APPENDIX

Chk1 loss creates replication barriers that compromise survival independently of origin firing levels

Marina A. González Besteiro, Nicolás L. Calzetta, Sofía M. Loureiro, Martín Habif, Rémy Bétous, Marie-Jeanne Pillaire, Antonio Maffia, Simone Sabbioneda, Jean-Sébastien Hoffmann and Vanesa Gottifredi

MATERIALS AND METHODS

siRNAs. siRNAs were purchased from Dharmacon or Eurofins Genomics: siLuc 5´-CG UACGCGGAAUACUUCGA-3´ (Speroni *et al*, 2012); siChk1 (100nM) 5´-GAAGCAGUC GCAGUGAAGA-3´ (Speroni *et al*, 2012); siPolη (50nM) 5´-CUGGUUGUGAGCAUUCG UGUA-3´ (Vallerga *et al*, 2015); siCDC7 (100nM) 5´-GCAGUCAAAGACUGUGGAU-3´ (Petermann *et al*, 2010); siCDT1 (50nM) 5´-GCGCAAUGUUGGCCAGAUC-3´ (Beck *et al*, 2012); siWEE1 (50nM) 5´-GGGAAUUUGAUGUGCGACA-3´ (Beck *et al*, 2010); siCDC45 (5nM) 5´-GCAAGACAAGAUCACUCAA-3´; siMUS81 5´-CAGCCCUGGUGG AUCGAUA-3´ (Wechsler *et al*, 2011); siMCM2 5´-GGAGCUCAUUGGAGAUGGCAUG GAA-3´ (Ibarra *et al*, 2008); siGENOME SMARTpool siRecQ1 5´-GAGCUUAUGUUA CCAGUUA-3´, 5´-CUACGGCUUUGGAGAUAUA-3´, 5´-GAUUAUAAGGCACUUGG UA-3´, 5´-GGGCAAGCAAUGAAUAUGA-3´ (Mendoza-Maldonado *et al*, 2011); siRad51 5´-AAGCUGAAGCUAUGUUCGCCA -3´ (Hicks *et al*, 2010).

Site directed mutagenesis. Serine (S) or Threonine (T) were mutated to Alanine (A) or Aspartate (D) using the following forward primers (reverse primers are the reverse complement of forward primers): S416D 5´-CTGGAATCCAGACAGAATGGGATCCTC CTCTCACAAT GC-3'; S512D 5´-CAGGCTCCCATGAGCAATGACCCATCCAAGCCC TC-3´; T591D 5´-CTAGAAGAATCCTCTAAAGCAGACCCTGCAGAGATGGA TTTG-3´; S687D 5´-AG AAATCCCAAGGACCCTTTGGCCTGCACTAATAAACGC-3´; S687A 5´-AGAAATCCCAAGGCCCCTTTGGCCTGCACTAATAAACGC-3´. The following forward primer was used to create the catalytically inactive mutant GFP-Polη-DEAD (D115A and E116A): 5´-GCTGTGATTGAACGTGCCAGCATTGCTGCGGCTTACGTAGATCTG ACCAGTGC-3´.

Primary antibodies for Western Blot. α-Chk1 (Santa Cruz Biotechnology, G-4), α-Polη (Santa Cruz Biotechnology, H-300), α-PCNA (Santa Cruz Biotechnology, PC10), α-γH2AX (Millipore), α-phosphoS296-Chk1 (Cell Signaling), α-fibrillarin (Sigma), α-

GFP (Santa Cruz Biotechnology, B-2), α-H2B (Santa Cruz Biotechnology, E-6), α-WEE1 (Santa Cruz Biotechnology, B-11), α-CDC7 (Santa Cruz Biotechnology, SPM171), α-CDC45 (Santa Cruz Biotechnology, H300), α-MCM2 (Abcam), α-MUS81 (Santa Cruz Biotechnology, B-12), α-Rbp1 NTD (Cell Signaling), α-Ku80 (Abcam), α-actin (Sigma).

