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Priego Moreno, Sara; Bailey, Rachael; Campion, Nicholas; Herron-Marx, Sandra; Gambus, Agnieszka

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Supplementary Materials for

Polyubiquitylation drives replisome disassembly at the termination of DNA replication

Sara Priego Moreno, Rachael Bailey, Nicholas Campion, Suzanne Herron, Agnieszka Gambus*

*Corresponding author. E-mail: a.gambus@bham.ac.uk

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This PDF file includes

Materials and Methods
Supplementary Text
Figs. S1 to S8
Full Reference List
Replication assay
The replication reactions were started with the addition of demembranated *Xenopus* sperm DNA to 10-15 ng/µl as described before (30). The synthesis of nascent DNA was measured by quantification of $\alpha^{32}$P-dATP incorporation into newly synthetised DNA as described before (30).

Chromatin and nuclei isolation
Chromatin assembled in *Xenopus* egg extract was isolated in ANIB/100 buffer as described before (30). A sample without sperm DNA addition (no DNA) was processed in an analogous way to provide a chromatin specificity control. The nuclei isolation was performed in Buffer A as in (31). Proteins were released from chromatin in ANIB/100 as described before (8).

Recombinant proteins
Recombinant untagged and His$_6$-tagged ubiquitin and ubiquitin mutants were purchased from Boston Biochem, dissolved in LFB1/50 buffer at 10 mg/ml and used at 0.5 mg/ml in the extract. Recombinant p97 and p97D1D2 mutant (E305Q, E578Q) were expressed and purified from *E.coli* as before (24). p27(KIP1) was expressed and purified from *E.coli* as in (32) and used at 100 nM, while geminin$^{\text{DEL}}$ was a kind gift from J.J. Blow laboratory (33).

Inhibitors and antibodies
Bortezomib (B-1408) was purchased from LC Laboratories, dissolved at 200 mM in DMSO and used at 100 µM, MLN4924 (A01139) from Active Biochem was dissolved in DMSO at 20 mM and used at 5 µM. ICRF193 (14659) from Scientific Lab Supplies was dissolved at 10 mM and used at 20 µM as previously described (2).
Aphidicolin (Aph) (Sigma) was dissolved in DMSO at 10 mM and used at 40 µM. Mitomycin C (MMC) (Calbiochem) was dissolved at 5 mM in water and used at 500 µM. Etoposide (Calbiochem) was dissolved in DMSO at 30 mM and used at 400 µM. Camptothecin (CPT) was dissolved in DMSO at 50 mM and used at 500 µM. EcoRI (Roche) was used at 0.05 U/µl egg extract. Caffeine (Sigma) was dissolved at 100 mM in water and used at 5 mM.
Monoclonal anti-PCNA (P8825) and monoclonal anti-PolyHistidine (H1029) antibodies were purchased from Sigma. Anti-Mcm2 (BM28) antibody was from BD Transduction Laboratories, anti-p97 ATPase (65278) from Progen Biotechnik and anti-P-Chk1 (S345) from Cell Signalling. Mcm3 affinity purified antibodies were previously described (34), as were Mcm4, Mcm5, Mcm6 and Mcm7 antibodies (35). Cdc45, Psf2, Sld5, Psf1, Ctf4 and Mcm10 antibodies were described before (8), as was Cyclin B2 antibody (18), ATR antibody (36) and Cdt1 antibody (37).

The antibody against *Xenopus* RecQ4L was raised in sheep against *E.coli* expressed N-terminally His6-tagged 1-260 aa fragment of RecQ4L and affinity purified (Fig. S9A), while anti-XCut5 antibody was raised in sheep against C-terminal 333 aa as previously described (38) and affinity purified (Fig. S9B).

To detect ubiquitylated Mcm7 membranes were incubated with Mcm7 antibody at 1:500 in 3% BSA solution overnight at 4°C, followed by 2-5h secondary antibody incubation at RT and visualized with SuperSignal West Pico Chemiluminescent Substrate ECL solution (Thermoscientific) after long exposures.

