Supplementary Information for " DNA end resection by CtIP and Exonuclease 1 prevents genomic instability"

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Supplementary Methods

Cell culture and transfections - HEK293T cells were maintained as described (El-Shemerly et al, 2005). Human U2OS osteosarcoma cells and U2OS cells stably expressing GFP-HA-EXO1 (kindly provided by S. P. Jackson, University of Cambridge, UK) were cultured in DMEM supplemented with 10% fetal calf serum, standard antibiotics and G-418 (0.5 mg/ml). Immortalized ATLD1 cells transduced with retrovirus expressing the wild-type MRE11 cDNA (ATLD1/MRE11) or retrovirus harboring the empty vector (ATLD1/vector) (a kind gift of M. Weitzman, Salk Institute, S. Diego, CA) were grown in DMEM supplemented with 20% FCS, streptomycin/penicillin (100 U/ml) and 1 µg/ml puromycin (Sigma). GFP-EXO1

(kindly provided by F. Marini, University of Milano, Italy) was transiently transfected in ATLD1 cells using Fugene HD (Roche).

All siRNA duplexes were purchased from Microsynth (Switzerland) with the exception of MRE11 siRNA, which was purchased from Dharmacon (USA). siRNA sequences are as follow: Luciferase (siCNTL) (CGUACGCGGAAUACUUCGATT) (Sartori et al, 2007), CtIP (GCUAAAACAGGAACGAAUCTT) (Sartori et al, 2007), EXO1 (CAAGCCUAUUCUCGUAUUUTT) (Gravel et al, 2008), and MRE11 (GAGCAUAACUCCAUAAGUATT) (Adams et al, 2006). siRNA duplexes were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) in two consecutive rounds to a final concentration of 80 nM as follows: control (80 nM luciferase siRNA), EXO1 (40 nM EXO1 siRNA + 40 nM control siRNA), CtIP (40 nM CtIP siRNA + 40 nM control siRNA), or combined EXO1 and CtIP (40 nM EXO1 siRNA + 40 nM CtIP siRNA). Experiments were typically performed 72h.

Antibodies and chemicals - Antibodies were purchased from S. Cruz Biotech. (goat polyclonal anti-CtIP and anti-OMNI, mouse monoclonal anti-CHK1 and anti-GFP, rabbit polyclonal anti-Cyclin A); Sigma (mouse monoclonal anti-beta-tubulin and anti-FLAG); Cell Signaling Tech. (rabbit monoclonal anti-γH2AX and anti-CHK1-pS345); Upstate Biotech. Inc. (mouse monoclonal anti-γH2AX); Novus Biologicals (rabbit polyclonal anti-MRE11); GeneTex (mouse monoclonal anti-MRE11); Calbiochem (mouse monoclonal anti-RPA2); NeoMarkers (mouse monoclonal anti-EXO1); Abcam (rabbit polyclonal anti-pS2056-DNA-PKcs); or described previously (rabbit polyclonal anti-EXO1, F-15) (El-Shemerly et al, 2005)

Polyclonal and mouse monoclonal anti-CtIP antibodies (Sartori et al, 2007) were provided by R. Baer (Columbia University, New York, NY). Secondary HRP-conjugated anti-mouse and anti-rabbit antibodies were form GE-Healthcare and the HRP-conjugated anti-goat was from S. Cruz Biotech. Alexa Fluor-488, -594, and -647-conjugated secondary antibodies were from Invitrogen.

Camptothecin (Sigma) and AZD2281 (Olaparib, Selleck Chemicals) were dissolved in DMSO at 1 mM and 10 mM stock concentrations, respectively. Hydroxyurea (Sigma) was dissolved in water at 1 M stock concentration. EdU (5-ethynyl-2'-deoxyuridine) was from Invitrogen. The DNA-PKcs inhibitor NU7441 (Tocris Bioscience) (Leahy et al, 2004) was dissolved in DMSO at 5 mM stock concentration. The *E. coli* exonuclease EXOIII was from New England Biolabs.

Immunofluorescence staining and analyses - Cells grown on cover slips were either fixed directly in ice-cold methanol for 15 min or pre-extracted for 5 min on ice using 25 mM HEPES pH 7.4, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose and 0.5% Triton X-100 before fixation in 4% formaldehyde (w/v) in PBS for 15 min at room temperature (RT). Cover slips were incubated overnight at 4 °C with primary antibodies and Alexa–conjugated secondary antibodies for 1h at RT. The cover slips were mounted with Vectrashield® (Vector Laboratories) containing DAPI. Images were acquired either using a Leica confocal SP2 or an Olympus IX81 fluorescence microscope.

Western blotting and Immunoprecipitation – Cells lysis, immunoprecipitation and immunoblot analysis were performed as described previously (El-Shemerly et al, 2008). To ensure that the observed interactions were not DNA-mediated, ethidium bromide was included in all samples.

In vitro **protein interaction -** 200 ng of purified, recombinant CtIP (Sartori et al, 2007) and EXO1 (El-Shemerly et al, 2005) were incubated either alone or together for 30 min at 4 ^{o}C in 1 ml TNE buffer containing 50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA and 10 μg/ml BSA. Proteins were immunoprecipitated with the antibody F-15 for 2h at 4 ^{o}C. Protein-A sepharose (GE Healthcare) immunocomplexes were analyzed as described (El-Shemerly et al, 2005).