Quantitative Real-Time PCR. Cells were lysed and total RNA was extracted using TRIzol Reagent (Invitrogen). An oligo-dT primer and the M-MLV Reverse Transcriptase (Invitrogen) were used for cDNA synthesis from total RNA (1μg). The Light Cycler 480 System (Instrument and SYBR Green I Master, Roche) was used. Two independent biological samples were analyzed, and one representative set of results is shown. Primer sequences were: CDT1-forward 5'-GAACGGCTGCCTGAGCT-3', CDT1-reverse 5'-CCATTTCCCCAGGGCTCA-3'; GADPH-forward 5'-AGCCTCCCGCTTCGC TCTCT-3', GADPH-reverse 5'-GAGCGATGTGGCTCGGCTGG-3' (Vallerga *et al*, 2015); MCM2-forward 5'-GTGGATAAGGCTCGTCAGAT-3', MCM2-reverse 5'-GTCGT GGCTGAACTTGTT-3' (Hua *et al*, 2014).

2D gel electrophoresis. 2D-PAGE was performed as in (Bertoletti *et al*, 2017). Briefly, cell extracts were prepared in Rehydration Buffer (Bio-Rad) with protease and phosphatase inhibitors and sonicated for 3 cycles/30 seconds with a Diagenode Bioruptor before incubation with Benzonase (50U, Merck Millipore). After centrifugation, the samples were quantified and 120μg of total protein extracts were loaded on a 7cm IPG strip (pH3-10, Bio-Rad). After passive (30') and active (14 h) rehydration, the first dimension was performed on a voltage gradient at 50mA. After focusing, the strips were washed in Equilibration Buffer (Bio-Rad) with 20mg/ml DTT or 25mg/ml iodoacetamide (Sigma-Aldrich), respectively. The second dimension was run on 10% polyacrylamide gels and analyzed by western blot with specific antibodies.

FIGURES

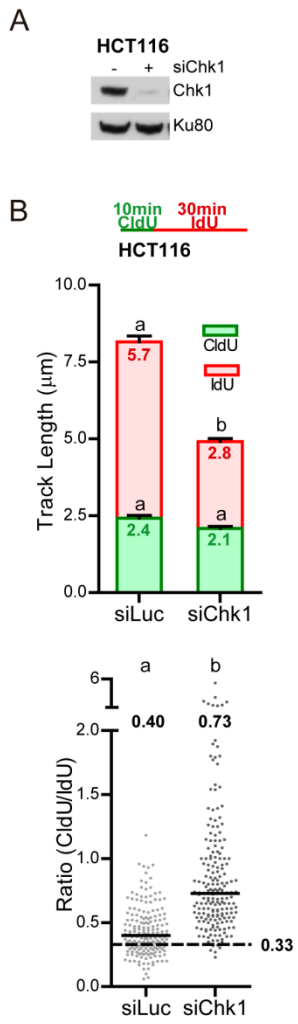

Figure S1 – Replication fork progression is unsteady after Chk1 loss.

A Western Blot of Chk1 in HCT116 cells, 48 h after transfection with siRNA. Ku80 was used as a loading control.

B HCT116 cells were labelled with CldU and IdU for 10 and 30 min, respectively. Mean (+SEM) track lengths (top) and CldU/IdU ratios (bottom) are shown. Data come from 2 independent experiments and a total of 190 (siLuc) and 196 (siChk1) fibers were scored.

