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PLK1 regulates CtIP and DNA2 interplay in long-range DNA end resection

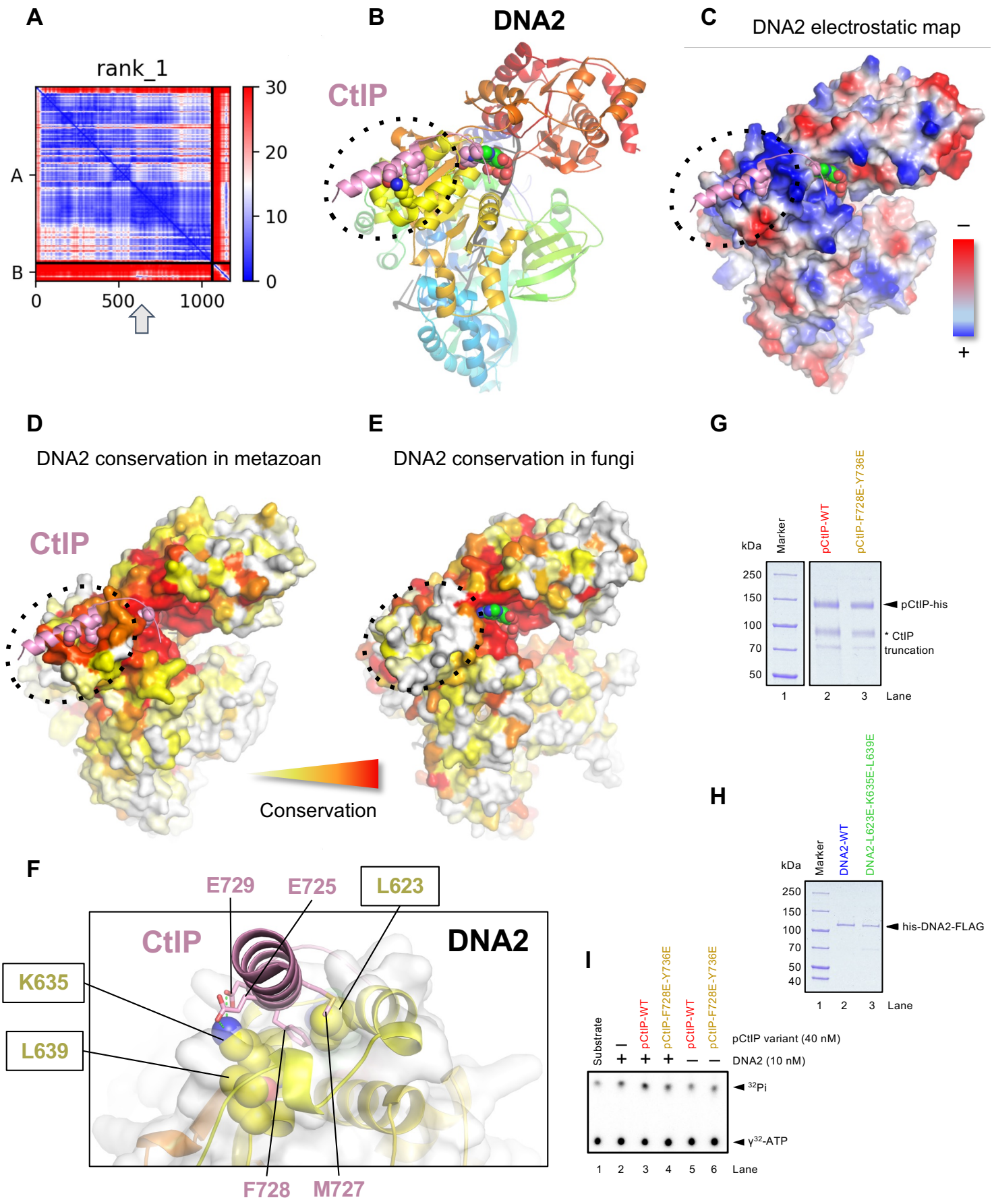
Supplementary Information

Supplementary Figure 1-6

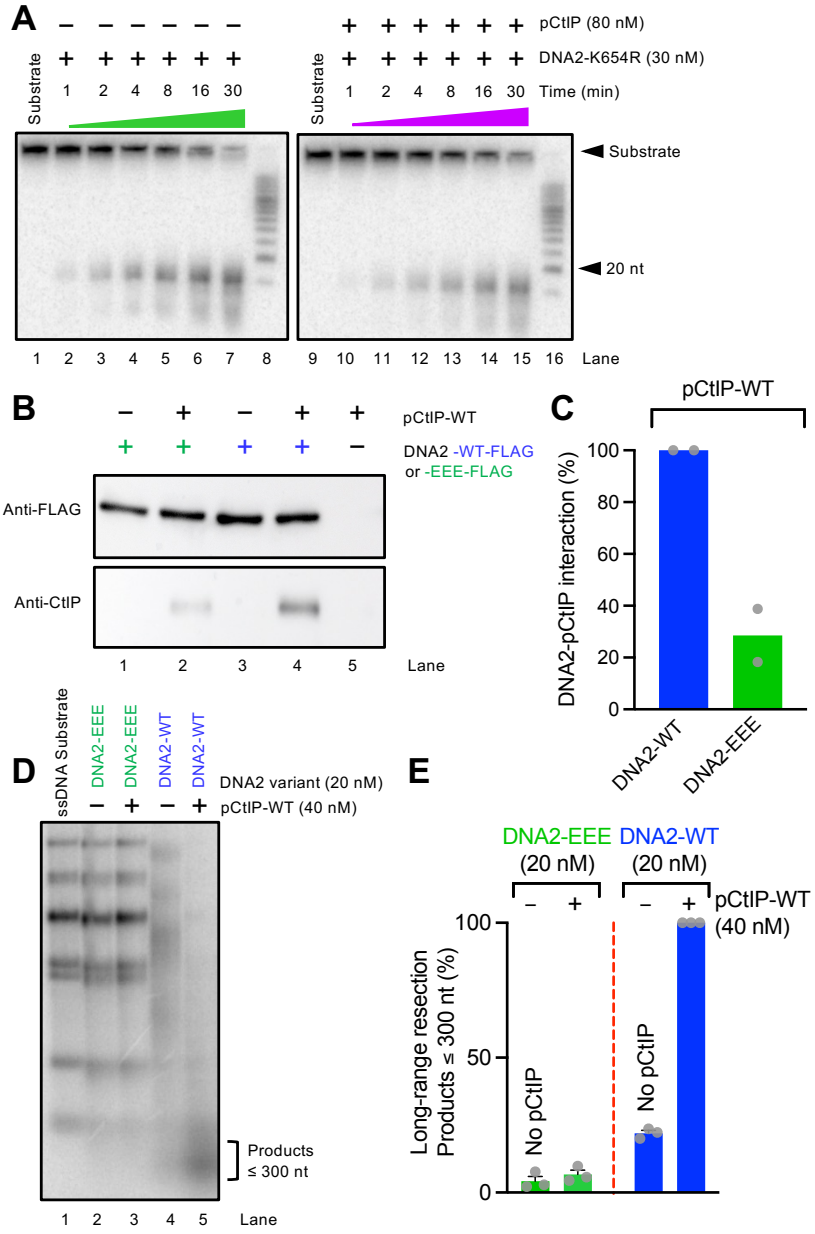
Supplementary Table 1-4

Supplementary Material and Methods

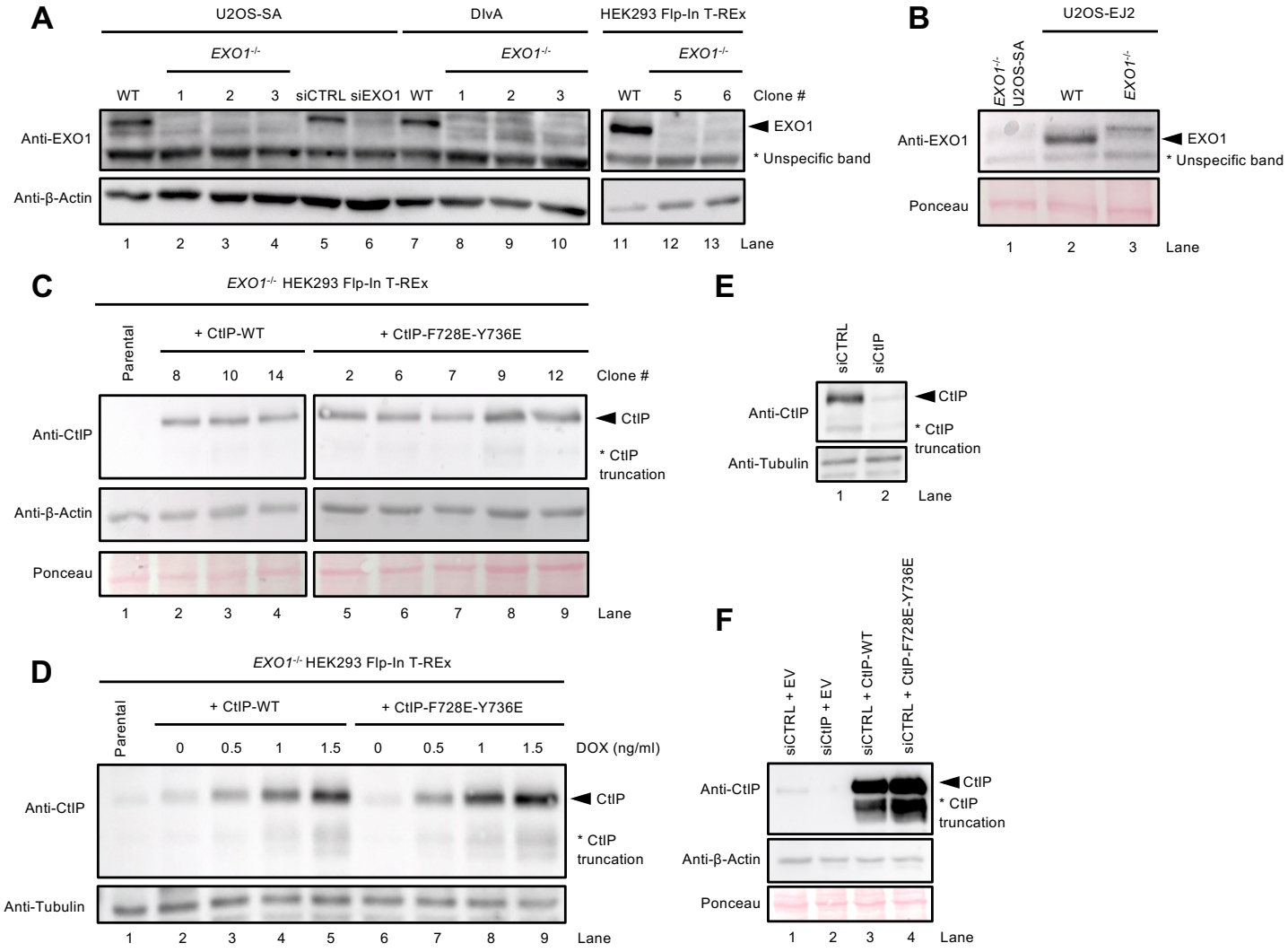
Ceppi_Supplemental FigS1



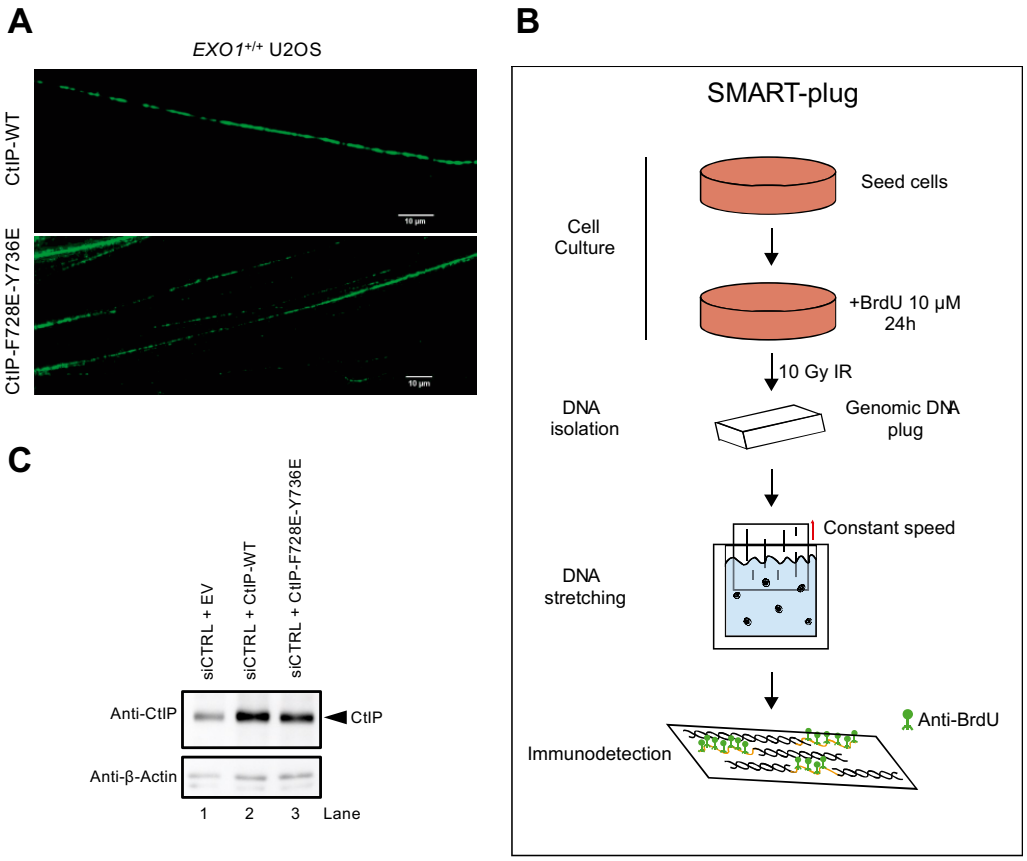
Supplemental Figure S1 (related to Figure 1). (*A*) Predicted Alignment Error (PAE) map generated by AlphaFold2 method with the arrow highlighting the confidence in the interaction in the region centered on CtIP F728 with the helices 614-624 and 628-639 of DNA2. (*B*) Structural model of DNA2 generated by AlphaFold2, in which DNA2 is colored from blue (N-ter) to red (C-ter) with the nucleotide bound in the active site of the helicase domain shown as green spheres. A model of the helical motif in CtIP (720-737) bound to DNA2 obtained with AlphaFold2 is shown as a pink cartoon and the location of the binding surface is indicated by a dashed-line circle. The position of ssDNA in DNA2 as observed in the structure of the mouse DNA2 (PDB 5EAX) is indicated as grey sticks. (*C*) Structural model of DNA2 generated by AlphaFold2 mapping the coulombic electrostatic potential highlighting the positively charged