. (**I**) qPCR analysis of *RHNO1* mRNA expression after knockdown in cells used for the TLR assay. Relative gene expression was normalized using *ACT1* as a housekeeping gene.

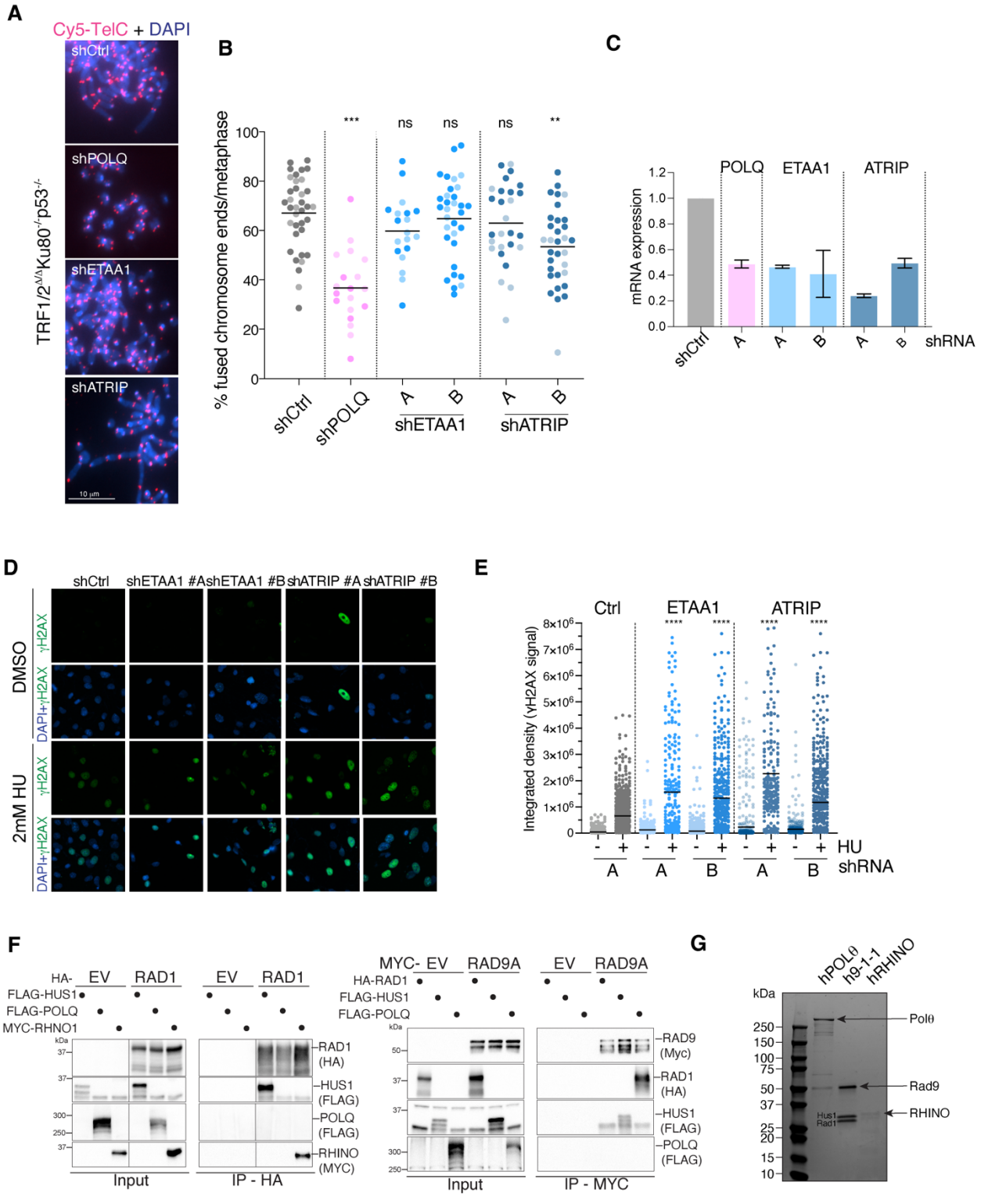

Fig. S3. 9-1-1/RHINO function in ATR signaling is insufficient to promote MMEJ.

(**A**) Representative images of metaphase spreads from *TRF1/2*$^{\Delta/\Delta}$*Ku80*$^{-/-}$ cells depleted for *ETAA1*, *ATRIP* and *POLQ*. Telomeres are labeled with a FISH probe (red), and chromosomes are counterstained with DAPI (blue). (**B**) Quantification of telomere fusions mediated by MMEJ as shown in panel A. Data represent the mean of two independent experiments, with different colors representing results from separate experiments. Statistical analysis was performed using one-way ANOVA compared to shCtrl (****p<0.0001, **p<0.01, *p<0.05). (**C**) qPCR analysis of *RHNO1* mRNA expression after knockdown with shRNA in *TRF1/2*$^{\Delta/\Delta}$ *Ku80*$^{-/-}$ cells used for the metaphase spreads. Relative gene expression was normalized using *ACT1* as a housekeeping gene. (**D**) Functional validation of *ETAA1* and *ATRIP* depletion in *TRF1/2*$^{\Delta/\Delta}$*Ku80*$^{-/-}$ cells. Cells with the indicated shRNAs were treated with 2 mM hydroxyurea (HU) for three hours and subjected to immunofluorescence analysis for γ-H2AX staining. Representative images from one of two independent experiments. (**E**) Quantification of γ-H2AX signal from two independent experiments. Data represent the mean of two independent experiments, and statistical analysis was performed using one-way ANOVA between the HU-treated samples (****p<0.0001, **p<0.01, *p<0.05). (**F**) Immunoprecipitation and western blot analysis of whole-cell extracts from HEK293T cells co-transfected with plasmids expressing FLAG-POLQ and subunits of 9-1-1 (MYC-RAD9, HA-RAD1, MYC-RHINO, and FLAG-HUS1). The indicated antibodies were used. **(G)** Representative Coomassie-stained SDS-PAGE analysis of purified human Polθ, 9-1-1 complex, and RHINO.