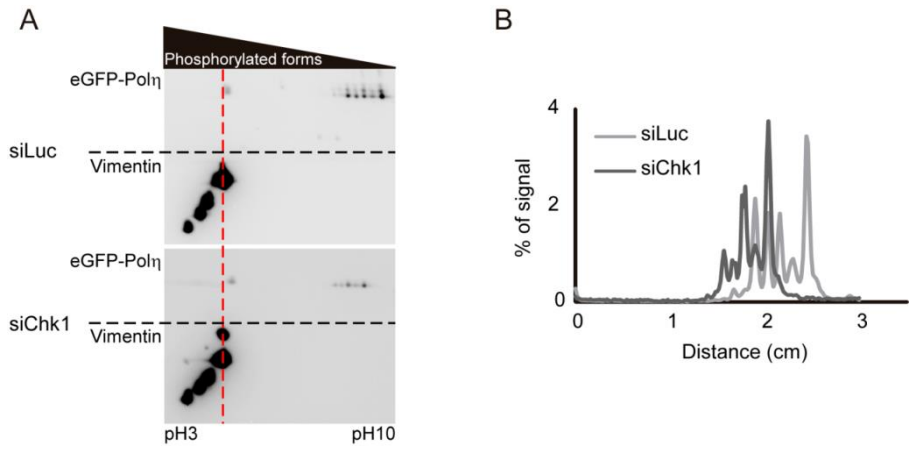

Figure S2 – Polη is hyper-phosphorylated after Chk1 loss.

A Representative (n=3) 2D-PAGE analysis of XP30RO cells expressing eGFP-Polη after Chk1 silencing. 2D-PAGE gels were aligned by using Vimentin (shown) and PCNA (not shown) as triangulation markers. The red dotted line is used as a reference for the alignment.

B Densitometric analysis of the blots shown in A.

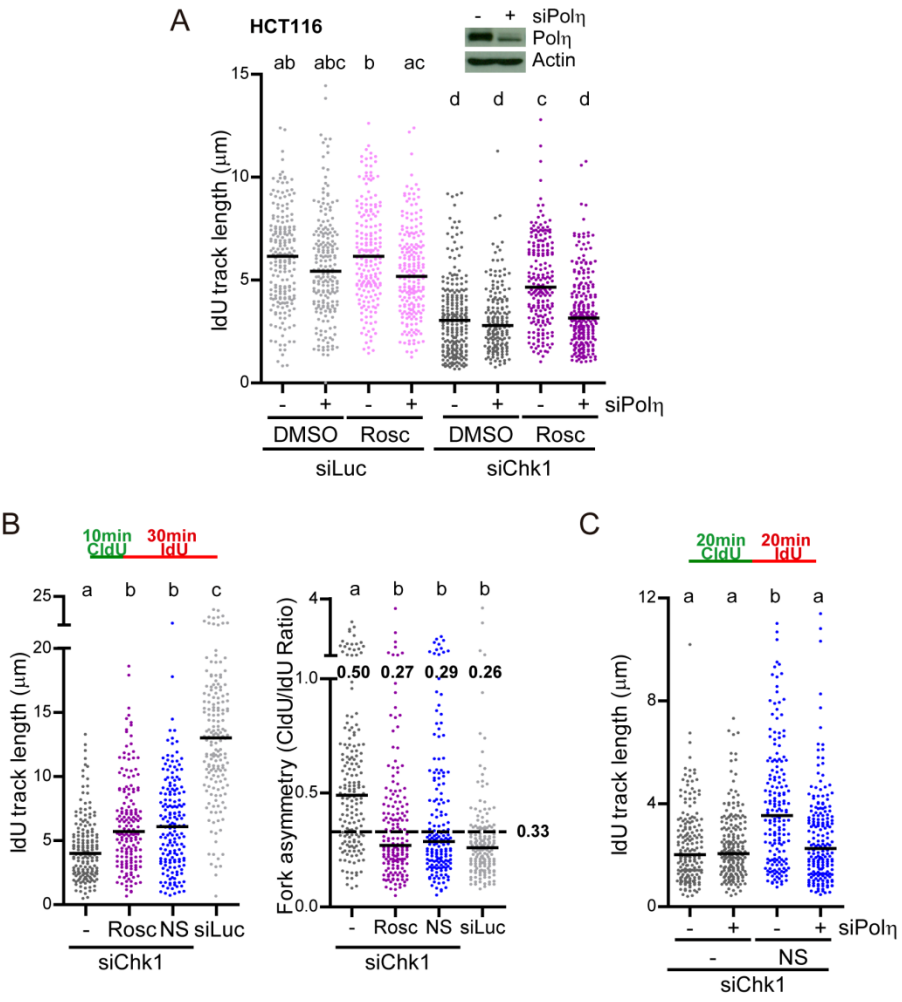

Figure S3 – High CDK activity and low NS availability prevent Polη-dependent bypass of replication barriers created by Chk1 loss.