character of the patch involved in the interaction with CtIP (dashed circle). (*D*) Surface representation of DNA2 mapping the conservation of positions in a multiple sequence alignment restricted to metazoan sequences, and calculated using Rate4Site, showing a strong conservation of the positions predicted to contact CtIP in the structural model of the complex. (*E*) Surface representation of a structural model of *S. cerevisiae* Dna2 mapping the conservation of positions in a multiple sequence alignment, restricted to fungal sequences, and calculated using Rate4Site. The data show that the region proposed to mediate the interaction with CtIP in vertebrates is not conserved in low eukaryotes. (*F*) Focused view of the structural model of DNA2 generated by AlphaFold2 in which the helical motif in CtIP (720-737) is shown as a pink cartoon bound to the light grey transparent surface of DNA2. The cartoon representation of the DNA2 region is shown in yellow as in *B*. The three residues mutated as glutamate in DNA2 (L623, K635 and L639) are shown as yellow spheres and labelled with black boxes and their contacting residues in CtIP are shown as pink sticks. (*G*) Recombinant phosphorylated wild type CtIP (pCtIP) and phosphorylated CtIP-F728E-Y736E (pCtIP-F728E-Y736E) used in this study. The polyacrylamide gel was stained with Coomassie Brilliant Blue. (*H*) Recombinant DNA2 variants used in this study. The polyacrylamide gel was stained with Coomassie Brilliant Blue. (*I*) Representative ATPase assay with DNA2 (10 nM) in the presence or absence of pCtIP-WT or pCtIP-F728E-Y736E (all 40 nM). The reactions contain RPA (395.5 nM) and ssDNA as cofactors.



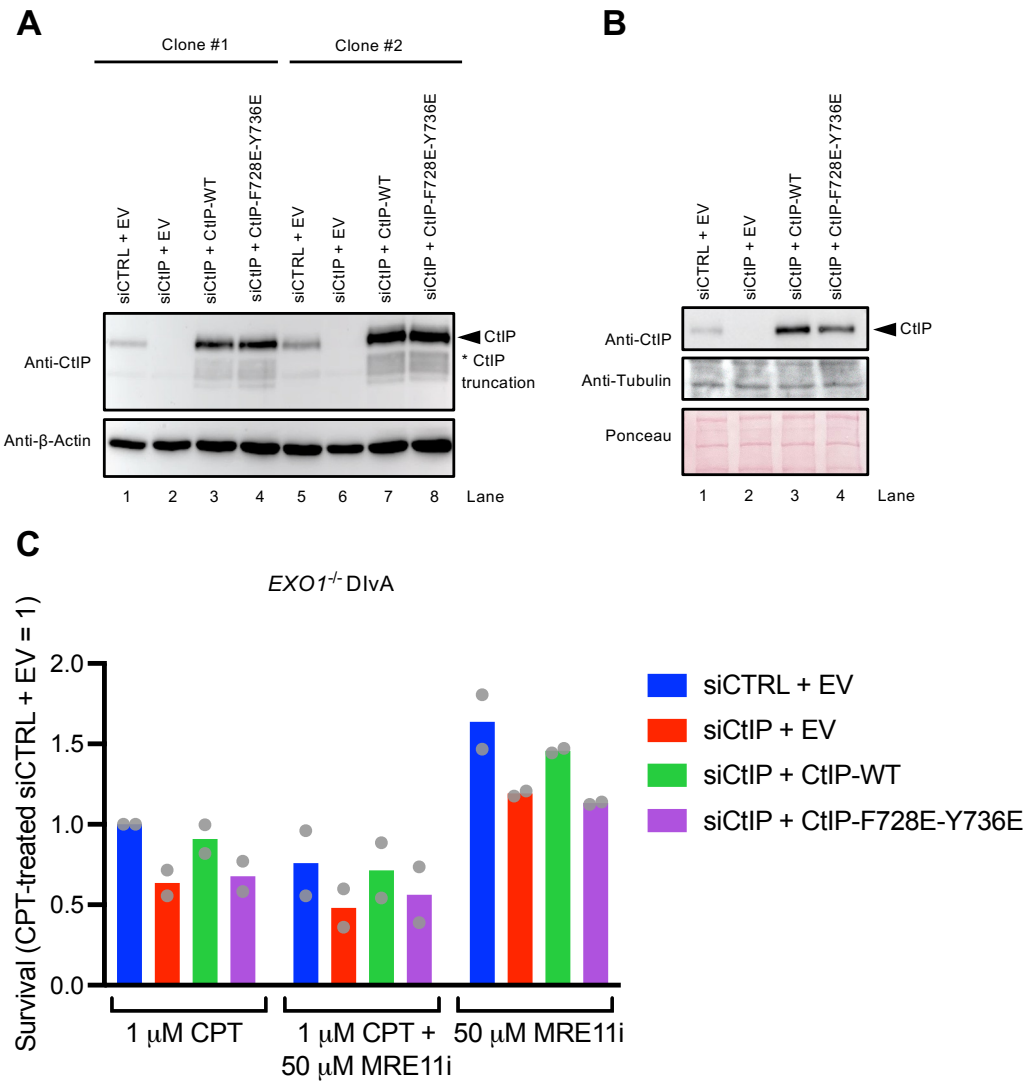
Supplemental Figure 2 (related to Figure 2). (*A*) Degradation of randomly labeled 2.2 knt-long ssDNA by helicase-deficient DNA2-K654R (30 nM) in the absence (*left*) or presence (*right*) of pCtIP (80 nM), analyzed by 20% polyacrylamide denaturing gels. Lanes 8 and 16 showed radioactively labeled marker. (*B*) Analysis of DNA2 wild type or DNA2-EEE interaction with pCtIP. The indicated DNA2-FLAG variant was immobilized on M2 anti-FLAG affinity resin and incubated with recombinant pCtIP. The Western blot was performed with anti-FLAG and anti-CtIP antibodies. (*C*) Quantitation of the DNA2-pCtIP interaction from experiments such as shown in *D*. Average shown, *n* = 2. (*D*) Degradation of ssDNA fragments of various length by DNA2 wild type or DNA2-EEE mutant alone (20 nM) or with pCtIP (40 nM) in the presence of RPA (864 nM). (*E*) Quantitation of small degradation products (≤ 300 nt) from experiments such as shown in *D*. *n* = 3; error bars, SEM.