Exonuclease assays - The nicked substrate was generated by incubating the pGEM-13Zf(+) plasmid derivative with *N.Bst*NBI (Fischer et al, 2007) and purified by gel extraction (Qiagen). Exonuclease activities were assayed in a buffer containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml BSA. Where indicated, final concentrations of purified recombinant proteins were as follows: 15 nM EXO1, 15 nM CtIP and 300 nM RPA. Notably, the presence of purified RPA in the reaction resulted in a defined pattern of nucleolytic processing (Fig 2B) as compared to reactions without RPA (supplementary Fig 2E). Reactions were stopped by incubation in 10 mM EDTA, 0.25 % SDS and 100 μg/ml Proteinase K for 10 min at 37° C. DNA products separated on 0.8% agarose were stained with

either ethidium bromide or SYBR Gold and analyzed with a Typhoon PhosphorImager (GE Healthcare).

Hairpin exonuclease assays were performed in 20 µl with 1 nM DNA substrate (annealed 3' labeled HL1: 5'-TCATTGCCTATCCTGACAGTCCGACACATCGGACTGTCAGGATAGGCAAT GATCTTTTTTTTT -3'), 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 50 ng/µl BSA at 37ºC with the indicated protein concentrations and time-points. Reactions were terminated by addition of an equal volume of 99% (v/v) formamide 0.1% (w/v) bromophenol blue and heating at 95°C for 5 min. Products were resolved by electrophoresis in 15% (w/v) polyacrylamide gel containing 8 M urea (acrylamide/bis-acrylamide 19:1) run in 1xTBE buffer at 25 mA. Gels were dried and analyzed with a Typhoon PhosphorImager (GE Healthcare).

Linearized plasmids were generated by incubating the pGEM-13Zf(+) plasmid derivative with either Ban*II* (5' overhangs), Hind*III* (3' overhangs) or Sca*I* (blunt ends) followed by column purification (Qiagen). Proteins used in the assay were mixed and incubated on ice prior to addition into reaction tubes containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml BSA. DNA products were separated as described above.

DNA-Binding assays - Gel mobility shift assays were performed in a volume of 20 µl containing 5 nM of annealed 5' radiolabeled DNA oligonucleotide substrates, 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 ng/µl BSA. The reactions were pre-incubated with the corresponding proteins, 10 nM EXO1, 50 nM CtIP for 15 min at RT before addition of the substrate and further incubated for 60 min. After addition of glycerol to a final 10% concentration, separation was performed in a 6% (w/v) native polyacrylamide gel run in 1xTBE buffer at 25 mAmp. Gels were analyzed as described above.

Pulse-field gel electrophoresis - Subconfluent cultures of U2OS were mock-treated (DMSO) or camptothecin-treated for 4h. Cells were harvested by trypsinization, and agarose plugs of 10^6 cells were prepared in a disposable plug mold (BioRad). Plugs were then incubated in lysis buffer (100 mM EDTA, 1% (w/v) sodium lauryl sarcosyl, 0.2% (w/v) sodium deoxycholate, 1mg/ml proteinase K) at 37 ^oC for 72h. Plugs were then washed four times in 20 mM Tris-HCl pH 8.0, 50 mM EDTA before loading

onto an agarose gel. Electrophoresis was performed for 23h at 14 ^{o}C in 0.9% (w/v) Pulse Field Certified Agarose (BioRad) containing Tris-borate/EDTA buffer according to the conditions described in (Hanada et al, 2007) and adapted to a BioRad CHEF DR III apparatus. The gel was then stained with ethidium bromide and analyzed using an Alpha Innotech Imaging system.

Colony formation assay - Cells were either mock-treated (DMSO) or treated with the indicated doses of camptothecin 72h after the first siRNA transfection. The drug was removed 1h upon treatment and cells were cultured for 10–14 days at 37°C. For the PARP-inhibitor AZD2281, continuous exposure to the compound was ensured by a first addition 72h after the siRNA transfection, and a second addition 72 h after the first. Colonies were stained with a crystal violet/ethanol (0.5%/20%) solution and counted.

Chromosome analysis - After treatment with camptothecin, cells were allowed to recover for 8h in complete medium before chromosome preparation. Caffeine (2 mM) was added for the last 5h to override the G2/M checkpoint, and colcemid (0.1 μg/ml) was added for the last 3h to arrest cells in metaphase. Metaphase chromosomes were stained with DAPI.

Supplementary References

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Supplementary Figure Legends

Supplementary Figure S1 - CtIP- and MRN-dependent recruitment of EXO1 to DSBs

(A) Recruitment of GFP-EXO1 to laser lines visualized by fluorescence microscopy at early (5 min) and late (240 min) time points.

(B) Live cell imaging of microirradiated GFP-EXO1 cells transfected with control (CNTL), CtIP or MRE11 siRNA. Scale bar = 10 μm.

(C-E) CtIP recruitment to DSBs in ATLD1 cells is insufficient for DNA end resection. MRE11-deficient ATLD1 fibroblasts, stably transfected with an empty vector or with a vector directing the expression of full-length MRE11, were treated with camptothecin (1 μM, 1 h) and analyzed by immunoblotting with the indicated antibodies *(C)*. The same pair of cells was microirradiated, fixed after 30 min and co-stained with either γH2AX and RPA2 *(D)* or γH2AX and CtIP *(E)* antibodies. Nuclei were visualized with DAPI.

(F) Live cell imaging of vector- or wild-type MRE11-complemented ATLD1 fibroblasts, transiently transfected with GFP-EXO1 and microirradiated.

Supplementary Figure S2 - Biochemical characterization of the EXO1-CtIP interaction

(A) WCEs (5 mg) from DMSO or camptothecin-treated (2h, 2.5 μM) HEK293T cells were immunoprecipitated using an anti-EXO1 antibody. Immunocomplexes bound to

sepharose beads were incubated with 10 units of calf intestinal phosphatase (CIP) for 10 min at 37 ^{o}C, washed with 3x 1ml TNET buffer (El-Shemerly et al, 2005), resolved by SDS-PAGE and analyzed by immunoblotting.