A IdU track lengths from HCT116 cells. >180 fibers obtained from 2 independent experiments were scored for each condition. The upper panel shows a Western Blot of Polη in HCT116, 48 h after transfection with siRNA. Actin was used as loading control.

B U2OS cells were labelled with CldU and IdU for 10 and 30 min, respectively. IdU track lengths (left panel) and CldU/IdU ratios (right panel) are shown (NS, nucleosides). Data come from 2 independent experiments and a total of 171 fibers per condition were scored.

C IdU track lengths from U2OS cells. >200 fibers obtained from 2 independent experiments were measured for each condition.

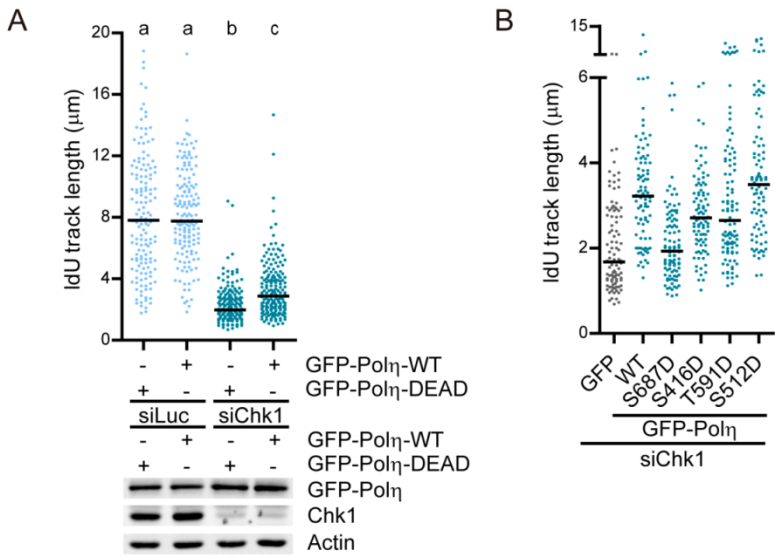

Figure S4 – Serine 687 phosphorylation status determines GFP-Polη function at replication barriers created by Chk1 loss.

A IdU track lengths from siChk1-depleted U2OS cells transfected with GFP-Polη-WT or GFP-Polη-DEAD. >150 fibers obtained from 2 independent experiments were measured for each condition. $p<0.01$ was considered significant. The lower panel shows the corresponding Western Blot of Chk1 and GFP-Polη. Actin was used as a loading control.

B IdU track lengths from siChk1-depleted U2OS cells transfected with GFP-Polη-WT/S687D/S512D/S416D/T591D. A representative experiment (n=2) is shown; >90 fibers/sample were scored.

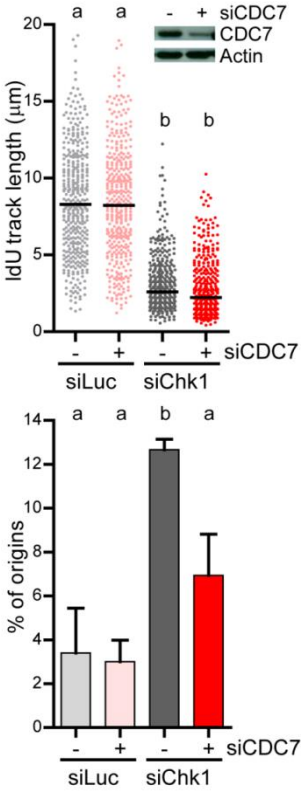

Figure S5 – Replication fork progression and origin firing are independent variables in Chk1-depleted cells.