A
U2OS-SA
DIvA
HEK293 Flp-In T-REx
EXO1-/-
EXO1-/-
EXO1-/-
WT 1 2 3 siCTRL siEXO1 WT 1 2 3 WT 5 6 Clone #
Anti-EXO1
Anti-β-Actin
EXO1
* Unspecific band
1 2 3 4 5 6 7 8 9 10 11 12 13 Lane
B
U2OS-EJ2
EXO1-/- U2OS-SA
WT
EXO1-/-
Anti-EXO1
Ponceau
EXO1
* Unspecific band
1 2 3 Lane
C
EXO1-/- HEK293 Flp-In T-REx
Parental
+ CtIP-WT
+ CtIP-F728E-Y736E
8 10 14 2 6 7 9 12 Clone #
Anti-CtIP
Anti-β-Actin
Ponceau
CtIP
* CtIP truncation
1 2 3 4 5 6 7 8 9 Lane
D
EXO1-/- HEK293 Flp-In T-REx
Parental
+ CtIP-WT
+ CtIP-F728E-Y736E
0 0.5 1 1.5 0 0.5 1 1.5 DOX (ng/ml)
Anti-CtIP
Anti-Tubulin
CtIP
* CtIP truncation
1 2 3 4 5 6 7 8 9 Lane
E
siCTRL
siCtIP
Anti-CtIP
Anti-Tubulin
CtIP
* CtIP truncation
1 2 Lane
F
siCTRL + EV
siCtIP + EV
siCTRL + CtIP-WT
siCTRL + CtIP-F728E-Y736E
Anti-CtIP
Anti-β-Actin
Ponceau
CtIP
* CtIP truncation
1 2 3 4 Lane

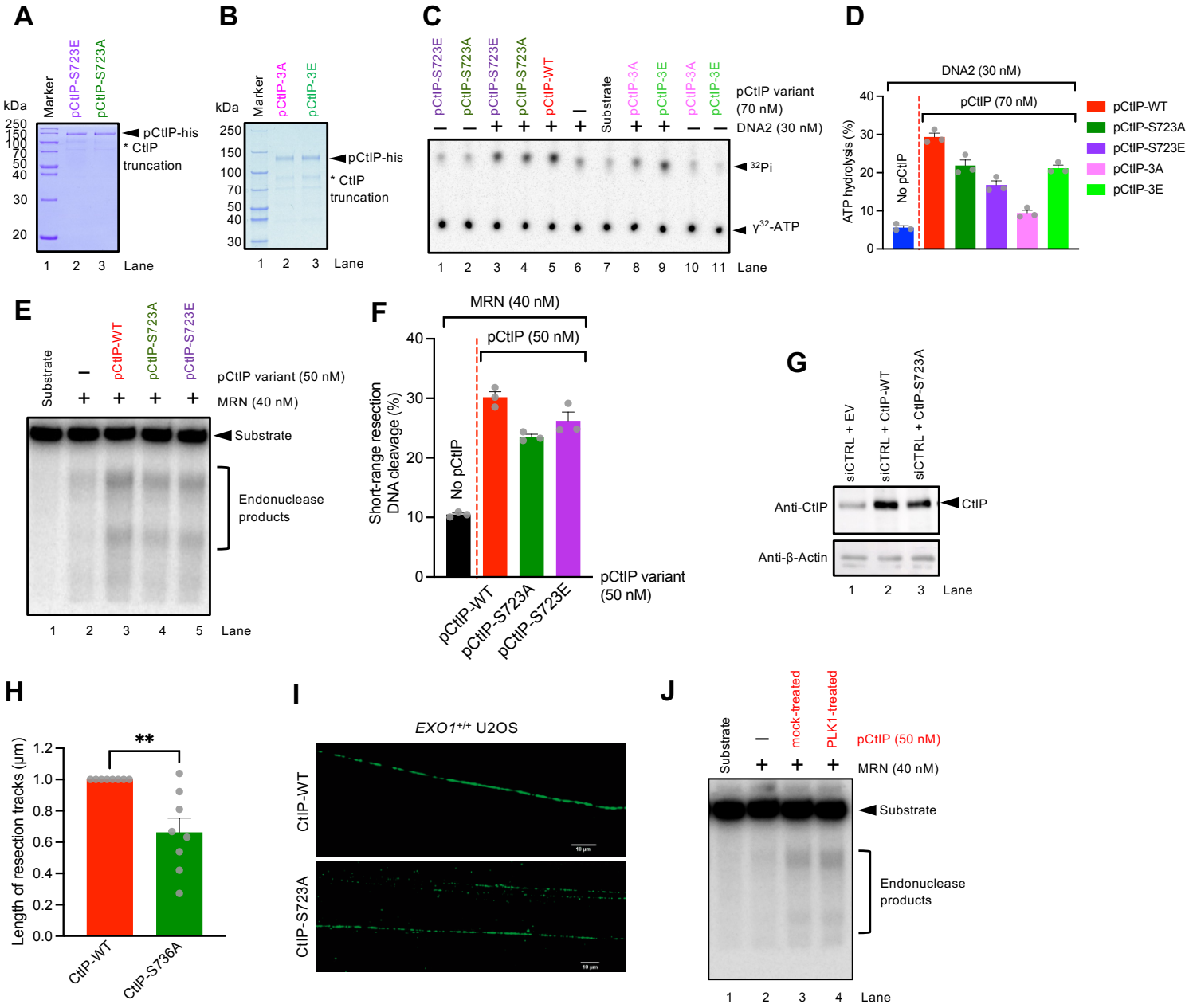
Supplemental Figure 3 (related to Figure 3). (*A*) Western blot analysis showing knock-out of endogenous *EXO1* by CRISPR/Cas9 in U2OS-SA, DIvA (*Asi*SI-ER-U2OS) and HEK293 Flp-In T-REx cells. Representative *EXO1*$^{-/-}$ clones are shown. *EXO1*$^{-/-}$U2OS-SA clone 1 and 2, *EXO1*$^{-/-}$ DIvA clone 1 and 3, and *EXO1*$^{-/-}$ HEK293 Flp-In T-REx clone 5 were used for subsequent experiments. (*B*) Western blot analysis showing knock-out of endogenous *EXO1* by CRISPR/Cas9 in U2OS-EJ2 cells. One representative *EXO1*$^{-/-}$ clone is shown. (*C*) Western blot analysis showing the inducible expression of FLAG-tagged wild type CtIP or CtIP-F728E-Y736E mutant upon the addition of 1 µg/ml doxycycline (DOX) for 48 h. Multiple representative *EXO1*$^{-/-}$ clones are shown. *EXO1*$^{-/-}$ HEK293 Flp-In T-REx clones 10 and 9 expressing wild type CtIP and CtIP-F728E-Y736E, respectively, were used for subsequent experiments. (*D*) Western blot analysis showing inducible expression of FLAG-tagged wild type CtIP or CtIP-F728E-Y736E mutant upon treatment with the indicated doxycycline (DOX) concentration for 48 h. One representative *EXO1*$^{-/-}$ clone for each cell line is shown. For subsequent experiments 0.5 ng/ml of DOX was used. (*E*) Western blot analysis showing depletion of endogenous CtIP by siCtIP in *EXO1*$^{-/-}$ HEK293 Flp-In T-REx cells. (*F*) Western blot analysis showing depletion of endogenous CtIP by siCtIP and complementation with the indicated siRNA-resistant FLAG-tagged CtIP variant in *EXO1*$^{-/-}$ DIvA cells. One representative *EXO1*$^{-/-}$ clone is shown. EV, empty vector.