(B) WCEs (5 mg) from HEK293T cells overexpressing OMNI-tagged EXO1, treated or not with hydroxyurea (2 mM, 16h) were immunoprecipitated with PI serum or an anti-CtIP antibody. Proteins were detected by immunoblotting with the indicated antibodies.

(C) Human recombinant $EXO1_{wt}$ and the catalytically-dead mutant $EXO1_{D173A}$ were expressed and purified as described (El-Shemerly et al, 2005). Peak fractions from the chitin affinity column were pooled, loaded on a Hi-Trap SP-FF (1 ml) cartridge and proteins eluted with a linear salt gradient (0.0 - 0.5 M NaCl). Aliquots of the load (L, 5 μl), flow-through (FT, 5 μl), wash (W, 5 μl) fractions and of the OD_{280} peak (20 μl) were resolved by 8% SDS-PAGE and stained with Coomassie brilliant blue.

(D) Purified wild-type EXO1 (15 nM; lanes 1-7) or catalytically-dead EXO1 (D173A, 15 nM; lanes 9-15) were incubated with 5 nM *N.BstNBI*-digested pGEM-13Zf(+) plasmid for the indicated time points and the products resolved on a 0.8% agarose gel before ethidium bromide staining.

(E) Gel-purified pGEM-13Zf(+)-nicked plasmid substrate (3.75 nM) was incubated at 37°C for the indicated time periods with purified EXO1 (15 nM) in the presence or absence of purified CtIP (15 nM) or MRE11-RAD50 (15 nM). The products were resolved as described in Supplementary Methods.

(F) Radiolabelled oligonucleotide (5 nM) was incubated for 60 min at room temperature with purified EXO1 (10 nM), CtIP (50 nM) or both together in a buffer containing 1 mM EDTA. The products were analyzed as described in Supplementary Methods. The migration patterns of free and EXO1-bound oligonucleotide are indicated.

(G) Purified linearized pGEM-13Zf(+) plasmid (2.5 nM, 5 nM DNA ends) containing either 3' overhangs, 5' overhangs or blunt ends was incubated for 30 min at 37°C with EXO1 (15 nM) in the presence or absence of RPA (300 nM) as indicated. The products were resolved as described in Supplementary Methods.

(H) Purified linearized pGEM-13Zf(+) plasmid containing 3' overhangs (2.5 nM, 5 nM DNA ends) was incubated at 37°C with EXO1 (15 nM) in the presence of RPA

(300 nM) and either CtIP (15 nM), MRE11-RAD50 (15 nM) or BLM (15 nM). The products were resolved as described in Supplementary Methods.

(I) Purified linearized pGEM-13Zf(+) plasmid (2.5 nM, 5 nM DNA ends) containing either blunt ends or 5' overhangs was incubated at 37°C with EXO1 (15 nM) in the presence of RPA (300 nM) or pre-incubated with either EXO1 (15 nM) or CtIP (15 nM) for 5 min at RT, followed by addition of CtIP or EXO1, respectively. The products were resolved as described in Supplementary Methods.

Supplementary Figure S3 - Effect of CtIP, EXO1 or CtIP/EXO1 downregulation on genome stability

(A) 72h after the transfection with the indicated siRNA oligonucleotides U2OS cells were subjected to propidium iodide (PI) staining for cell cycle analysis.

(B) U2OS cells grown on glass cover slips and transfected as described in *(A)* were incubated for 20 min with 10 μM EdU (5-ethynyl-2'-deoxyuridine). Immediately after fixation with 4% formaldehyde cover slips were processed following manufacturers instructions (Invitrogen). At least 150 cells were counted for each condition. Percentages indicate the number of EdU-positive cells.

(C) 72h after the transfection with the indicated siRNA oligonucleotides U2OS cells were treated with either DMSO or camptothecin (1h, 1 μM). WCEs were analyzed by immunoblot (*left panel*) or immunoprecipitation (*right panel*) with the indicated antibodies.

(D) Cells transfected as described in *(A)* were treated with the indicated doses of AZD2281 (Olaparib) and survival was determined by colony formation. Data represent the mean ± SEM of three independent experiments.

(E) Cells transfected as in (A) were treated with camptothecin (1 μM, 1h) and allowed to recover for 8h in complete medium before chromosome preparation. Caffeine (2 mM) was added for the last 5h to override the G2/M checkpoint, and colcemid (0.1 μg/ml) was added for the last 3h to arrest cells in metaphase. 50 metaphase spreads were analyzed for each sample. The percentages of metaphase spreads displaying the indicated numbers of broken chromosomes are shown.

(F) Representative images of chromosomal abnormalities detected in metaphase spreads of camptothecin treated cells: broken chromatids (arrowheads); radial chromosomes (ellipses). Scale bar, 10 μm.