Figure S4

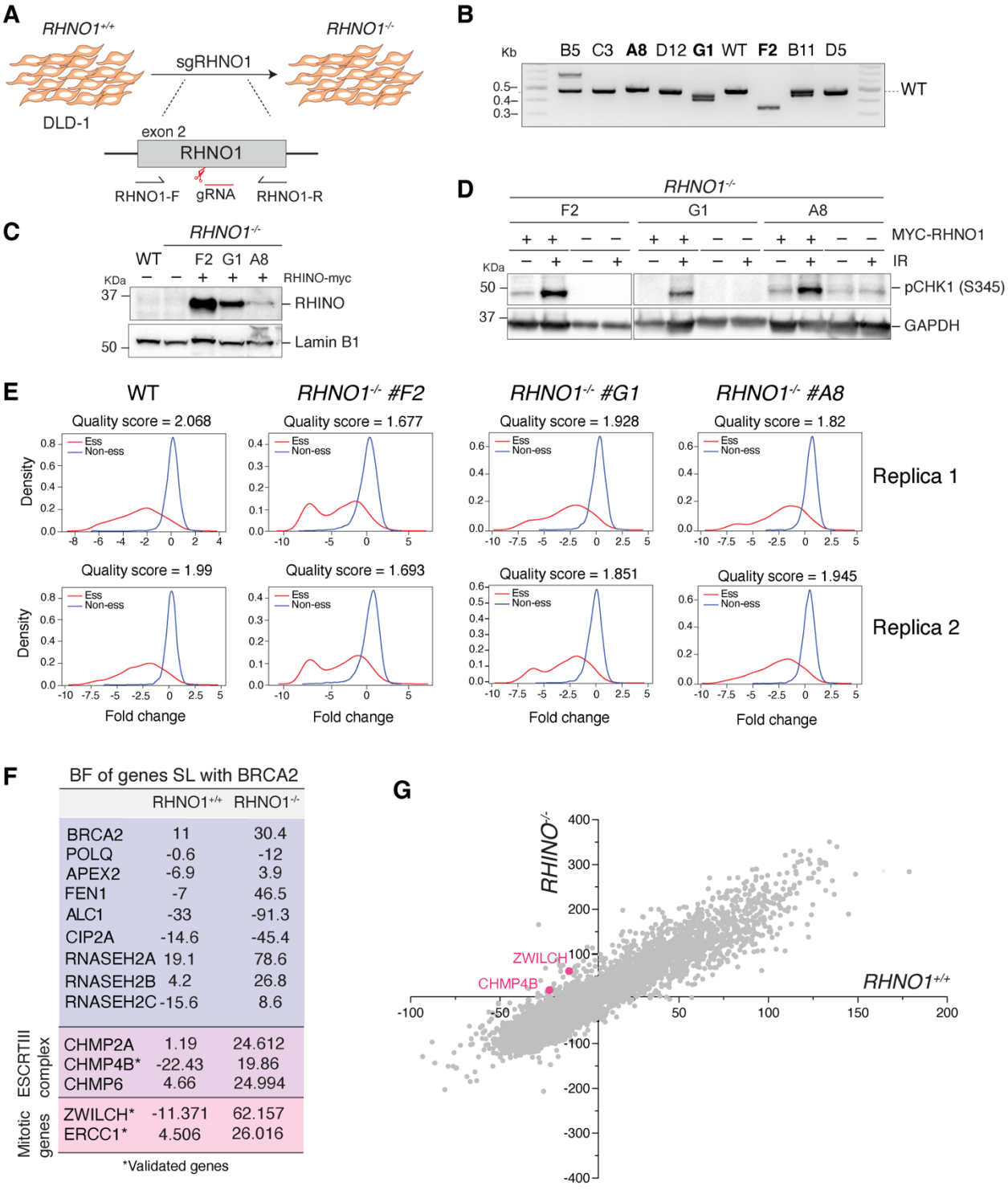
A
RHNO1+/+
RHNO1-/-
sgRHNO1
DLD-1
exon 2
RHNO1
RHNO1-F
gRNA
RHNO1-R
B
Kb
B5 C3 A8 D12 G1 WT F2 B11 D5
0.5
0.4
0.3
WT
C
RHNO1-/-
WT F2 G1 A8
KDa
RHINO-myc
37
RHINO
50
Lamin B1
D
RHNO1-/-
F2 G1 A8
MYC-RHNO1
IR
KDa
50
pCHK1 (S345)
37
GAPDH
E
WT
RHNO1-/- #F2
RHNO1-/- #G1
RHNO1-/- #A8
Quality score = 2.068
Quality score = 1.677
Quality score = 1.928
Quality score = 1.82
Quality score = 1.99
Quality score = 1.693
Quality score = 1.851
Quality score = 1.945
Ess
Non-ess
Density
Fold change
Replica 1
Replica 2
F
BF of genes SL with BRCA2
RHNO1+/+
RHNO1-/-
BRCA2 11 30.4
POLQ -0.6 -12
APEX2 -6.9 3.9
FEN1 -7 46.5
ALC1 -33 -91.3
CIP2A -14.6 -45.4
RNASEH2A 19.1 78.6
RNASEH2B 4.2 26.8
RNASEH2C -15.6 8.6
ESCRTIII complex
CHMP2A 1.19 24.612
CHMP4B* -22.43 19.86
CHMP6 4.66 24.994
Mitotic genes
ZWILCH* -11.371 62.157
ERCC1* 4.506 26.016
*Validated genes
G
RHINO-/-
RHNO1+/+
ZWILCH
CHMP4B

Fig. S4. RHINO is strictly expressed in mitosis.

(**A**) Schematic of the CRISPR/Cas9 strategy to knockout *RHNO1*. (**B**) Genotyping PCR on genomic DNA extracted from cells targeted with CRISPR/Cas9 to detect deletions in the *RHNO1* gene. Clones used in the screen are highlighted in bold (i.e. A8, G1, F2). (**C**) Western blot of *RHNO1*$^{-/-}$ clones complemented with RHINO-MYC-FLAG expressing vector. Lamin B1 is used as a loading control. (**D**) Functional validation of *RHNO1* deletion in three clones used in the CRISPR/Cas9 screen based on the accumulation of phospho-CHK1 following irradiation. Three *RHNO1*$^{-/-}$ clones were complemented with RHINO-MYC-FLAG expressing vector, subjected to western blot three hours post-irradiation (2 Gy), and monitored for phospho-CHK1. GAPDH antibody is used as a loading control. (**E**) Kernel density estimates of reference core-essential genes (red) and non-essential genes (blue) in each CRISPR/Ca9 screen replica. (**F**) BF scores for a selection of genes that exhibit synthetic lethality with *BRCA2*$^{-/-}$ cells or involved in mitotic pathways. (**G**) Result of the genome-wide CRISPR-Cas9 screen in *RHNO1*$^{-/-}$ and *RHNO1*$^{+/+}$ cells. Highlighted in purple are the mitotic genes validated with growth curves in Fig. S5A-B. Genes were considered essential above a threshold of 5. The x- and y-axes intersect at 5 to reflect this threshold.