**His6-Ubi Pulldowns**

Interphase egg extract was supplemented with 100 µM Bortezomib, 0.5 mg/ml His6-Ubi, 15 ng/µl demembranated sperm DNA and optionally with 100 µM p27(KIP1). Chromatin was isolated in the middle of S-phase (peak of fork proteins on chromatin), or as indicated, with ANIB/100 buffer as previously described (8) but with addition of 5 mM N-Ethylmaleimide (NEM) (Acros Organics) and 20 mM iodoacetamide (GE Healthcare).

Once isolated, chromatin was pelleted at 14000g for 2 min at 4°C, after which the remaining buffer was carefully removed and the pellet resuspended in 0.1 of the original extract volume of ANIB/100 (+ NEM, + iodoacetamide) buffer and 5 U/µl of Benzonase (Sigma) added and incubated at RT for 15 min with occasional mixing. After DNA digestion the chromatin mixture was denatured in 0.9 volume of the extract used of Urea buffer (8 M urea, 100 mM potassium phosphate, 10 mM Tris HCl, 10 mM 2-mercamptoethanol, pH 8.0) and mixed at RT for 30 min, before being mixed with 0.25 original extract volume of pre-washed HIS-Tag Isolation and Pulldown Dynabeads (10103D, Life Technologies) and rotated for 1 h at RT. The beads were then washed for 5 min with 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.2% triton, 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.1% triton, 1 ml of Urea buffer + 20 mM imidazole. Finally beads were eluted with Urea buffer + 400 mM imidazole for 1h at RT or boiled directly in the 1x NuPAGE LDS loading buffer (Life Technologies).

Monoubiquitylated PCNA served as a positive control.

**His6-Ubi Pulldowns**

Interphase egg extract was supplemented with 100 µM Bortezomib, 0.5 mg/ml His6-Ubi, 15 ng/µl demembranated sperm DNA and optionally with 100 µM p27(KIP1). Chromatin was isolated in the middle of S-phase (peak of fork proteins on chromatin), or as indicated, with ANIB/100 buffer as previously described (8) but with addition of 5 mM N-Ethylmaleimide (NEM) (Acros Organics) and 20 mM iodoacetamide (GE Healthcare).

Once isolated, chromatin was pelleted at 14000g for 2 min at 4°C, after which the remaining buffer was carefully removed and the pellet resuspended in 0.1 of the original extract volume of ANIB/100 (+ NEM, + iodoacetamide) buffer and 5 U/µl of Benzonase (Sigma) added and incubated at RT for 15 min with occasional mixing. After DNA digestion the chromatin mixture was denatured in 0.9 volume of the extract used of Urea buffer (8 M urea, 100 mM potassium phosphate, 10 mM Tris HCl, 10 mM 2-mercamptoethanol, pH 8.0) and mixed at RT for 30 min, before being mixed with 0.25 original extract volume of pre-washed HIS-Tag Isolation and Pulldown Dynabeads (10103D, Life Technologies) and rotated for 1 h at RT. The beads were then washed for 5 min with 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.2% triton, 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.1% triton, 1 ml of Urea buffer + 20 mM imidazole. Finally beads were eluted with Urea buffer + 400 mM imidazole for 1h at RT or boiled directly in the 1x NuPAGE LDS loading buffer (Life Technologies).

Monoubiquitylated PCNA served as a positive control.

**His6-Ubi Pulldowns**

Interphase egg extract was supplemented with 100 µM Bortezomib, 0.5 mg/ml His6-Ubi, 15 ng/µl demembranated sperm DNA and optionally with 100 µM p27(KIP1). Chromatin was isolated in the middle of S-phase (peak of fork proteins on chromatin), or as indicated, with ANIB/100 buffer as previously described (8) but with addition of 5 mM N-Ethylmaleimide (NEM) (Acros Organics) and 20 mM iodoacetamide (GE Healthcare).

Once isolated, chromatin was pelleted at 14000g for 2 min at 4°C, after which the remaining buffer was carefully removed and the pellet resuspended in 0.1 of the original extract volume of ANIB/100 (+ NEM, + iodoacetamide) buffer and 5 U/µl of Benzonase (Sigma) added and incubated at RT for 15 min with occasional mixing. After DNA digestion the chromatin mixture was denatured in 0.9 volume of the extract used of Urea buffer (8 M urea, 100 mM potassium phosphate, 10 mM Tris HCl, 10 mM 2-mercamptoethanol, pH 8.0) and mixed at RT for 30 min, before being mixed with 0.25 original extract volume of pre-washed HIS-Tag Isolation and Pulldown Dynabeads (10103D, Life Technologies) and rotated for 1 h at RT. The beads were then washed for 5 min with 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.2% triton, 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.1% triton, 1 ml of Urea buffer + 20 mM imidazole. Finally beads were eluted with Urea buffer + 400 mM imidazole for 1h at RT or boiled directly in the 1x NuPAGE LDS loading buffer (Life Technologies). Monoubiquitylated PCNA served as a positive control.