Supplemental Figure 4 (related to Figure 4). (*A*) Representative ssDNA fibers from SMART assay, obtained from irradiated *EXO1*$^{+/+}$ U2OS cells overexpressing wild type CtIP or CtIP-F728E-Y736E labelled with CldU. (*B*) Schematic representation of the SMART protocol using the DNA plug formation approach. (*C*) Western blot analysis showing expression of the indicated FLAG-tagged CtIP variant in *EXO1*$^{+/+}$ U2OS cells prior to SMART assay. EV, empty vector.



Supplemental Figure 5 (related to Figure 5). (*A*) Western blot analysis showing depletion of endogenous CtIP by siCtIP and complementation with the indicated siRNA-resistant FLAG-tagged CtIP variant in *EXO1*$^{-/-}$ U2OS-SA cells. Two representative *EXO1*$^{-/-}$ clones are shown. EV, empty vector. (*B*) Western blot analysis showing depletion of endogenous CtIP by siCtIP and complementation with the indicated siRNA-resistant FLAG-tagged CtIP variant in *EXO1*$^{-/-}$ U2OS-EJ2 cells. One representative *EXO1*$^{-/-}$ clone is shown. EV, empty vector. (*C*) Quantitation of clonogenic cell survival upon acute treatment with 50 μM MRE11i for 30 min followed by 1 μM CPT for 1 h in *EXO1*$^{-/-}$ DIvA cells, where endogenous CtIP was mock-depleted (siCTRL) or depleted (siCtIP) and complemented with the indicated CtIP variant. The values are normalized to siCTRL + EV treated with 1 μM CPT. Average shown, *n* = 2. EV, empty vector.


A
kDa
Marker
pCtIP-S723E
pCtIP-S723A
250
150
100
70
50
40
30
20
pCtIP-his
* CtIP truncation
1 2 3 Lane
B
kDa
Marker
pCtIP-3A
pCtIP-3E
250
150
100
70
50
40
30
pCtIP-his
* CtIP truncation
1 2 3 Lane
C
pCtIP-S723E
pCtIP-S723A
pCtIP-S723E
pCtIP-S723A
pCtIP-WT
Substrate
pCtIP-3A
pCtIP-3E
pCtIP-3A
pCtIP-3E
pCtIP variant (70 nM)
DNA2 (30 nM)
32Pi
γ32-ATP
1 2 3 4 5 6 7 8 9 10 11 Lane
D
DNA2 (30 nM)
pCtIP (70 nM)
ATP hydrolysis (%)
No pCtIP
pCtIP-WT
pCtIP-S723A
pCtIP-S723E
pCtIP-3A
pCtIP-3E
E
Substrate
pCtIP-WT
pCtIP-S723A
pCtIP-S723E
pCtIP variant (50 nM)
MRN (40 nM)
Substrate
Endonuclease products
1 2 3 4 5 Lane
F
MRN (40 nM)
pCtIP (50 nM)
Short-range resection DNA cleavage (%)
No pCtIP
pCtIP-WT
pCtIP-S723A
pCtIP-S723E
pCtIP variant (50 nM)
G
siCTRL + EV
siCTRL + CtIP-WT
siCTRL + CtIP-S723A
Anti-CtIP
CtIP
Anti-β-Actin
1 2 3 Lane
H
Length of resection tracks (μm)
**
CtIP-WT
CtIP-S736A
I
EXO1+/+ U2OS
CtIP-WT
CtIP-S723A
J
Substrate
mock-treated
PLK1-treated
pCtIP (50 nM)
MRN (40 nM)
Substrate
Endonuclease products
1 2 3 4 Lane