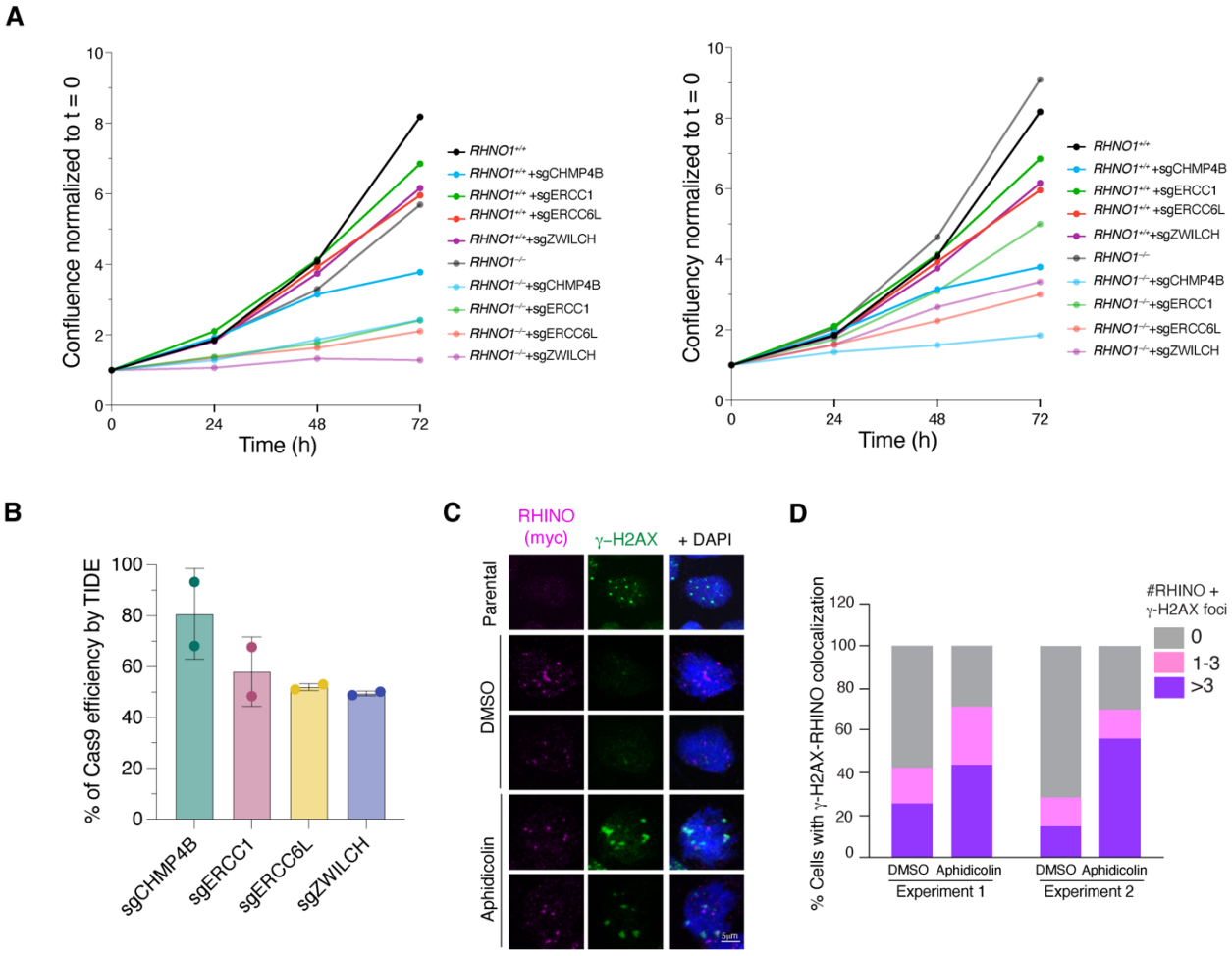

Fig. S5. Hits validation of RHINO knockout CRISPR-Cas9 synthetic lethal screen

(**A**) Growth curve of two *RHNO1*$^{-/-}$ clones and *RHNO1*$^{+/+}$ cells treated with sgRNA against the genes *ERCC6L*, *ERCC1*, *ZWILCH* and *CHMP4B*. Data are the mean of two independent experiments normalized to time point zero (one day after seeding). (**B**) Editing efficiency of the sgRNAs used in panel A. Genomic DNA was isolated from WT cells expressing FLAG-Cas9 and the indicated sgRNAs. The region flanking the sgRNA cut site was amplified by PCR and sequenced. Results from the TIDE analysis (*75*) are shown. (**C**) Representative immunofluorescence images of DLD1 cells stably overexpressing RHINO-MYC-FLAG synchronized in mitosis in the presence of low-dose Aphidicolin (400 nM) stained with anti-MYC and γ-H2AX antibodies. (**D**) Quantification of RHINO foci in mitosis and its colocalization with γ-H2AX foci as shown in panel C.

Figure S6

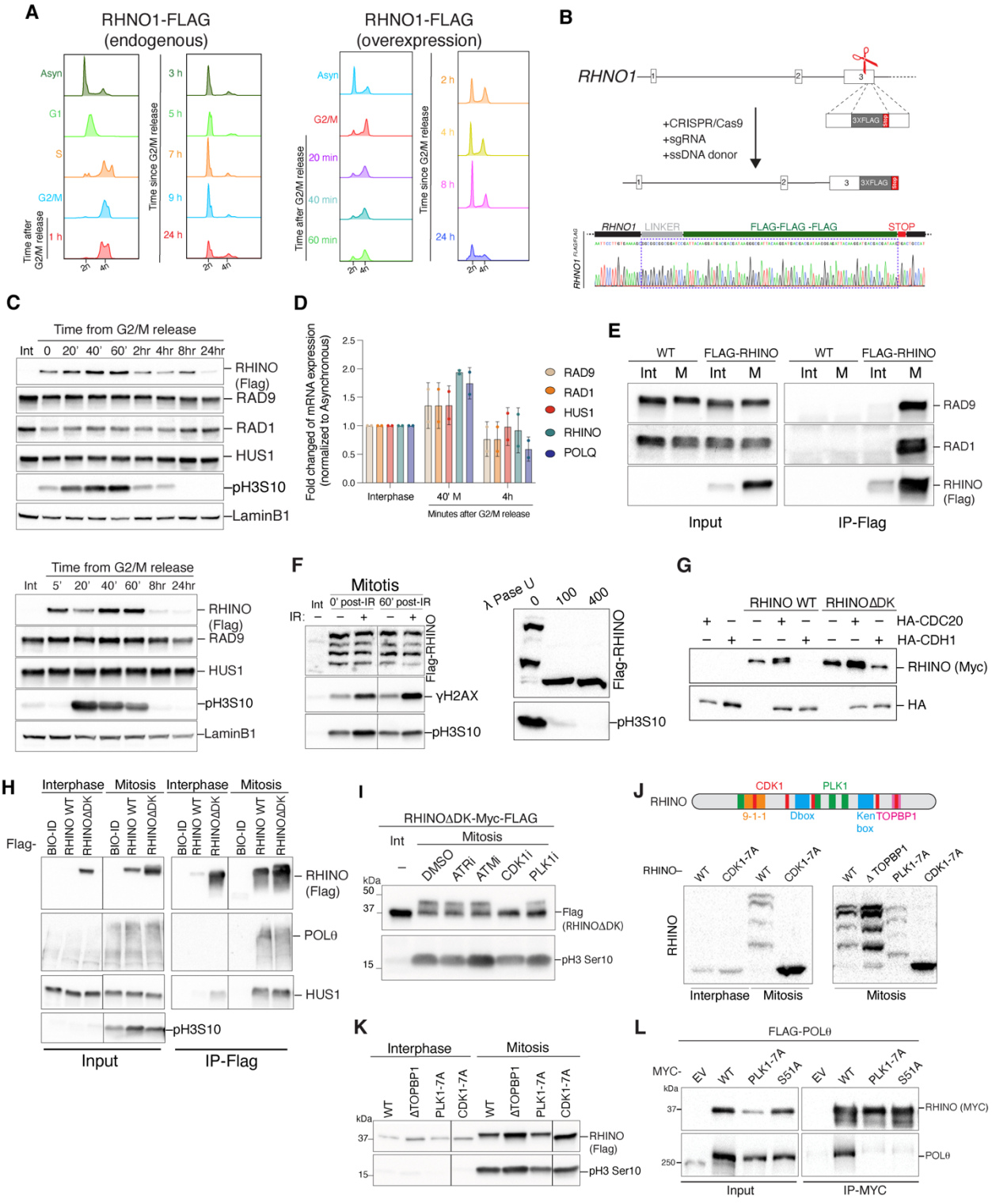
A
RHNO1-FLAG (endogenous)
RHNO1-FLAG (overexpression)
Asyn
G1
S
G2/M
Time after G2/M release
Time since G2/M release
B
RHNO1
+CRISPR/Cas9
+sgRNA
+ssDNA donor
3XFLAG
LINKER
FLAG-FLAG -FLAG
STOP
C
Time from G2/M release
Int 0 20' 40' 60' 2hr 4hr 8hr 24hr
RHINO (Flag)
RAD9
RAD1
HUS1
pH3S10
LaminB1
D
Fold changed of mRNA expression (normalized to Asynchronous)
Interphase
40' M
4h
Minutes after G2/M release
RAD9
RAD1
HUS1
RHINO
POLQ
E
WT
FLAG-RHINO
Int M
RAD9
RAD1
RHINO (Flag)
Input
IP-Flag
Time from G2/M release
Int 5' 20' 40' 60' 8hr 24hr
F
Mitotis
Int 0' post-IR 60' post-IR
IR:
Flag-RHINO
γH2AX
pH3S10
λ Pase U
0 100 400
G
RHINO WT
RHINOΔDK
HA-CDC20
HA-CDH1
RHINO (Myc)
HA
H
Interphase
Mitosis
Flag-
BIO-ID
RHINO WT
RHINOΔDK
RHINO (Flag)
POLθ
HUS1
pH3S10
Input
IP-Flag
I
RHINOΔDK-Myc-FLAG
Int
Mitosis
DMSO
ATRi
ATMi
CDK1i
PLK1i
kDa
Flag (RHINOΔDK)
pH3 Ser10
J
RHINO
CDK1
PLK1
9-1-1
Dbox
KenTOPBP1 box
RHINO–
WT
CDK1-7A
ΔTOPBP1
PLK1-7A
Interphase
Mitosis
K
Interphase
Mitosis
WT
ΔTOPBP1
PLK1-7A
CDK1-7A
RHINO (Flag)
pH3 Ser10
L
FLAG-POLθ
MYC-
EV
WT
PLK1-7A
S51A
RHINO (MYC)
POLθ
Input
IP-MYC