**His6-Ubi Pulldowns**

Interphase egg extract was supplemented with 100 µM Bortezomib, 0.5 mg/ml His6-Ubi, 15 ng/µl demembranated sperm DNA and optionally with 100 µM p27(KIP1). Chromatin was isolated in the middle of S-phase (peak of fork proteins on chromatin), or as indicated, with ANIB/100 buffer as previously described (8) but with addition of 5 mM N-Ethylmaleimide (NEM) (Acros Organics) and 20 mM iodoacetamide (GE Healthcare).

Once isolated, chromatin was pelleted at 14000g for 2 min at 4°C, after which the remaining buffer was carefully removed and the pellet resuspended in 0.1 of the original extract volume of ANIB/100 (+ NEM, + iodoacetamide) buffer and 5 U/µl of Benzonase (Sigma) added and incubated at RT for 15 min with occasional mixing. After DNA digestion the chromatin mixture was denatured in 0.9 volume of the extract used of Urea buffer (8 M urea, 100 mM potassium phosphate, 10 mM Tris HCl, 10 mM 2-mercamptoethanol, pH 8.0) and mixed at RT for 30 min, before being mixed with 0.25 original extract volume of pre-washed HIS-Tag Isolation and Pulldown Dynabeads (10103D, Life Technologies) and rotated for 1 h at RT. The beads were then washed for 5 min with 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.2% triton, 1 ml of Urea buffer + 20 mM imidazole, 1 ml of Urea buffer + 20 mM imidazole + 0.1% triton, 1 ml of Urea buffer + 20 mM imidazole. Finally beads were eluted with Urea buffer + 400 mM imidazole for 1h at RT or boiled directly in the 1x NuPAGE LDS loading buffer (Life Technologies). Monoubiquitylated PCNA served as a positive control.
**Immunoprecipitations**

Interphase egg extract was supplemented with 15 ng/µl demembranated sperm DNA and optionally with indicated inhibitors. Chromatin was isolated in the middle of S-phase with ANIB/100 with addition of 5 mM NEM (Acros Organics) and 20 mM iodoacetamide (GE Healthcare), and digested with Benzonase as described before (8). Chromatin samples were supplemented with affinity purified sheep Mcm3 antibodies (34) or IgG from sheep serum (I5131 Sigma) at 5 µg per 100 µl of extract used. After an hour-long incubation on ice, immunoprecipitation samples were mixed with washed Protein G Dynabeads (Life Technologies) at 20 µl per 100 µl original extract and incubated with mixing at 4°C for 1 h. Beads were washed twice with ANIB/100 buffer as above, once with the buffer supplemented with additional 0.1% triton X-100 and again twice with the buffer alone. Immunoprecipitated proteins were eluted off beads by boiling in 2x NuPAGE LDS loading buffer (Life Technologies).

**Gel filtration analysis**

The chromatin samples of middle of S-phase or His6-UbiNOK late S-phase (when forks are not detectable on chromatin in control) were prepared and separated through Superose 6 column as described before (8).

**DNA fibre preparation**

Metaphase-arrested *Xenopus* egg extracts were prepared and induced to interphase as above. Extracts were also supplemented with 400 µM BrdUTP and 15 ng/µl demembranated *Xenopus* sperm and incubated at 23°C for 37.5 min (early S-phase).

For isolation of nuclei, the method used was adapted from that described previously (39). Extract was diluted in 350 µl Nuclear Isolation buffer supplemented with 10 µg/ml aphidicolin and then underlayed with the same buffer containing 15% sucrose. Chromatin was pelleted at 2100 g for 5 min at 4°C, after which the overlying buffer was carefully removed and the nuclear pellet resuspended in 400 µl PBS/0.1% Triton X-100. This was underlaid with PBS containing 15% sucrose and spun at 2100g for 5 mins at 4°C. The cushion was removed and the pellet was resuspended in PBS.