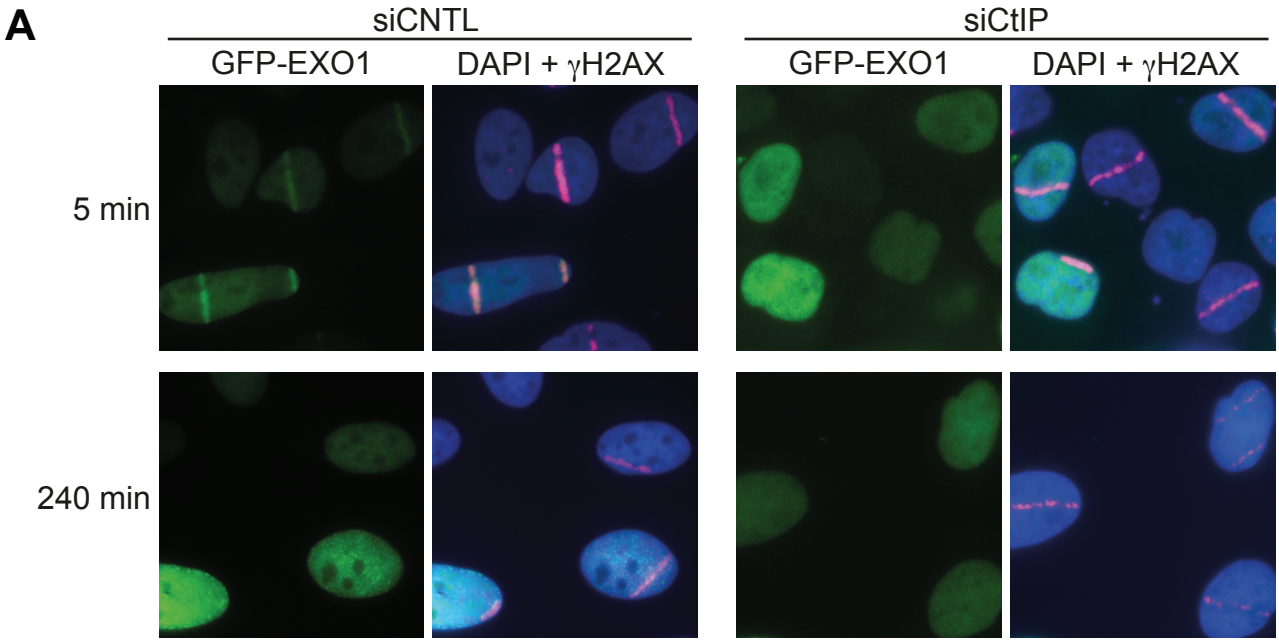
Supplementary Figure S4 - Inhibition of DNA-PKcs rescues camptothecin-induced radials formation in CtIP/EXO1 depleted cells

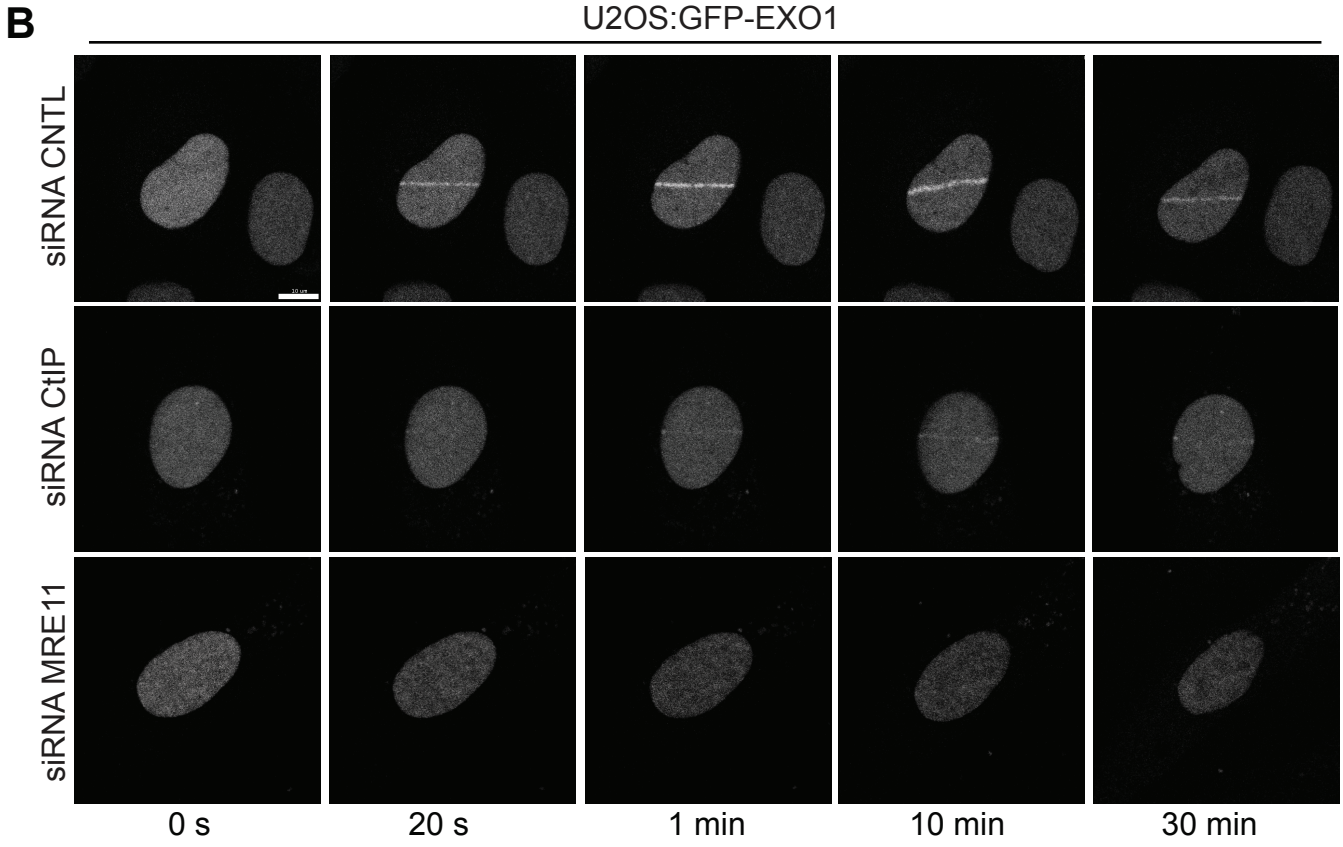
(A) WCEs obtained from U2OS that were treated with camptothecin (1h, 1 μM) in the presence or the absence of NU7441 (10 μM), were analyzed by immunoblot with the indicated antibodies. Autophosphorylation at S2056 was use as read-out for inhibition of DNA-PKcs activity by NU7441.