Fig. S6. RHINO is phosphorylated in mitosis by CDK1 and PLK1 kinases and degraded by the APC/CDH1 complex.

(A) Cell cycle analysis by FACS of the synchronization experiments with endogenously tagged RHINO shown in Fig. 3F and for the synchronization experiments with overexpressed RHINO showed in panel Fig. S6C. **(B)** CRISPR-Cas9 targeting strategy to tag RHINO with 3X-FLAG endogenously. **(C)** Western blot analysis of 9-1-1/RHINO expression during the cell cycle. Cells overexpressing RHINO-MYC-FLAG underwent a double thymidine block and were then released into CDK1 inhibitor (RO-3306, 9uM) for 16h to induce a G2/M arrest. Cells were collected at the indicated time points via trypsinization or mitotic shake-off (only 20', 40', 60'), lysed, and subjected to western blot to detect RHINO and endogenous components of the 9-1-1 complex. The phosphoantibody against serine 10 in H3 (pS10H3) was used as a mitotic marker. Lamin B1 was used as a loading control. **(D)** qPCR analysis of 9-1-1 complex members, *RHNO1*, and *POLQ* mRNA expression. DLD1 WT cells were synchronized at the G2/M boundary using CDK1 inhibitor and then released into mitosis. Cells were harvested after 40 minutes (mitotic shake-off) and 4 hours (trypsinization) post-CDK1 inhibitor release. Asynchronous cells were also harvested as a reference value. Relative gene expression was normalized using *ACT1* as a housekeeping gene. Fold change was calculated by normalizing to asynchronous values. Data are the mean of two independent experiments. **(E)** Results from immunoprecipitation of cells stably overexpressing RHINO-MYC-FLAG and WT DLD1 cells. Cells were harvested asynchronously (Int) or synchronized via double thymidine block, CDK1 inhibitors, and released into mitosis in the presence of nocodazole (M). Mitotic cells were harvested via mitotic shake-off. Both interphase and mitotic cells were lysed, and lysates were subjected to anti-FLAG immunoprecipitation. Western blot analysis depicts FLAG-RHINO as well as endogenous RAD9 and RAD1. **(F)** Left panel: Phosho-tag gel of cells overexpressing RHINO-MYC-FLAG in interphase (Int) and synchronized in mitosis and irradiated with 2 Gy. Cells were collected immediately after irradiation and after 60 minutes. γ-H2AX is used as a marker of DNA damage, and pS10H3 of mitosis. Right panel: Phosho-tag gel of cells expressing RHINO-MYC-FLAG synchronized in mitosis and treated with 100 or 400 units of lambda (λ) phosphatase. pS10H3 is used as a marker of mitosis as well as positive control for dephosphorylation. In both panels, mitotic cells were harvested via mitotic shake-off. **(G)** Co-expression of RHINO-MYC-FLAG and HA-CDC20 or HA-CDH1 in HEK293T. **(H)** Immunoprecipitation analysis in cells expressing RHINO-MYC-FLAG, RHINOΔDK-MYC-FLAG, and FLAG-BioID to assess for POLθ interaction. Cells were synchronized using a double thymidine block followed by CDK1 inhibition and released into nocodazole to enrich mitotic cells. Mitotic cells were harvested via mitotic shake-off. "Interphase" indicates cells that did not undergo

any synchronization. FLAG-BioID is used as a negative control. Cell lysates were subjected to anti-FLAG immunoprecipitation. **(I)** Assessment of kinases responsible for phosphorylation of RHINOΔDK. Cells overexpressing RHINOΔDK-MYC-FLAG were harvested in interphase (Int) or subjected to a single thymidine block, CDK1 inhibitors, and nocodazole (Mitosis). After 15 minutes in nocodazole, cells were supplemented with either DMSO, 5uM ATRi (VE821), 10uM ATMi (KU-55933), 10uM CDK1i (RO-3306), or 0.1uM PLK1i (BI-2536) in addition to nocodazole. Cells were incubated for another 40 minutes and then harvested via trypsinization (interphase samples) or mitotic shake-off (mitotic samples) for western blot analysis. **(J)** Top: schematic of RHINO phosphorylation motifs. Bottom left: phosho-tag gel of cells overexpressing RHINO-MYC-FLAG (WT) or RHINO-CDK1(S/T)7A synchronized in mitosis or not synchronized (Interphase). Right panel: phosho-tag gel of cells expressing RHINO-WT, -ΔTOPBP1, -CDK1(S/T)7A or -PLK1(S/T)7A mutants and synchronized in mitosis. PLK1(S/T)7A = PLK1-7A, CDK1(S/T)7A = CDK1-7A. For both panels, mitotic cells were harvested via mitotic shake-off. **(K)** Cells overexpressing MYC-FLAG tagged RHINO-WT, -ΔTOPBP1, -PLK1(S/T)7A, or -CDK1(S/T)7A were harvested either asynchronously (Interphase) or after double thymidine block, CDK1 inhibitors, and nocodazole (Mitosis). Mitotic cells were harvested via mitotic shake-off. Cells were lysed and subjected to western blot analysis. **(L)** Co-IP experiments in HEK293T cells co-transfected with plasmids expressing FLAG-Polθ and the RHINO-MYC mutants (PLK1(S/T)7A and S51A).