To prepare DNA fibres, 2 µl aliquots of BrdUTP-labelled nuclei were spotted onto a glass slide and lysed with 0.5% SDS, 200 mM Tris-HCl, pH 7.4, 50 mM EDTA (5 min, 20°C). Slides were tilted 15° horizontally to allow DNA to run slowly down the slide, air dried, and fixed in methanol/acetic acid (3:1).

**Immunolabelling of DNA fibres**

Spread fibres were incubated with 2.5 M HCl (1 h 15 min, 20°C) to denature DNA, then washed in PBS and incubated with a PBS solution containing 1% BSA and 0.1% Tween 20 for 30 min at 20°C. Slides were incubated (1 h, 20°C) with a mixture of rat anti-BrdUTP IgG (1:1000, Bio-rad
AbD Serotec) and mouse anti-ssDNA IgG (1:500, Millipore), washed three times with PBS and three times with a PBS/1% BSA/0.1% Tween solution, then incubated (1 h 30 min, 20°C) with a mixture of anti-rat AlexaFluor 555 IgG (1:500, Life Technologies) and anti-mouse AlexaFluor 488 IgG (1:500, Life Technologies). Finally, slides were washed twice in PBS, three times in PBS/1% BSA/0.1% Tween solution, and mounted in Fluoroshield (Sigma).

Fibres were examined using the AMG Evos Fl imaging system with a 100X oil-immersion objective. Lengths of red (AF 555) or green (AF 488) labelled tracks were measured using the Image J software (National Institutes of Health; http://rsbweb.nih.gov/ij/), with arbitrary lengths converted into micrometers using scale bars created by the microscope.

**Effects of His$_6$-UbiNOK on termination by DNA fibre analysis**

Metaphase-arrested *Xenopus* egg extracts were prepared and supplemented as above. Extracts were also supplemented with 400 µM BrdUTP and 15 ng/µl demembranated *Xenopus* sperm and incubated at 23°C for 90 min to allow BrdUTP incorporation, in the presence of either 40 µM ICRF193 (2) or His$_6$-UbiNOK. Chromatin was isolated and the incorporation of a second nucleotide analogue, Dig-dUTP for a further 30 min in a new extract (in the presence of geminin and cyclohexamide to prevent re-initiation) was used to estimate DNA replication termination.

DNA fibres were immunolabelled as above, using a rat anti-BrdUTP IgG (1:1000, Bio-rad AbD Serotec) and a mouse anti-digoxigenin IgG (1:500, Abcam). The short termination tracks were measured in the same way as longer fibres using conversion 1 µm = 2.6 kb based on (40), which is commonly used for fibre experiments. Jackson et al. (40) amplified with BrdU a 36 kb viral genome and measured Br-labeled DNA molecules of 13.9 ± 1.3 µm to work out the conversion. The mean track length we measured was 0.69 µm, with resolution of our microscope setting at 0.262 µm.