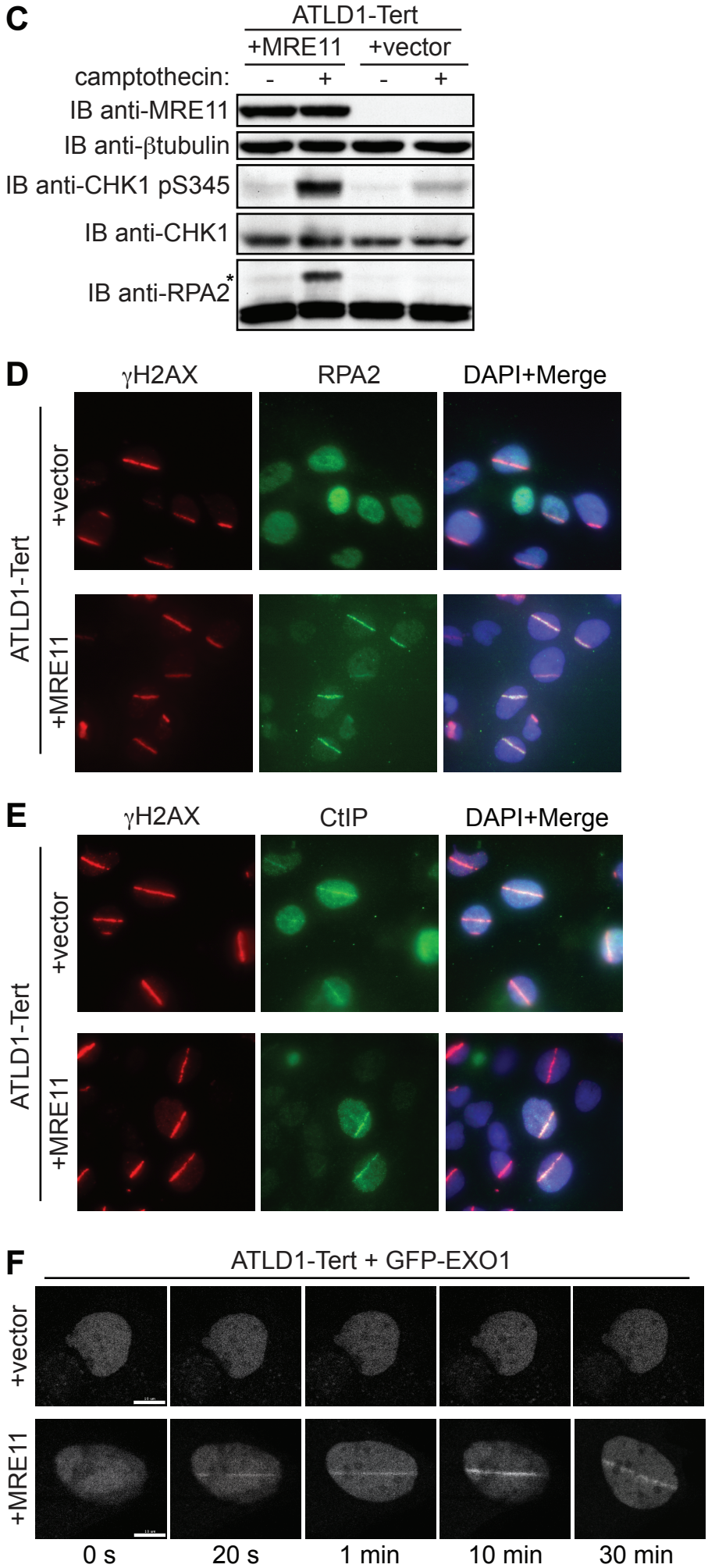
(B) 72h after the transfection with the indicated siRNA oligonucleotides, U2OS cells were treated with DMSO or camptothecin (2.5 μM, 4h) in the presence or the absence of the DNA-PKcs inhibitor NU7441 (10 μM). Representative images of chromosomal abnormalities detected in metaphase spreads are shown. Radial chromosomes (ellipses) and broken chromatids (arrowheads) are indicated.

(C) Cells transfected as in *(B)* were treated with DMSO or camptothecin (1h, 1 μM). The amount of broken DNA was assessed by PFGE as described in supplementary Methods.