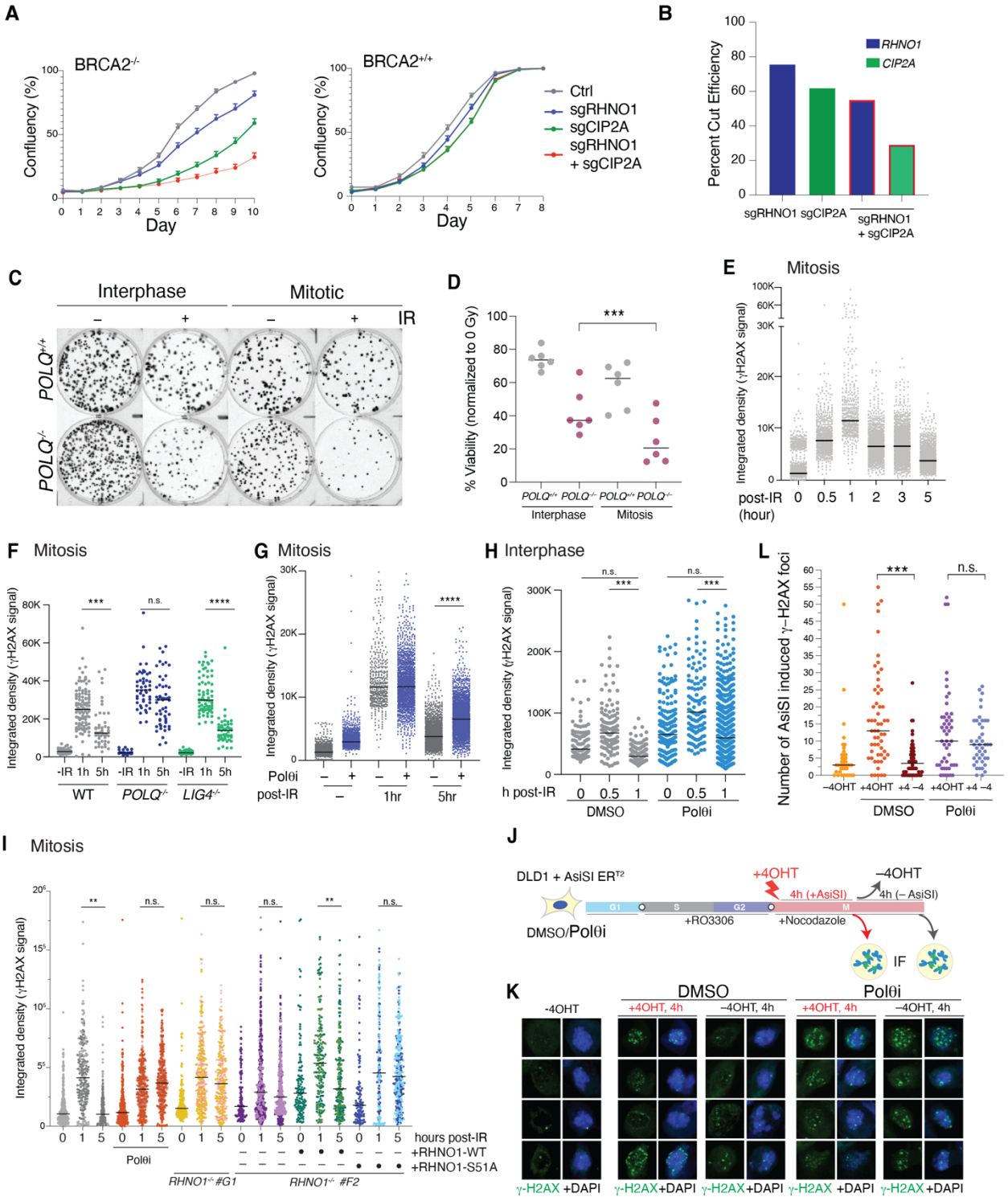

Fig. S7. Polθ resolves DSBs during mitosis.

(**A**) Growth curve of *BRAC2*$^{-/-}$ and *BRAC2*$^{+/+}$ cells treated with sgRNAs against *RHNO1* and *CIP2A*. Data are normalized to time point zero (one day after seeding). (**B**) Editing efficiency of the *RHNO1* and *CIP2A* sgRNAs used in panel A. Genomic DNA was isolated from *BRCA2*$^{+/+}$ cells

expressing FLAG-Cas9 and the indicated sgRNAs. The region flanking the sgRNA cut site was amplified by PCR and sequenced. Results from the TIDE analysis are shown. (**C**) Representative images of clonogenic survival of *POLQ*$^{+/+}$ and *POLQ*$^{-/-}$ cells irradiated with 2 Gy in interphase and mitotic cells. (**D**) Quantification of colony formation of cells irradiated with 2 Gy in interphase or mitosis. Samples were normalized to the non-irradiated cells. Bars represent the mean of six independent experiments. An unpaired t-test was run for statistical significance (*p<0.05). (**E**) Quantification of γ-H2AX signal intensity from IF in control cells synchronized and irradiated with 2 Gy in mitosis. IF were performed at the indicated time points. 0h is the no-irradiation control sample. At least 400 nuclei per sample were analyzed. Statistical analysis was performed using a non-parametric unpaired t-test (****p<0.0001) comparing time point 1h and 5h. (**F**) Quantification of γ-H2AX signal intensity from IF in control cells and *POLQ* and *LIG4* deficient cells synchronized in mitosis and irradiated with 2 Gy. IF were performed at the indicated time points. Statistical analysis was performed using a non-parametric unpaired t-test (****p<0.0001) comparing time points 1h and 5h. At least 50 nuclei per sample were imaged for the experiment. (**G**) Quantification of γ-H2AX signal intensity by IF in cells synchronized in mitosis, as in panel F-H, and treated with Polθ inhibitor RP6685. Statistical analysis was performed using a non-parametric unpaired t-test (****p<0.0001) comparing time point 5h of control and Polθi-treated cells. (**H**) Quantification of γ-H2AX signal intensity from IF in control cells and cells treated with Polθ inhibitor RP6685 in interphase and irradiated at 2 Gy in mitosis. IF were performed at the indicated time points. (**I**) Quantification of γ-H2AX signal intensity from IF in control cells, Polθi-treated cells and *RHNO1*-deficient cells synchronized in mitosis and irradiated with 2 Gy. IF were performed at the indicated time points. Data represent the mean of two independent experiments, with different colors representing results from separate experiments. Statistical analysis was performed using a non-parametric unpaired t-test (****p<0.0001) comparing 1h and 5h time points. (**J**) Schematic representation of the experimental pipeline for DLD1 cells expressing the inducible AsiSI and synchronized in mitosis. (**K**) Representative images from the IF of AsiSI-expressing cells treated with DMSO and Polθi after AsiSI induction and after washout. (**L**) Quantification of γ-H2AX foci in cells treated as described in panel H. Statistical analysis was performed using a non-parametric unpaired t-test (****p<0.0001) comparing 1h and 5h time points.