Supplemental Figure 6 (related to Figure 6). (*A*) Recombinant pCtIP-S723E and pCtIP-S723A used in this study. The polyacrylamide gel was stained with Coomassie Brilliant Blue. (*B*) Recombinant pCtIP-3A and pCtIP-3E used in this study. The polyacrylamide gel was stained with Coomassie Brilliant Blue. (*C*) Representative ATPase assay with DNA2 (30 nM) alone or in the presence of the indicated pCtIP variant (70 nM). The reactions contain RPA (395.5 nM) and ssDNA as cofactors. (*D*) Quantitation of ATP hydrolysis from experiments such as shown in *C*. n = 3; error bars, SEM. (*E*) Representative 15% denaturing polyacrylamide gels showing an endonuclease assay with recombinant MRN complex (40 nM) and the indicated pCtIP variant (50 nM) on 3'-end-labeled 70 bp dsDNA with all ends blocked with streptavidin. (*F*) Quantitation of endonuclease products from experiments such as shown in *E*. n = 3; error bars, SEM. (*G*) Western blot analysis showing the overexpression of the indicated FLAG-tagged CtIP variant in *EXO1*$^{+/+}$ U2OS cells. EV, empty vector. (*H*) Quantification of the length of ssDNA fibers. Cultures were irradiated (10 Gy) and harvested after 1 h. The values are normalized to CtIP-WT. n = 8; error bars, SEM. ** (P < 0.01), two-tailed t-test. CtIP-WT is again shown as in Figure 4B as reference. (*I*) Representative ssDNA fibers of SMART assay from irradiated *EXO1*$^{+/+}$ U2OS cells overexpressing CtIP-S723A marked with CldU. CtIP-WT is again shown as in Supplemental Figure S4A as reference. (*J*) Representative 15% denaturing polyacrylamide gels showing an endonuclease assay with recombinant MRN complex (40 nM) and pCtIP either mock-treated or pre-phosphorylated by PLK1 (all 50 nM) on 3'-end-labeled 70 bp dsDNA with all ends blocked with streptavidin.

Supplemental Table 1. crRNAs used in this study.

Name	**Sequence (5’ to 3’)**
Hs.Cas9.EXO1.1.AC	AltR1/rArArGrCrUrCrGrArGrArGrUrGrUrUrUrCrArCrCrGrUrUrUrArGrArGrCrUrArUrGrCrU/AltR2
Hs.Cas9.EXO1.1.AD	AltR1/rArCrGrArCrArArGrCrCrArArUrCrUrUrCrUrUrArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AltR2

Supplemental Table 2. Primers used in this study.

Name	**Sequence (5’ to 3’)**
CtIP_1_F	GTAAGTGGATCCATGAACATCTCGGGAAGCAGC
CtIP_2_R	TGGCGAGCGGCCGCCCCGGGTCACTTATCGTCGTCATCCTTGTAATCCTTATCGTCGTCATCCTTGTAATCTGTCTTCTGCTCCTTGCC
DSB-II-200_F	ACCATGAACGTGTTCCGAAT
DSB-II-200_R	GAGCTCCGCAAAGTTTCAAG
DSB-V-740_F	GTCCCCTCCCCCACTATTT
DSB-V-740_R	ACGCACCTGGTTTAGATTGG
CtIP_F728E_For	AATGATAGCTTGGAAGATATGGAAGATCGGACAACACATGAAGAG
CtIP_F728E_Rev	CTCTTCATGTGTTGTCCGATCTTCCATATCTTCCAAGCTATCATT
CtIP_Y736E_For	GATCGGACAACACATGAAGAGGAAGAATCCTGTTTGGCAGACAG
CtIP_Y736E_Rev	CTGTCTGCCAAACAGGATTCTTCCTCTTCATGTGTTGTCCGATC
CtIP_S723A_F	GAAGAAAGAAAAATGAATGATGCTTTGGAAGATATGTTTGATCGG
CtIP_S723A_R	CCGATCAAACATATCTTCCAAAGCATCATTCATTTTTCTTTCTTC
CtIP_S723E_F	GAAGAAAGAAAAATGAATGATGAGTTGGAAGATATGTTTGATCG

CtIP_S723E_R	CGATCAAACATATCTTCCAACTCATCATTCATTTTTCTTTCTTC
CtIP_T693A_F	GAGACAGTGGACATGGACTGTGCTTTGGTTAGTGAAACCGTTCTC
CtIP_T693A_R	GAGAACGGTTTCACTAACCAAAGCACAGTCCATGTCCACTGTCTC
CtIP_T731A_F	GGAAGATATGTTTGATCGGGCTACACATGAAGAGTATGAATCCTG
CtIP_T731A_R	CAGGATTCATACTCTTCATGTGTAGCCCGATCAAACATATCTTCC
CtIP_T693E_F	GAGACAGTGGACATGGACTGTGAGTTGGTTAGTGAAACCGTTCTC
CtIP_T693E_R	GAGAACGGTTTCACTAACCAACTCACAGTCCATGTCCACTGTCTC
CtIP_T731E_F	GGAAGATATGTTTGATCGGGAGACACATGAAGAGTATGAATCCTG
CtIP_T731E_R	CAGGATTCATACTCTTCATGTGTCTCCCGATCAAACATATCTTCC
DNA2_L623E_F	AGGATACCGTGGCCTGCATCGAAAAGGGTTTGAACAAGCCACAG
DNA2_L623E_R	CTGTGGCTTGTTCAAACCCTTTTCGATGCAGGCCACGGTATCCT
DNA2_K635E_L639E_F	AAGCCACAGAGGCAAGCCATGGAGAAGGTCCTGGAAAGCAAGGACTA CACCCTGATC
DNA2_K635E_L639E_R	GATCAGGGTGTAGTCCTTGCTTTCCAGGACCTTCTCCATGGCTTGCCTCT GTGGCTT
NBS1_F	GGCTAGATGGATCCTCTAGAGCTAGCATGTGGAAACTGCTGCCCGC
NBS1_R	GATTTGCG CTCGAG TTA CCCGGG TCTTCTCCTTTTTAAATAAG

Supplemental Table 3. siRNA used in this study.

Name	**Sequence (5’ to 3’)**
siCTRL	TGGTTTACATGTCGACTAA
siCtIP	GCTAAAACAGGAACGAATC
siDNA2	CAGUAUCUCCUCUAGCUAGTT

siEXO1	CAGCCATTCTTACTACGCTUAA

Supplemental Table 4. Oligonucleotides used in this study. The bold T represents the site of the biotin modification.

Name	**Sequence (5′ to 3′)**
PC210	G**T**AAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCA CCTCATGCA**T**C
PC211	GA**T**GCATGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCACC GCGGCACT**T**AC

Supplemental Methods

Plasmids and cloning for transfection in mammalian cells

The constructs for the transient expression of wild type CtIP or the F728E-Y736E mutant in *EXO1*$^{-/-}$ U2OS-SA, *EXO1*$^{-/-}$ U2OS-EJ2 or *EXO1*$^{-/-}$ DIvA cells were prepared by PCR with the CtIP_1_F and CtIP_2_R primers, which added 2xFLAG tag at the C-terminus, and the sequences were cloned into BamHI and NotI sites of the pcDNA3.1 vector. The pcDNA5/FRT/TO plasmids expressing either CtIP_WT-2XFLAG or CtIP_F728E-Y736E-2XFLAG were cloned by digestion of the corresponding pcDNA3.1 plasmid with KpnI and ApaI restriction endonucleases (New England BioLabs) and ligated into the pcDNA5/FRT/TO vector using the same restriction enzymes. The pEGFP-C1 plasmid containing CtIP wild type for the expression in HEK293T cells was previously described (Sartori et al. 2007). The pEGFP-C1 plasmid containing CtIP F728E-Y736E was generated by two steps of site-directed mutagenesis of CtIP wild type. The pDEST pCR3 plasmid for the expression of FLAG-DNA2 was a gift from Kerstin Gari (ZHAW School of Life Sciences). The sequence of all primers used in this study is listed in Supplemental Table 2.