Eid *et al.*, Supplemental Figure 1



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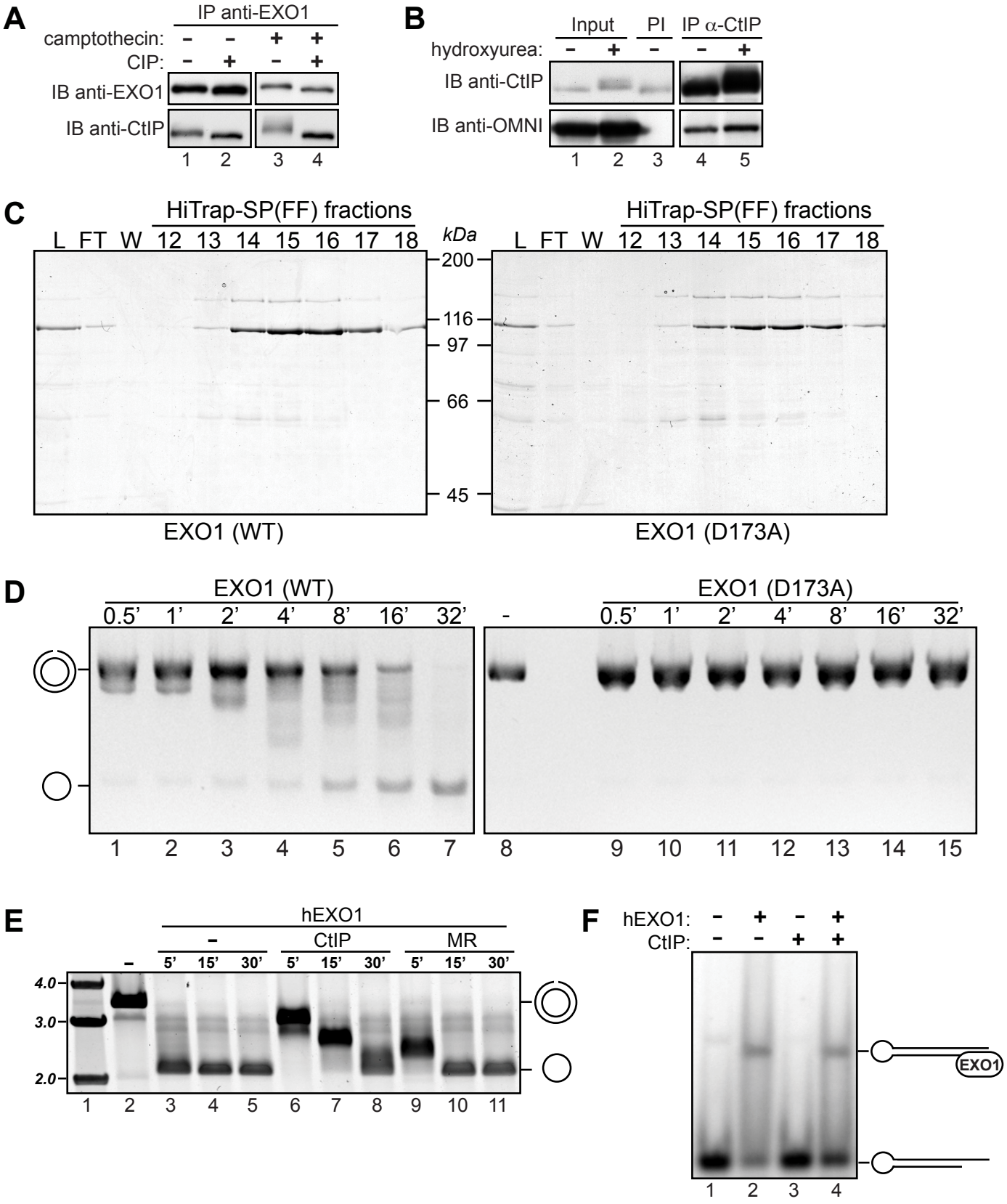
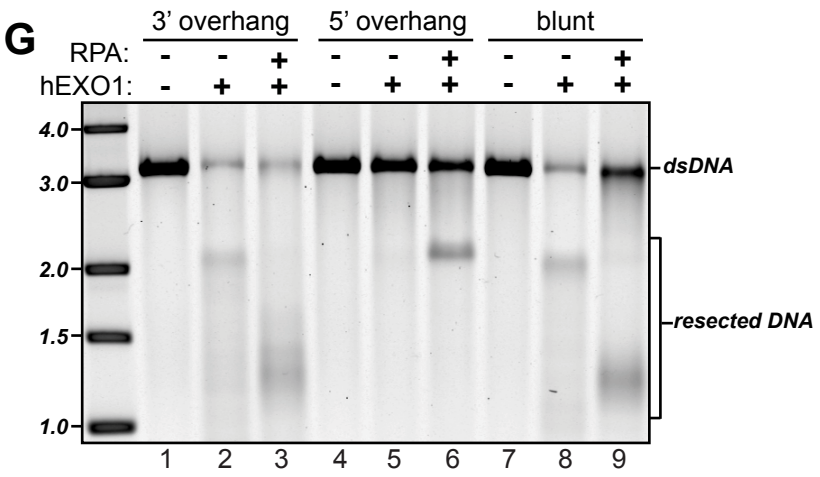
Eid *et al.*, Supplemental Figure 2