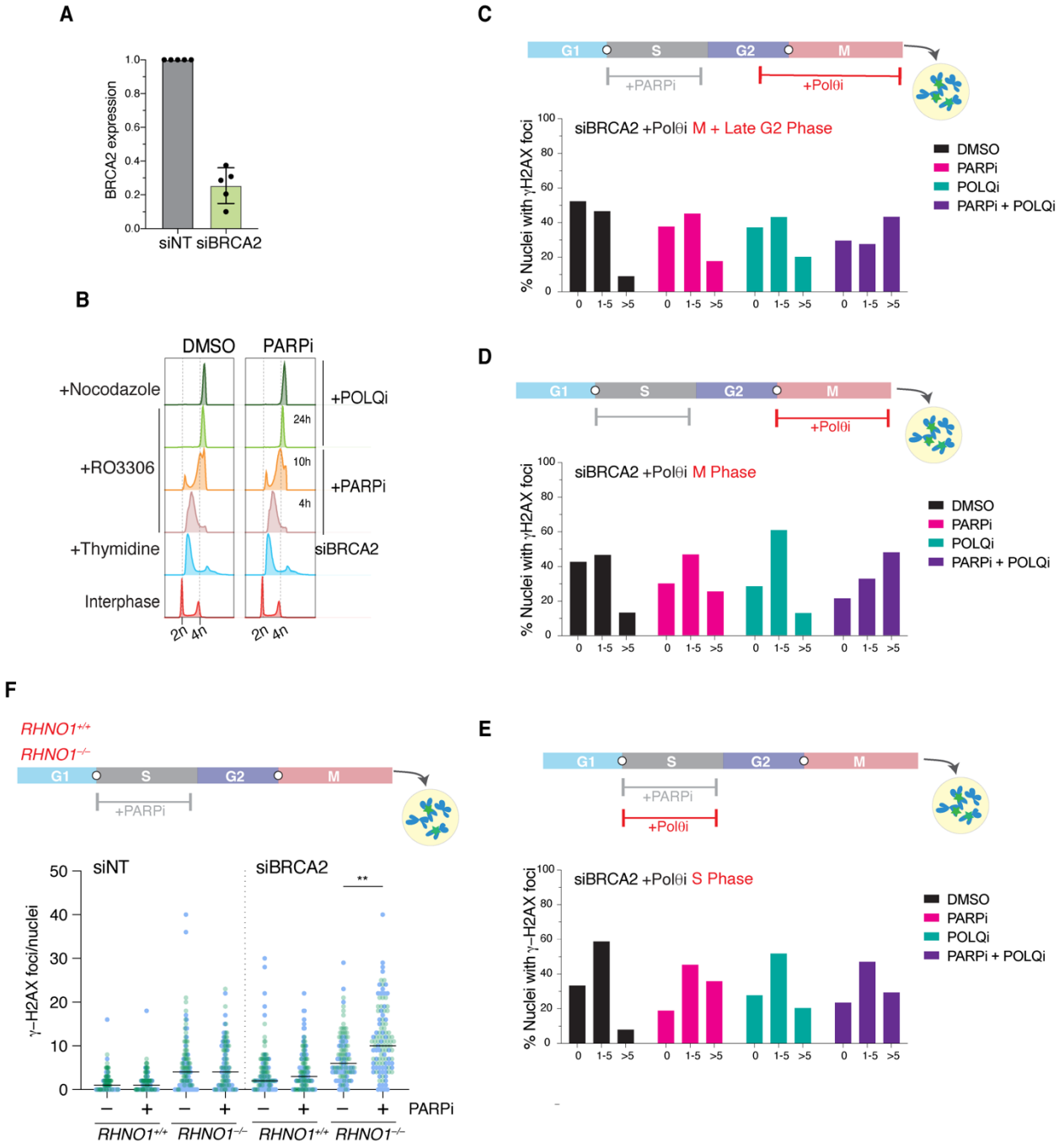

Fig. S8. In BRCA2-deficient cells, PARP inhibitors synergize with Polθ inhibitors in the G/M phase but not in S phase

(**A**) qPCR analysis of *BRCA2* mRNA expression after siRNA knockdown in samples analyzed in Figure 4F-H and Figure S8C-E. Relative gene expression was normalized using *ACT1* as a

housekeeping gene. (**B**) Cell cycle analysis by FACS of the samples analyzed in Figure 4F-H. (**C-E**) Quantification of γ-H2AX foci in mitotic cells treated as follows: Cells treated with siRNA against control (siNT) and *BRCA2* were synchronized at the G1/S interphase using a thymidine block and released into S phase with PARP inhibitor (Olaparib). PARP inhibitor was withdrawn upon exit from S phase, and CDK1 inhibitor was added (RO3306). Cells were then washed and treated with nocodazole, fixed, and stained with γ-H2AX antibody one hour after release into mitosis. Polθ inhibitor was added at late G2 through M (**C**) or exclusively in M (**D**) or S phase (**E**). At least 50 nuclei were scored for each condition. (**F**) Quantification of γ-H2AX foci in *RHNO1*$^{-/-}$ mitotic cells and treated as in panel C. Data represent the mean of two independent experiments, with different colors representing results from separate experiments. Statistical analysis was performed using a non-parametric unpaired t-test (****$p<0.0001$) comparing 1h and 5h time points.

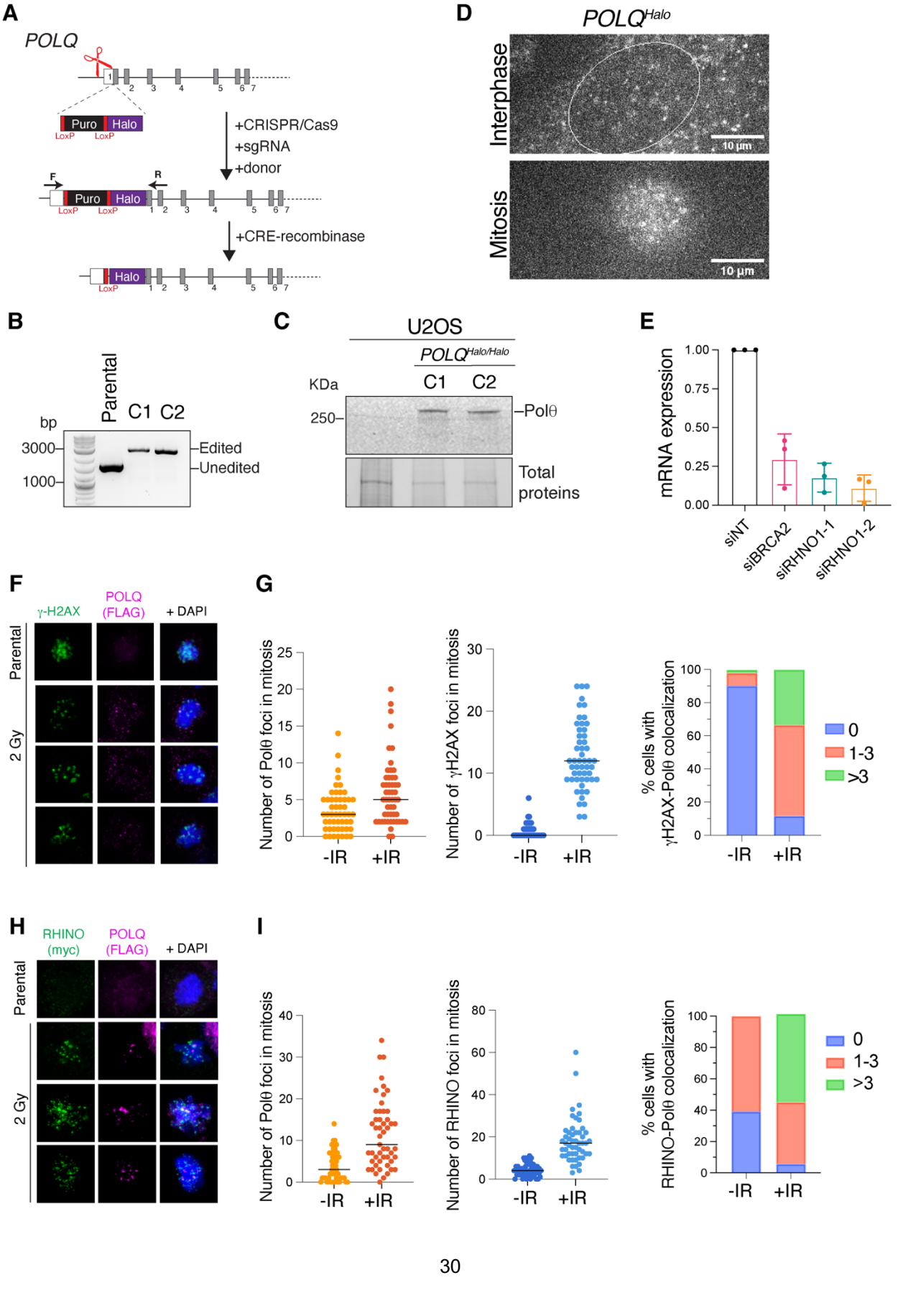
A
POLQ
Puro
Halo
LoxP
+CRISPR/Cas9
+sgRNA
+donor
+CRE-recombinase
D
POLQHalo
Interphase
Mitosis
10 µm
B
Parental
C1 C2
bp
3000
1000
Edited
Unedited
C
U2OS
POLQHalo/Halo
KDa
250
Polθ
Total proteins
E
mRNA expression
siNT
siBRCA2
siRHNO1-1
siRHNO1-2
F
γ-H2AX
POLQ (FLAG)
+ DAPI
Parental
2 Gy
G
Number of Polθ foci in mitosis
Number of γH2AX foci in mitosis
% cells with γH2AX-Polθ colocalization
-IR
+IR
0
1-3
>3
H
RHINO (myc)
POLQ (FLAG)
+ DAPI
I
Number of Polθ foci in mitosis
Number of RHINO foci in mitosis
% cells with RHINO-Polθ colocalization