IdU track lengths and percentage of origin firing (mean+SD) from U2OS cells. >380 (track length) and >450 (origin frequency) fibers obtained from 4 (track length) or 3 (origin frequency) independent experiments were measured for each condition. Some of the data plotted here (siLuc and siChk1) are equal to those shown in Fig 5A. The upper panel shows a Western Blot of CDC7, 48 h after transfection with siRNA. Actin was used as a loading control.

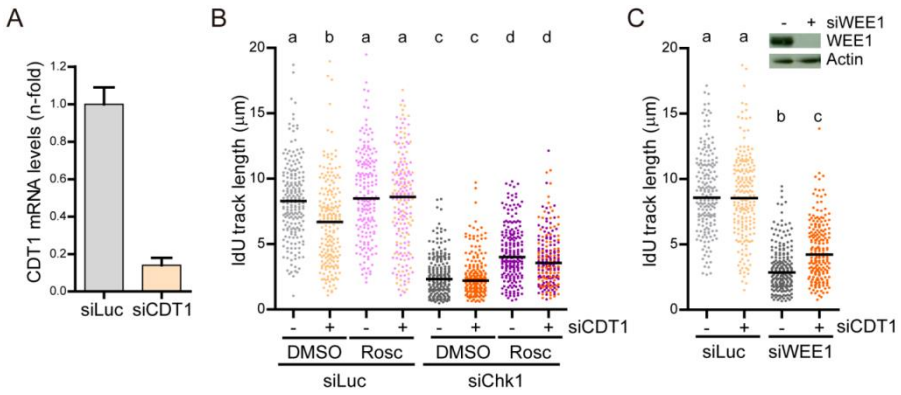

Figure S6 – CDT1 downregulation *per se* does not impair fork elongation.

A Quantitative Real-Time PCR of CDT1 normalized to GAPDH in U2OS cells, 48 h after transfection with siRNA. A representative experiment is shown (n=2). Error bars represent the SD of 2 technical replicates.

B IdU track lengths from U2OS cells. 200 fibers obtained from 2 independent experiments were measured for each condition.

C IdU track lengths from U2OS cells. 200 fibers obtained from 2 independent experiments were measured for each condition. The upper panel shows a Western Blot of WEE1, 48 h after transfection with siRNA. Actin was used as a loading control.

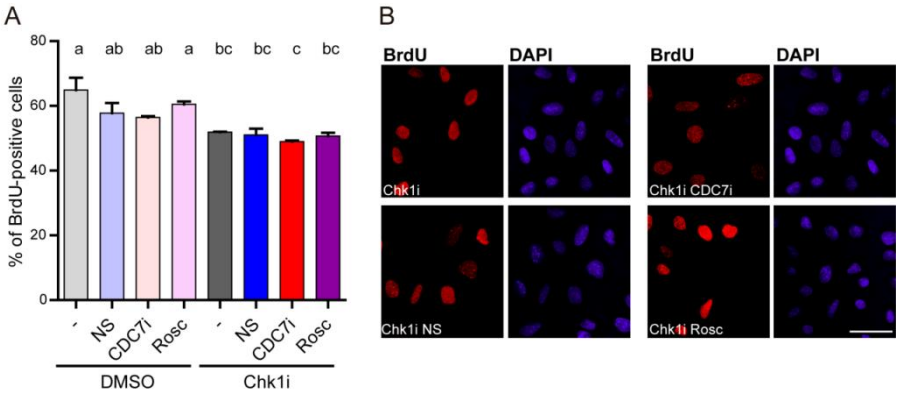

Figure S7 – Modulation of replication fork progression and origin firing in Chk1-inhibited cells does not affect the percentage of S-phase cells in short-term experiments.

A Percentage of S-phase (BrdU-positive) U2OS cells (mean+SD) treated as described in Fig 6. >600 cells/sample were analyzed in 2 independent experiments.