**Figure S1. His$_6$-Ubi and His$_6$-UbiNOK in Xenopus egg extract.**

(A) Recombinant His$_6$-tagged ubiquitin (His$_6$-Ubi) is used efficiently to ubiquitylate proteins in egg extract in the presence of endogenous ubiquitin. Chromatin was isolated in the middle of S-phase from extract supplemented with 0.5 mg/ml His$_6$-Ubi or untagged ubiquitin (Ubi). PCNA is efficiently ubiquitylated by the recombinant ubiquitin as indicated by the size shift of the monoubiquitin-PCNA due to His$_6$ tag on His$_6$-Ubi. (B) His$_6$-Ubi addition does not affect efficiency and kinetics of DNA replication in the extract. Sperm nuclei were incubated at 10 ng/µl in the extract supplemented with α-[³²P]dATP and His$_6$-Ubi or buffer. At indicated times DNA synthesis was assessed by TCA precipitation and scintillation counting. (C) His$_6$-Ubi addition does not affect temporal order of replication factors binding to chromatin. Interphase egg extract was supplemented with sperm nuclei and His$_6$-Ubi or buffer, chromatin was isolated at indicated times and subjected to western blotting with indicated antibodies. (D) Unloading of a small proportion of Mcm2-7 is delayed after inhibition of polyubiquitylation when lower concentration of sperm DNA is replicated. 5 ng/µl of sperm DNA was replicated in the extract.
supplemented optionally with His<sub>6</sub>-UbiNOK, chromatin was isolated at indicated times and analysed by western blotting. (E) Inhibition of polyubiquitylation does not affect bulk of Mcm2-7 complexes on chromatin. Extract was supplemented optionally with His<sub>6</sub>-UbiNOK, chromatin isolated and analysed by western blotting. The western blot signal for Mcm7 was quantified and normalized to the loading control. The plots from 4 independent experiments in different extracts are presented. (F) CMG subunits accumulated on chromatin upon inhibition of polyubiquitylation form large size protein complexes. Extract was supplemented with His<sub>6</sub>-UbiNOK, chromatin isolated at 90 min, proteins released from chromatin by DNA digestion and separated through a gel filtration column. S-phase chromatin was also analysed for comparison.

**Figure S2. Using His<sub>6</sub>-Ubi and bortezomib in Xenopus egg extract.**

(A) Proteasome driven Cyclin B2 degradation upon exit from meiosis is inhibited by bortezomib in *Xenopus* egg extract. Cyclin B2 is degraded in the extract when extracts exit meiosis upon Ca<sup>2+</sup> stimulation (+ Ca<sup>2+</sup>) but not without such stimulation (- Ca<sup>2+</sup>) (upper panel). Bortezomib is much more efficient across tested concentrations in blocking Cyclin B2 degradation at 15 min post Ca<sup>2+</sup> addition than MG132 proteasome inhibitor (lower panel). (B) Addition of bortezomib to the extract leads to accumulation of ubiquitylated products both in the extract and on chromatin. Interphase extract was supplemented with recombinant His<sub>6</sub>-Ubi and sperm nuclei and optionally with bortezomib, chromatin was isolated at indicated times and subjected to western blotting with anti-His antibody to detect ubiquitylated proteins. (C) Blocking proteasome activity does not affect active helicase chromatin binding. DNA synthesis (left panel) and chromatin binding (right panel) was analysed as in Fig. 1A, but after supplementing extract with bortezomib to block proteosomal activity. (D) Quantification of accumulated active helicase subunit Psf2 and non-helicase replisome component PCNA after polyubiquitylation or proteasome inhibition. The level of accumulated Psf2 and PCNA western blot signal on chromatin at 90 min post sperm nuclei DNA addition was quantified, normalised to histone loading control, compared to control and fold increase over control plotted. An average of 4 experiments with SEM is presented. (E) Inhibition of polyubiquitylation by His<sub>6</sub>-UbiNOK addition delays Cdt1 degradation by 15 min. Interphase extract was supplemented with sperm nuclei and His<sub>6</sub>-UbiNOK or His<sub>6</sub>-Ubi and Cdt1 degradation in the extract was analysed by western blotting. As controls, extract supplemented with bortezomib or p27(KIP1) after 60 min of incubation was analysed.

**Figure S3. Inhibition of polyubiquitylation impacts on unloading of active helicase from chromatin at the end of replication.**