Fig. S9. Polθ visualization at DSB sites in mitosis.

(**A**) Strategy for endogenous Halo-Polθ targeting using CRISPR/Cas9 in U2OS. (**B**) Genotyping PCR on genomic DNA extracted from targeted cells as in panel A. PCR was conducted on cells after excision of the Puromycin cassette using Cre Recombinase. (**C**) Western blot analysis on two Halo-Polθ clones and the parental line as a negative control. Protein loading was detected using the Stain-Free filter on a BioRad Chemidoc. (**D**) Representative still images of Halo-Polθ in live-cell movies of interphase and mitotic cells. (**E**) qPCR analysis of *BRCA2* and *RHNO1* mRNA expression after siRNA knockdown of the samples analyzed in Fig. 5A-C. Relative gene expression was normalized using *GAPDH* as a housekeeping gene. (**F**) Representative immunofluorescence images of DLD1 expressing POLQ-FLAG and synchronized in mitosis with CDK1 inhibitor for 16h. Cells were stained with anti-FLAG (magenta) and γ-H2AX (green) antibodies. (**G**) Quantification of POLθ, γ-H2AX foci and their colocalization as in panel F. At least 50 mitotic cells were scored. (**H**) Representative immunofluorescence images of cells expressing POLQ-FLAG and RHINO-MYC and synchronized in mitosis with CDK1 inhibitor for 16h. Cells were stained with anti-FLAG (magenta) and γ-H2AX (green) antibodies. (**I**) Quantification of POLθ foci, RHINO foci, and their colocalization. At least 50 mitotic cells were scored.

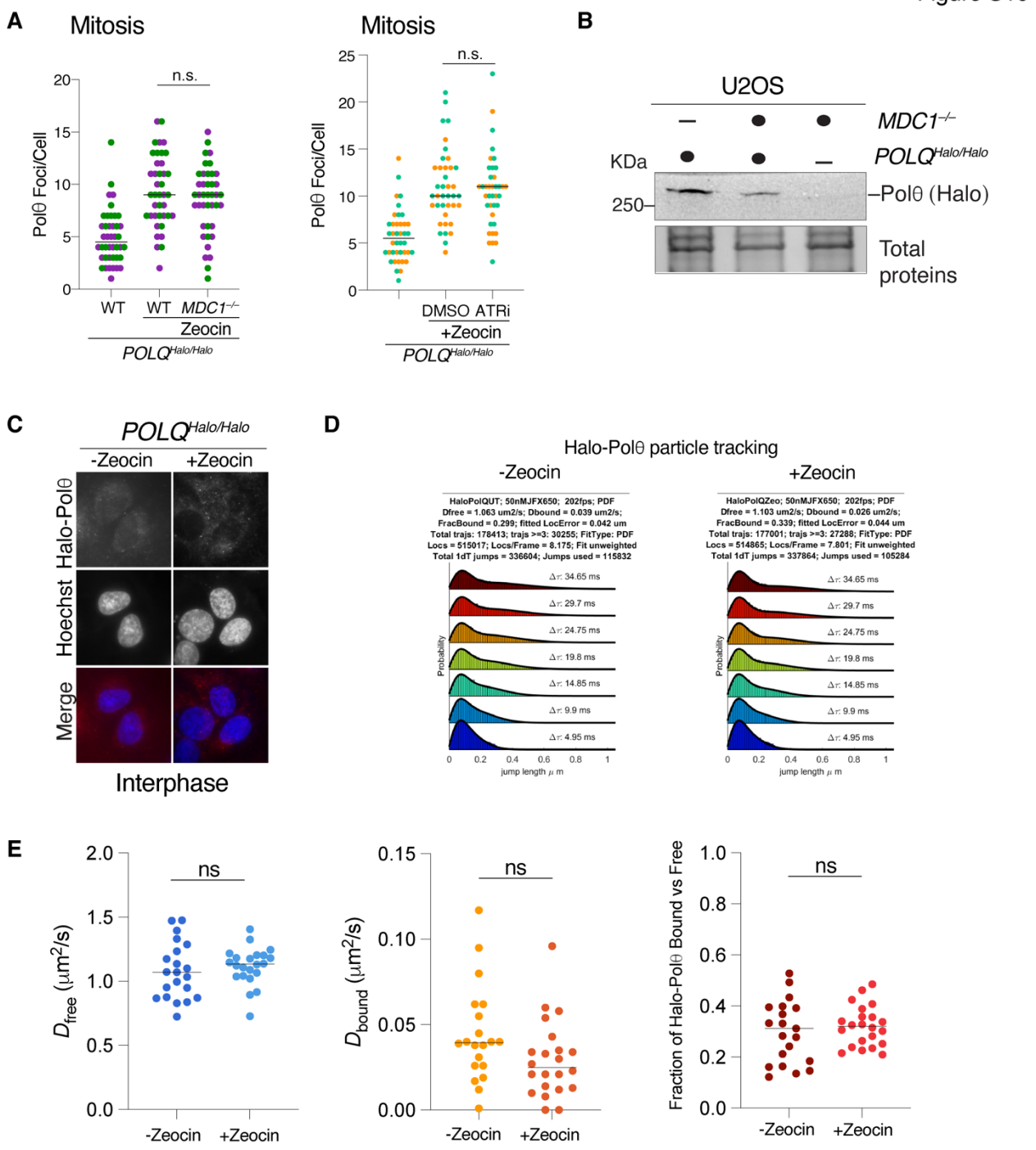

Fig. S10. Monitoring Polθ dynamics in interphase cells.