B Representative images of data shown in A. Scale bar: 50 μm.

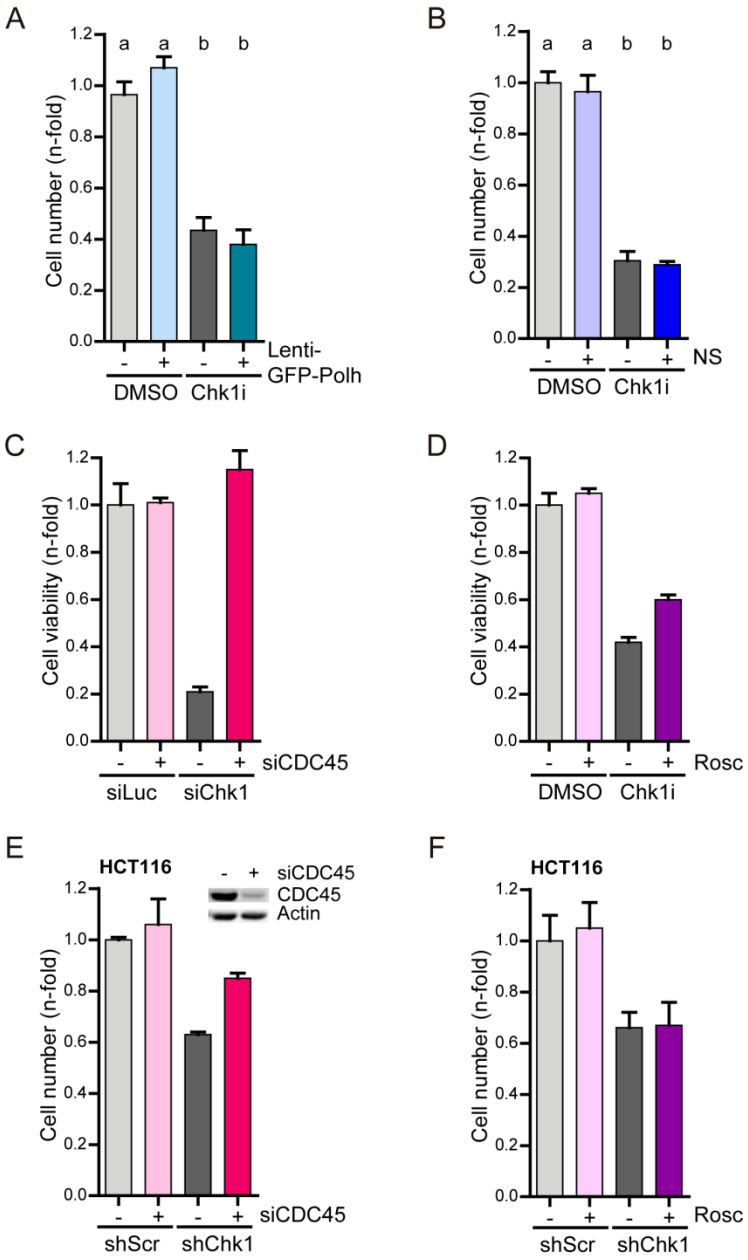

Figure S8 – CDC45-dependent generation of replication barriers, not their bypass, compromise the fitness of Chk1-deficient cells.

A Sensitivity of U2OS cells transduced with Lenti-GFP-Polη or Lenti-GFP (-) to Chk1 inhibition. Cell number was determined 4 days after a 24 h (5 days in total) treatment with Chk1i. Data represent the mean (+SD) of 2 independent experiments.

B Sensitivity of U2OS cells to Chk1 inhibition and NS. Cell number was determined 4 days after a 24 h (5 days in total) treatment with Chk1i±NS. Data represent the mean (+SD) of 2 independent experiments.

C Sensitivity of U2OS cells to Chk1 and CDC45 depletion. Cell viability was determined 7 days after transfection (5 days after maximal downregulation). A

representative experiment is shown (n=3). Error bars represent the SD of 3 technical replicates.