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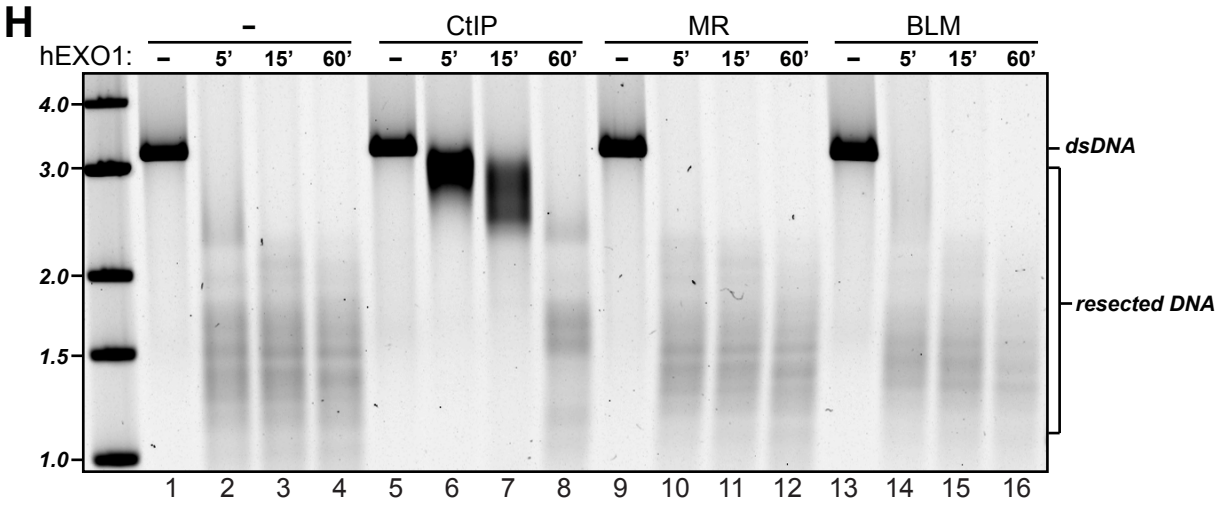
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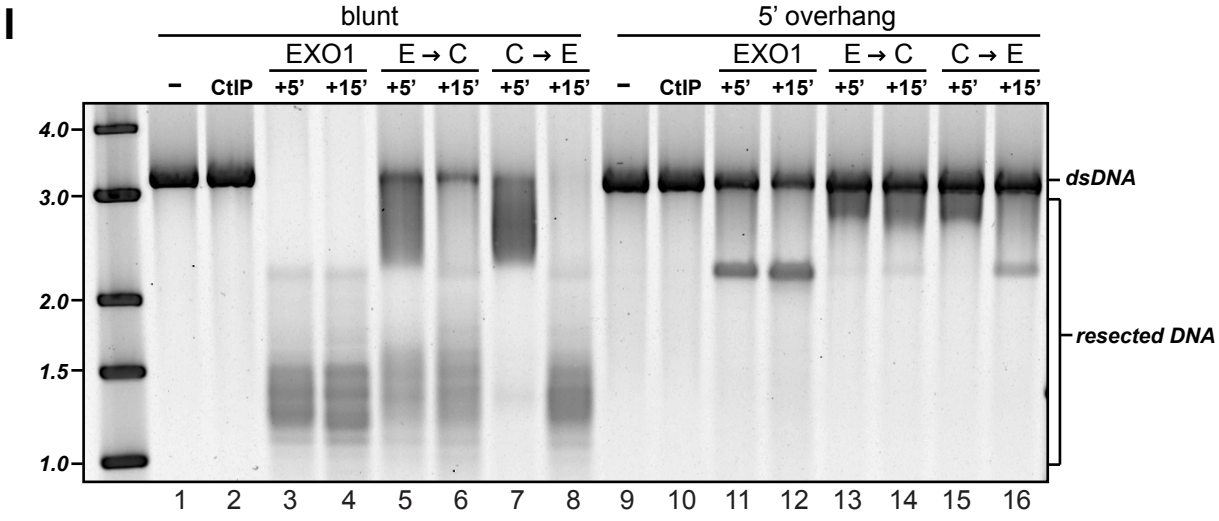
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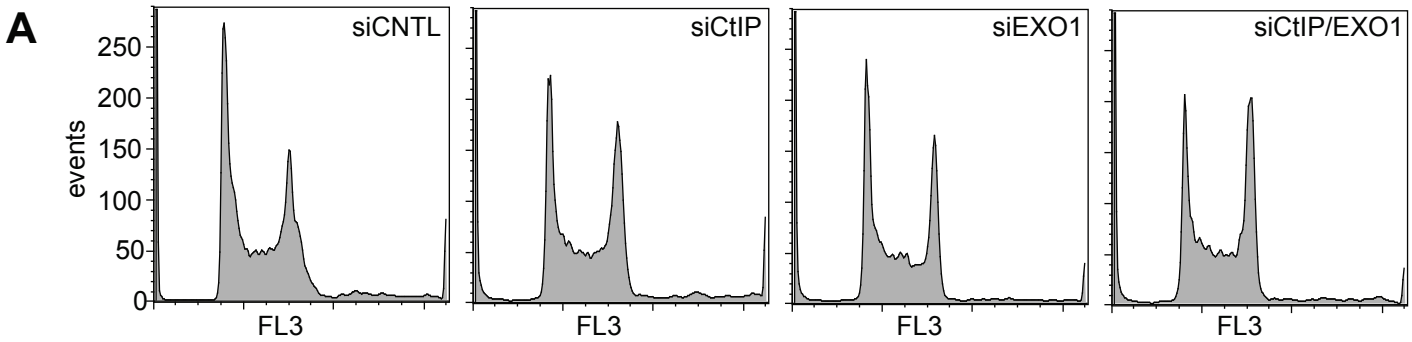
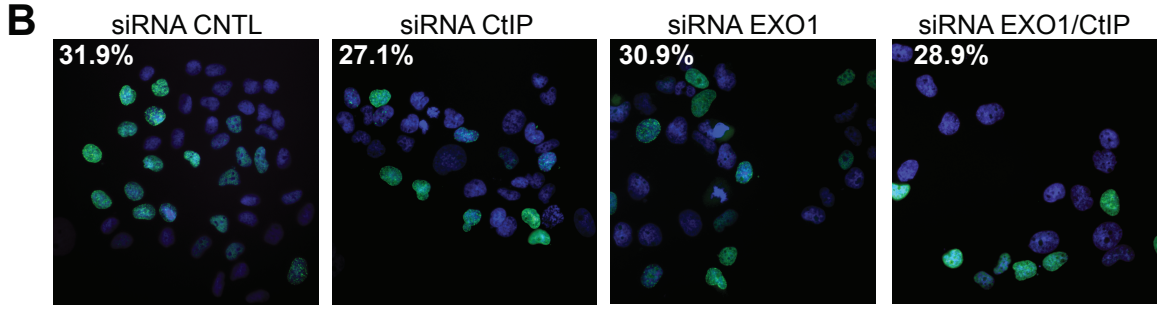
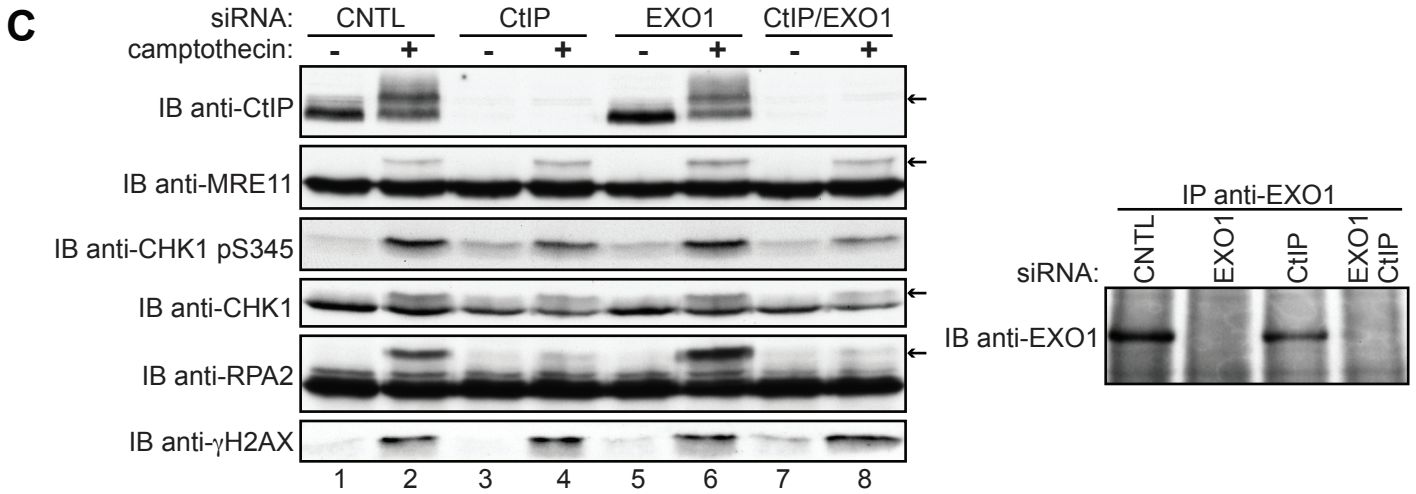
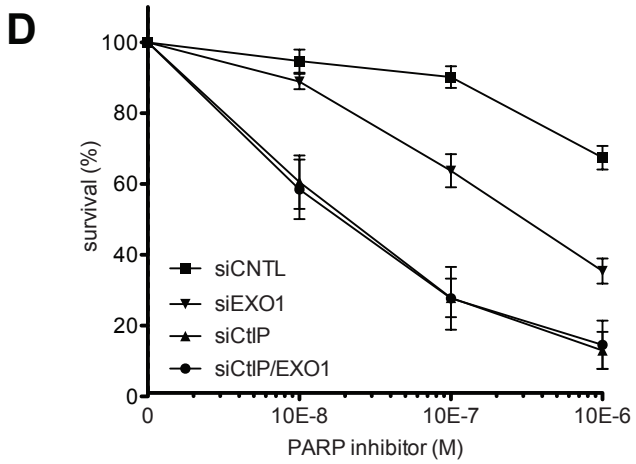
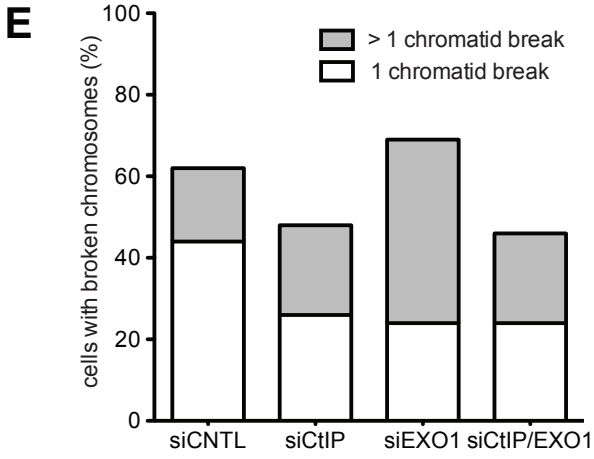
Eid *et al*., Supplemental Figure 3