Co-immunoprecipitation

HEK293T cells were seeded in 10-cm dishes at a confluency of 20-25%. The next day, cells were co-transfected with 3 μg of FLAG-DNA2 wild type or empty vector (EV) and 3 μg of GFP-CtIP wild type or F728E-Y736E mutant using calcium phosphate. Two days later, cells were washed with cold PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl,

1 mM EDTA, 6 mM EGTA, 15 mM sodium pyrophosphate, and 1% IGEPAL CA-630 [Sigma]), supplemented with phosphatase inhibitors (20 mM NaF and 0.5 mM Na_3VO_4) and protease inhibitors (1 mM benzamidine and 1 mM PMSF). Lysates were syringed, incubated for 2 h rolling at 4°C with 500 units of DENARASE (c-LEcta) and cleared by centrifugation at 18'000 g for 20 min at 4°C. Protein concentration was measured by Bradford Protein Assay (BioRad) according to manufacturer's instructions and 3 mg of lysates were incubated with 25 µl of anti-FLAG M2 beads slurry (Sigma) over night at 4°C. Beads were then washed three times with NP-40 buffer and once with TEN100 buffer (50 mM Tris-HCl pH 7.5, 140 mM NaCl, and 5 mM EDTA). All washing steps were conducted by spinning cells at 2'500 g for 2 min at 4°C.

Generation of *EXO1* knock-out cell lines

To knock-out *EXO1* gene in U2OS-SA and U2OS-EJ2 (Stark et al. 2004), DIvA (Zhou et al. 2014) and 293 Flp-In T-REx cells (Invitrogen), oligonucleotides corresponding to crRNAs and tracrRNA (IDT) were mixed at a final concentration of 40 µM in 10 µl of nuclease-free duplex buffer (IDT), followed by annealing upon boiling and cool-down to obtain the crRNA-tracrRNA duplex targeting *EXO1*. The sequence of the two crRNAs targeting *EXO1* used in this study is listed in Supplemental Table 1. Immediately before transfection, 0.75 µl of each duplex was mixed with 18 µg of SpCas9 Nuclease V3 (IDT) in a total volume of 3 µl followed by incubation for 20 min at room temperature to allow the formation of the ribonucleotide-protein (RNP) complex. Transfection was performed with the 10 µl Neon Transfection System kit (Invitrogen). 1 x 10^6 of the desired cell type was resuspended in 10 µl of the Neon electroporation buffer R (Invitrogen) before the addition of 3 µl of the RNP complex. Cells were then transfected with the Neon Transfection System (Invitrogen) according to the

recommended voltage. Particularly, 4 pulses at 1230 V with a width of 10 ms were used for U2OS-SA, U2OS-EJ2 and DIvA cells, while 3 pulses at 1325 V with a width of 10 ms were used for HEK293 Flp-In T-REx cells. After nucleofection, cells were plated in 1 ml antibiotic-free media onto 12-well plates and left for 24 h. Fresh medium was then added to the cells, which were immediately subcloned for clonal selection. To verify the deletion of *EXO1*, cells were lysed in RIPA buffer; proteins in the cell extract were quantitated with *DC* protein assay (BioRad) according to manufacturer's instructions. Subsequent SDS-PAGE and Western blotting were used to analyzed the EXO1 expression (refer to the Immunoblotting methods section for further details).

Generation of stable CtIP-expressing HEK293 Flp-In T-REx cells

The Flp-In T-REx system was used to generate HEK293 cell lines stably expressing different siRNA-resistant FLAG-tagged CtIP constructs under the control of a doxycycline-inducible promoter. In brief, HEK293 Flp-In T-REx (Invitrogen) cells were transfected with siRNA-resistant expression vectors pcDNA5/FRT/TO-CtIP_WT-2XFLAG or pcDNA5/FRT/TO-CtIP_F728E-Y736E-2XFLAG and the Flp recombinase expression plasmid, pOG44, mixed in a 1:9 ratio using Lipofectamine 2000 transfection reagent (Invitrogen). 24 h post transfection, the cells were plated at different dilutions for clonal selection. Once the cells were attached, fresh medium supplemented with 150 μg/ml Hygromycin B Gold (Invivogen) was added. Cell selection was performed for approximately 10 days with the medium changed every 2-3 days. Resistant colonies were picked and single-cell clones were analyzed for CtIP expression by immunoblotting. CtIP expression was induced with 1 μg/ml doxycycline for 48 h.

Cloning, expression and purification of recombinant proteins

Wild type DNA2 and helicase-deficient DNA2-K654R were expressed in *Spodoptera frugiperda* 9 (*Sf*9) insect cells and purified by affinity chromatography taking advantage of the N-terminal 6X his-tag and the C-terminal FLAG-tag (Pinto et al. 2016; Anand et al. 2018). DNA2-L623E-K636E-L640E mutant was prepared by mutating the respective pFB-6Xhis-hDNA2co-FLAG plasmid by QuickChange II XL site-directed mutagenesis kit following manufacturer's instructions (Agilent Technology) and purified following the same procedure used for the wild type protein. The sequence of all primers used in this study is listed in Supplemental Table 2. MRN was prepared using the 10Xhis- and FLAG-tags at the C-termini of MRE11 and RAD50, respectively (Anand et al. 2016; Anand et al. 2018). Wild type pCtIP was obtained taking advantage of the maltose-binding protein (MBP)-tag at the N-terminus and 10X his-tag at the C-terminus (Anand et al. 2016). The constructs for the expression of CtIP-F728E-Y736E, CtIP-S723A, CtIP-S723E, CtIP-3A and CtIP-3E were prepared by mutating the respective pFB-2XMBP-CtIP-10Xhis plasmid by QuickChange II XL site-directed mutagenesis kit following manufacturer's instructions (Agilent Technology). For expression of phosphorylated CtIP (pCtIP) variants, *Sf*9 cells were treated with 50 nM of the phosphatase inhibitor Okadaic acid (APExBIO) for 3 h prior to collection to preserve proteins in their phosphorylated state, and 1 μM CPT (Sigma) for 1 h prior collection to further activate protein phosphorylation cascade (Anand et al. 2016; Anand et al. 2018). Human RPA was expressed in *E. coli* and purified using ÄKTA pure (GE Healthcare) with HiTrap Blue HP, HiTrap desalting and HiTrap Q chromatography columns (all GE Healthcare) (Anand et al. 2018).