(A) The length of synthesised individual nascent DNA tracks is not affected by polyubiquitylation inhibition. BrdU TP labelled replicated DNA fibres from the early S-phase were visualised and measured as described in the Supplementary Methods. Data from 3 independent experiments. The mean track length values were 4.3 kbp (0.5 SEM) for His<sub>6</sub>-Ubi and 5.0 kbp (1.1 SEM) for His<sub>6</sub>-UbiNOK. (B) Polyubiquitylation inhibition does not affect fork progression in later S-phase. BrdU TP labelled replicated DNA fibres were analysed as in (A) but the BrdU TP was added to the extract for 10 min at indicated times during S-phase. The measured values of 100 tracks per treatment with median is presented. (C) Polyubiquitylation inhibition does not lead to Chk1 phosphorylation. Interphase extract was supplemented with His<sub>6</sub>-Ubi or
His$_6$-UbiNOK, nuclei isolated at indicated times and subjected to western blotting. As a positive control, nuclei were isolated from extract treated with aphidicolin or aphidicolin and caffeine at 60 min post sperm and inhibitor addition. (D) Addition of geminin to egg extract at 3 min after sperm nuclei addition (experiment in Fig. 1B) leads to much reduced levels of chromatin loaded Mcm2-7. Chromatin at 30 min post sperm nuclei addition was isolated from extract optionally supplemented with geminin, His$_6$-Ubi, His$_6$-UbiNOK as indicated and subjected to western blotting. (E) Inhibition of late origin firing does not affect active helicase accumulation after polyubiquitylation inhibition. Late origin firing was inhibited in extracts supplemented with His$_6$-Ubi or His$_6$-UbiNOK by addition of p27(KIP1) at 40 min. Such inhibition marginally reduces the total level of replicated DNA in the extracts as assessed at 120 min after sperm DNA addition (left panel). Throughout the replication reaction chromatin was isolated from all four extracts and analysed by western blotting (right panel). (F) Polyubiquitylation inhibition does not alter interorigin distances. BrdUTP labelled replicated DNA tracks were isolated as in (A) and the distance between middles of two BrdUTP tracks on the same DNA fibre measured. Data from 3 independent experiments are presented. The average inter-origin distance was 7.7 kbp (1.1 SEM) for His$_6$-Ubi and 8.4 kbp (1.7 SEM) for His$_6$-UbiNOK. (G) Active helicase is locked down on chromatin. Chromatin was isolated from extract in the middle of S-phase and resuspended in the isolation buffer with optional Benzonase addition. After 15 min incubation samples were spun down and soluble (released from chromatin) and insoluble (chromatin associated) proteins analysed by western blotting. (H) The termination fibre experiment was performed as in Fig. 1C but the incubation time in the first extract varied as indicated. The length of 100 green tracks per treatment was measured and plotted with marked median value.

**Figure S4. Lysine-48-linked ubiquitin chains modification is needed for active helicase disassembly.**

(A) Interphase extract was supplemented with ubiquitin mutants indicated and the ability of extract to synthesise DNA within 120 min assessed (left panel) while chromatin was isolated at 90 min (late S-phase), analysed by western blotting, Psf2 and Cdc45 signal was quantified, normalised to histone loading control and compared to control (no mutant addition) signal. Average values with SEM are presented. (B) The same as in (A), but the complementary set of ubiquitin mutants has been tested. (C) Chromatin was isolated at indicated times from extract supplemented with His$_6$-Ubi, His$_6$-UbiNOK or His$_6$-UbiK48R and subjected to western blotting.

**Figure S5. Active helicase chromatin unloading depends on activity of cullin family of ubiquitin ligases.**

(A) MLN4924 blocks cullin E3 activity in Xenopus egg extract. Increasing concentrations of MLN4924 were added to the extract at the same time as sperm nuclei DNA addition and cullin ubiquitin ligase dependent degradation of Cdt1 monitored at 60 min by western blotting. (B) MLN4924 blocks cullin activity also when added 15 min after sperm nuclei DNA addition. 5 µM MLN4924 was added to the extract 15 min after sperm addition (to avoid problems with nuclear envelope formation and replication initiation) and Cdt1 degradation was monitored by western blotting. (C) Addition of 5 µM MLN4924 does not block ubiquitin E1 activity in the extract. MLN4924 was added to the extract at 15 min after sperm DNA addition and chromatin isolated in the middle of S-phase to compare the level of monoubiquitylation of PCNA. (D) Blocking cullins leads to active helicase accumulation on chromatin. 5 µM MLN4924 was added at 15 min to the egg extract and its effect on nascent DNA synthesis was assessed (left panel),
while chromatin was isolated at indicated times and subjected to western blotting (middle panel). The accumulation of Psf2 and PCNA on chromatin at 90 min was quantified, normalised to loading control and compared with control. The average of 3 experiments with SEM is presented.

Figure S6. Polyubiquitylation of Mcm7 on replicating chromatin and active helicase accumulation after topoisomerase II inhibition.
(A) Chromatin was isolated at indicated times from extract supplemented with His6-Ubi or His6-UbiNOK and subjected to western blotting. (B) Active helicase subunits accumulate on chromatin upon termination inhibition with ICRF193. The chromatin bound Psf2 and PCNA were quantified at 60 min after sperm DNA addition (late S-phase in used extract), normalised to loading control and the signal fold increase in ICRF193 treated samples over control presented (average of 3 experiments with SEM).