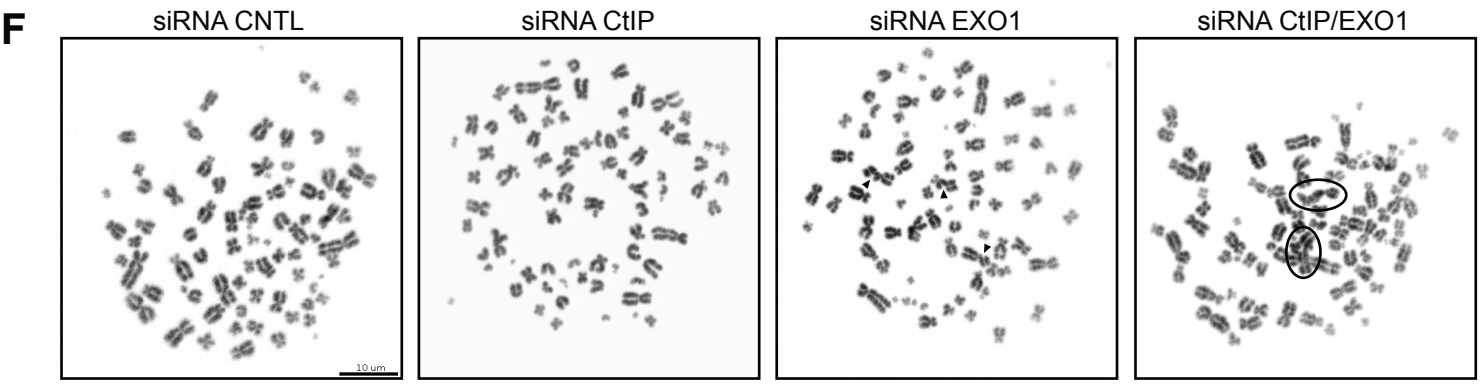












Eid *et al.,* Supplemental Figure 4