(**A**) Quantification of mitotic Halo-Polθ foci in live cells treated with Zeocin or ATRi in WT and *MDC1*$^{-/-}$ cells. At least 20 nuclei were counted per experiment. The different colored dots represent the results of two independent experiments. Statistical analysis was performed using one-way

ANOVA (***p<0.001, **p<0.01). (**B**) Western blot analysis on an *MDC1*$^{-/-}$ clone where Polθ was targeted with Halo. *MDC1*$^{-/-}$ cells were used as a negative control, and a clone of *MDC1*$^{+/+}$ Halo-Polθ as a positive control. Protein loading was detected using the Stain-Free filter on a BioRad Chemidoc. (**C**) Representative images of Halo-Polθ foci from live-cell imaging experiment in interphase cells treated with DMSO and Zeocin. (**D**) Plots from SpotOn analysis for a single live-cell single-molecule experiment (>20 cells) for Halo-Polθ ± Zeocin. Spot-On is an open-source package that allows kinetic modeling of single-particle tracking data. (**E**) Results of live-cell single-molecule analysis of Halo-Polθ molecules analyzed with single particle tracking on SpotOn. Each dot represents the indicated D_{free}, D_{bound}, or Fraction Bound for Halo-Polθ molecules appearing in at least three consecutive frames within a single cell. Data were analyzed by unpaired t-test.

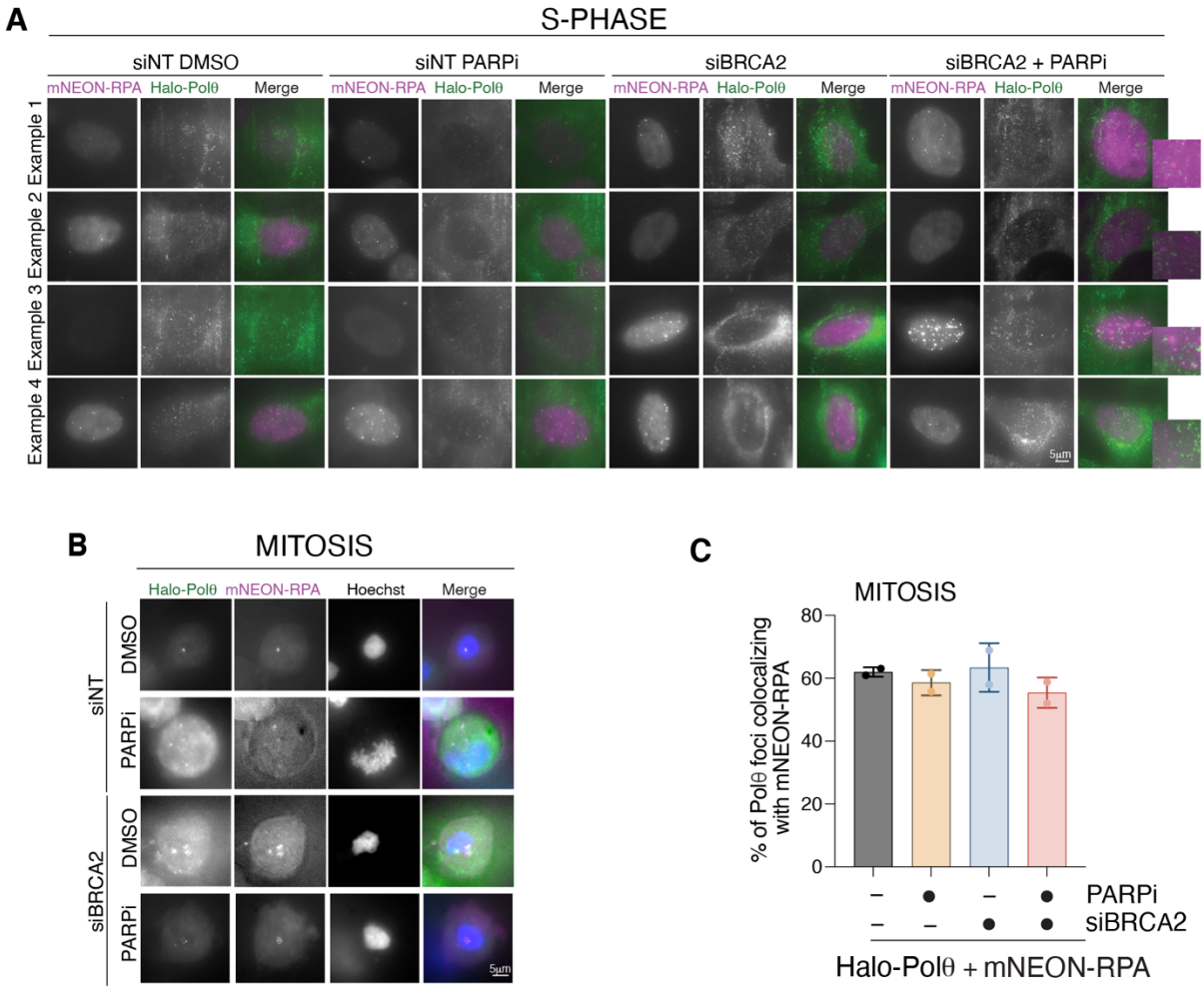

Fig. S11. Inhibition of HR and treatment with PARPi does not alter Polθ dynamics in interphase.

(**A**) Representative images of Halo-Polθ and mNEON-RPA in live-cell imaging experiments in S phase cells. Cells treated with siRNA against control (siNT) and *BRCA2* were transfected with a vector for mNEON-RPA32 and synchronized in G1/S using thymidine block and released into S phase in the presence of PARP inhibitor (Olaparib, 10 μm). Images were taken four hours after release from the thymidine block. mNEON-RPA is depicted in magenta and Halo-Polθ in green. (**B**) Representative images of Halo-Polθ and mNEON-RPA in live-cell imaging experiment in M phase cells. Cells treated with siRNA against control and *BRCA2* were transfected with a vector for mNEON-RPA32 and synchronized in G1/S using thymidine block and released into S phase

in the presence of PARP inhibitor (Olaparib, 10 μm) followed by CDKi, and then release in nocodazole to enrich for mitotic cells. mNEON-RPA is depicted in magenta and Halo-Polθ in green. (**C**) Quantification of Halo-Polθ foci colocalization with mNEON-RPA foci. Bars represent the mean of two independent experiments. At least 20 nuclei were counted for each sample.

Table S1. (Separate file)

Tables with BF and Z-scores calculated for the CRISPR-Cas9 screens conducted in TKO and *RHNO1*$^{-/-}$ cells.

Table S2. (Separate file)

Tables of sgRNA, shRNA, and primers for qPCR, TIDE, and gene targeting experiments.

Movie S1. (Separate file)

Representative live-cell single-molecule imaging movie of U2OS cells expressing 3XFLAG-Halo-Polθ in interphase labeled with JFX650 and acquired at 147 frames per second. An oval was drawn to define the nucleus based on brightfield imaging.

Movie S2. (Separate file)

Representative live-cell single-molecule imaging movie of U2OS cells expressing 3XFLAG-Halo-Polθ in mitotic cells arrested with nocodazole, labeled with JFX650, and acquired at 147 frames per second.