D Sensitivity of U2OS cells to Chk1 inhibition and Rosc (2.5 μM). Cell viability was determined 4 days after a 24 h (5 days in total) treatment with Chk1i±Rosc. A representative experiment is shown (n=3). Error bars represent the SD of 3 technical replicates.

E Sensitivity of HCT116 cells to Chk1 downregulation and CDC45 depletion. Cell number was determined 3 days after infection/transfection. A representative experiment is shown (n=2). Error bars represent the SD of 3 technical replicates. The upper panel shows a Western Blot of CDC45, 48 h after transfection with siRNA. Actin was used as a loading control.

F Sensitivity of HCT116 cells to Chk1 downregulation and Rosc. Rosc was added 24 h after infection and maintained until the end of the experiment, 5 days afterwards. A representative experiment is shown (n=2). Error bars represent the SD of 3 technical replicates.

REFERENCES

Beck H, Nahse-Kumpf V, Larsen MS, O'Hanlon KA, Patzke S, Holmberg C, Mejlvang J, Groth A, Nielsen O, Syljuasen RG, Sorensen CS (2012) Cyclin-dependent kinase suppression by WEE1 kinase protects the genome through control of replication initiation and nucleotide consumption. *Mol Cell Biol* 32**:** 4226-4236

Beck H, Nahse V, Larsen MS, Groth P, Clancy T, Lees M, Jorgensen M, Helleday T, Syljuasen RG, Sorensen CS (2010) Regulators of cyclin-dependent kinases are crucial for maintaining genome integrity in S phase. *J Cell Biol* 188**:** 629-638

Bertoletti F, Cea V, Liang CC, Lanati T, Maffia A, Avarello MDM, Cipolla L, Lehmann AR, Cohn MA, Sabbioneda S (2017) Phosphorylation regulates human poleta stability and damage bypass throughout the cell cycle. *Nucleic Acids Res* 45**:** 9441-9454

Hicks JK, Chute CL, Paulsen MT, Ragland RL, Howlett NG, Gueranger Q, Glover TW, Canman CE (2010) Differential roles for DNA polymerases eta, zeta, and REV1 in lesion bypass of intrastrand versus interstrand DNA cross-links. *Mol Cell Biol* 30**:** 1217-1230

Hua C, Zhao G, Li Y, Bie L (2014) Minichromosome Maintenance (MCM) Family as potential diagnostic and prognostic tumor markers for human gliomas. *BMC cancer* 14**:** 526

Ibarra A, Schwob E, Mendez J (2008) Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. *Proc Natl Acad Sci U S A* 105**:** 8956-8961

Mendoza-Maldonado R, Faoro V, Bajpai S, Berti M, Odreman F, Vindigni M, Ius T, Ghasemian A, Bonin S, Skrap M, Stanta G, Vindigni A (2011) The human RECQ1 helicase is highly expressed in glioblastoma and plays an important role in tumor cell proliferation. *Molecular cancer* 10**:** 83

Petermann E, Woodcock M, Helleday T (2010) Chk1 promotes replication fork progression by controlling replication initiation. *Proc Natl Acad Sci U S A* 107**:** 16090-16095

Speroni J, Federico MB, Mansilla SF, Soria G, Gottifredi V (2012) Kinase-independent function of checkpoint kinase 1 (Chk1) in the replication of damaged DNA. *Proc Natl Acad Sci U S A* 109**:** 7344-7349

Vallerga MB, Mansilla SF, Federico MB, Bertolin AP, Gottifredi V (2015) Rad51 recombinase prevents Mre11 nuclease-dependent degradation and excessive PrimPol-mediated elongation of nascent DNA after UV irradiation. *Proc Natl Acad Sci U S A* 112**:** E6624-6633

Wechsler T, Newman S, West SC (2011) Aberrant chromosome morphology in human cells defective for Holliday junction resolution. *Nature* 471**:** 642-646