Preparation of DNA substrates

Oligonucleotide-based DNA substrates were ^{32}P-labeled at the 3' terminus with [α-^{32}P]dCTP (Perkin Elmer) and terminal transferase (New England BioLabs) according to the manufacturer's instructions (Pinto et al. 2018). Unincorporated nucleotides were removed using Micro Bio-Spin P-30 Tris chromatography columns (Bio-Rad). To prepare the quadruple blocked 70 bp-long dsDNA substrate, the oligonucleotides PC210 and PC211 were used (Cannavo and Cejka 2014). The sequence of all oligonucleotides used for DNA substrates preparation is listed in Supplemental Table 4. The randomly labeled 2.2-knt-long substrate was prepared amplifying the human *NBS1* gene by PCR from pFB-MBP-NBS1-his plasmid (Anand et al. 2018) using Phusion high-fidelity DNA polymerase (New England BioLabs) and the NBS1_F and NBS1_R primers. The sequence of all primers used in this study is listed in Supplemental Table 2. 66 nM [α-^{32}P]dCTP was added to the PCR reaction together with the standard dNTPs concentration (200 μM each). The PCR reaction product was purified using the QIAquick PCR purification kit (Qiagen) and Chroma Spin TE-200 columns (Clontech). Purified DNA was quantitated by comparing the radioactive DNA fragment with known amounts of a cold PCR product on an agarose gel stained with GelRed (Biotium). The randomly labeled 2.2-kbp-long substrate was then boiled at 95°C for 5 min to obtain ssDNA. The HindIII digest of λ DNA (New England BioLabs) was labeled at the 3' end with [α-^{32}P]dATP (Perkin Elmer) and the Klenow fragment of DNA polymerase I (New England BioLabs). Free nucleotides were removed with Micro Bio-Spin P-30 Tris chromatography columns (BioRad). Prior to each experiment, the substrate was denatured by heating at 95°C for 5 min to obtain ssDNA. For the ATPase assay, the 10.3-kbp-long pFB-MBP-hMLH3 plasmid (Ranjha et al. 2014) was linearized with NheI restriction enzyme (New England Biolabs) and the reaction product was purified with QIAquick PCR purification kit (Qiagen). The substrate was denatured at 95°C for 5 min before each experiment.

ATPase assays

The ATPase assays were performed in 10 µl volume in 25 mM Tris-acetate pH 7.5, 3 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 1 mM ATP, 6 nM of [γ-32P] adenosine 5'-triphosphate (Perkin Elmer) and 0.32 nM (in molecules) of the heat-denatured 10.3-kbp-long DNA substrate. 395.5 nM RPA and the indicated recombinant proteins were added on ice and samples were incubated at 37°C for 10 min. Reactions were stopped with 1.1 µl of 0.5 M EDTA and separated using TLC plates (Merk) and 0.3 M LiCl and 0.3 M formic acid as the mobile phase. Dried plates were exposed to storage phosphor screens (GE Healthcare) and scanned by a Typhoon 9500 phosphorimager (GE Healthcare).

Single-molecule analysis of resection tracks

To measure the length of the resected DNA fragments, SMART (single-molecule analysis of resection tracks) assay was performed either embedding the DNA fibers in agarose (SMART-plug) (Huertas and Cruz-Garcia 2018) or directly lysing the cells and stretching the DNA on coverslip (SMART-tilt) (Altieri et al. 2020). On the first case, SMART was performed as previously described (Huertas and Cruz-Garcia 2018). Briefly, U2OS cells stably expressing different versions of CtIP (WT, CtIP-F728E-Y736E or CtIP-S723A) were grown in the presence of 10 µM BrdU (Sigma) for less than 24 h. Cultures were then irradiated (10 Gy) and harvested after 1 h. Cells were embedded in low-melting agarose (BioRad), followed by DNA extraction. DNA fibers were stretched on silanized coverslips (Genomic Vision, COV-002-RUO), baked for 2 h at 65ºC and incubated directly without denaturation with an anti-BrdU (Abcam, ab6326, 1:100). After washing with PBS, coverslips were incubated with the secondary antibody (Alexa

Fluor 488 anti-rat, Molecular probes, A-11006, 1:500). Finally, coverslips were mounted with ProLong Gold Antifade Reagent (Molecular Probes, P36930). Samples were observed with a Nikon NI-E microscope and PLAN FLOUR40 3 /0.75 PHL DLL objective and images were taken and processed with the NIS ELEMENTS Nikon Software. For each experiment, at least 150 DNA fibers were analyzed, and the length of the fibers was measured with Fiji Image software analysis. For the new adapted SMART, cultures were grown in the presence of 32 µM CldU (Sigma) for 24 h. They were then irradiated (10 Gy) for 1 h at 37°C and harvested using accutase (eBioscience, 00-4555-56). Cells were centrifuged at 500 g for 4 min, resuspended in PBS and mixed in a 1:8 proportion with unlabeled cells. For DNA stretching, 3 µl of this mix were lysed using a spreading buffer (50 mM EDTA, 0.5% SDS, 200 mM Tris-HCl pH 7.4) to later stretch the nucleic acid fibers on silanized slides (Sigma) tilting the slides to a 15° angle. After air-drying the DNA fibers for 10 min, they were fixed in 3:1 methanol/acetic acid at -20ºC for 15 min. Slides were then washed twice in PBS, incubated in 70% ethanol overnight at 4ºC and washed twice again in PBS. For immunodetection, samples were blocked with 5% BSA (Sigma) in PBS for 30 min at RT and incubated with anti-BrdU (Abcam, ab6326, 1:100) for 1 h at 37ºC. Slides were washed with PBS twice and incubated with the fluorescent secondary antibody (Alexa Fluor 488 anti-rat, Molecular probes, A-11006, 1:500) for 1 h at RT. Finally, samples were washed again twice in PBS, dried and mounted with ProLong Gold Antifade Reagent (Molecular Probes). DNA fibers images were acquired with a Leica Thunder Microscope with automatized stage and a 63x oil immersion objective (Leica). For each experiment, at least 200 DNA fibers were measured using the Fiji image software analysis. The experiments presented in Fig. 4 and S6 contain data that were pooled from the BrdU and CldU labelling experiments.

Supplemental references

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