Figure S7. Mcm2-7 interacts with p97, which stimulates its chromatin unloading.
(A) Ubiquitylated Mcm7 is not degraded on chromatin. Chromatin was isolated in the middle of S-phase from extract supplemented with His6-Ubi and optionally bortezomib to block proteasome activity or p27(KIP1) to block replication. Ubiquitylated proteins were pulled down from isolated chromatin and subjected to western blotting. (B) p97 interacts with Mcm2-7 on chromatin. Chromatin was isolated in the later part of S-phase from extract optionally supplemented with p27(KIP1) or MLN4924 and protein complexes released off chromatin followed by Mcm3 immunoprecipitation. Immunoprecipitated samples were analysed by western blotting. Asterisk indicates nonspecific IgG signal. (C) Addition of p97D1D2 mutant does not affect Cdt1 degradation. Extract was supplemented with p97wt, p97D1D2mut (p97mut) or buffer, and Cdt1 degradation during S-phase monitored by western blotting. (D) Ubiquitylated forms of Mcm7 accumulate on chromatin after p97mut addition. Chromatin was isolated at indicated times from extract supplemented with buffer or p97mut and analysed by western blotting.

Figure S8. Characterisation of novel raised antibodies used in this study.
(A) A sample of Xenopus egg extract and chromatin isolated from middle of S-phase were subjected to western blotting with affinity purified anti-XRecQ4L antibodies. (B) As in (A) but with anti-XCut5 antibody.

Supplementary References

**Author contributions**

S.P.M. performed all of the experiments with p97 and some of the ubiquitin mutants experiments; R.B. performed DNA fibre experiments; N.C. performed MLN4942 experiments and Mcm3 IP / mass spectrometry experiment, S.H. helped with revision experiments; A.G. performed the rest of the experiments, designed the project and wrote the manuscript.
Priego Moreno et al Supp Fig 1

A

B

C

D

E

F

Mcm2
Mcm7
Psf2
histones
PCNA

His₆-Ubi

buffer

D Ubi

PCNA-His₆-Ubi

PCNA-Ubi

extract

15 30 45 60 90 120

no DNA

15 25 35 45

[min]

Replicated DNA [ng/μL]

Time [min]

His₆-Ubi

Mcm2
Mcm7
Cdc45
PCNA

extract

15 25 35 45

[min]

% of Mcm7 on chromatin

control

His₆-UbiNOK

control

His₆-UbiNOK

% of Mcm7 on chromatin

control

His₆-UbiNOK

control

His₆-UbiNOK

1200
669
200
66

MW [kD]

Mcm7
Mcm5
Mcm2
Cdc45
Psf2

S phase

His₆-UbiNOK
A

\[ \text{- CaCl}_2 \quad + \text{CaCl}_2 \]

Mcm2
Cyc B2

\[ \text{MG132} \quad \text{Bortezomib} \]

Mcm2
Cyc B2

B

chromatin control
chromatin bortezomib

His\textsubscript{6}-Ubi added, anti-His tag blot

Orc2

C

Replicated DNA [pg/μg]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \quad 120 \quad 140 \]

Time [min]

control bortezomib

D

Fold increase over control

\[ 0 \quad 5 \quad 10 \quad 15 \quad 20 \quad 25 \quad 30 \]

His\textsubscript{6}-UbiNOK bortezomib

E

His\textsubscript{6}-Ubi His\textsubscript{6}-UbiNOK +p27(KIP1) +bortezomib

Cdt1

Priego Moreno et al Supp Fig 2
Priego Moreno et al Supp Fig 3

A

![Graph showing the number of fibres versus track length for His\textsubscript{6}-Ubi and His\textsubscript{6}-UbiNOK](image)

B

![Graph showing track length distribution for different conditions](image)

C

![Western blot images for Mcm2, P-Chk1, and PCNA](image)

D

![Western blot images for Mcm2, Psf2, and PCNA](image)

E

![Graph showing replicated DNA versus time for different conditions](image)

F

![Graph showing the number of fibres versus interorigin distance for His\textsubscript{6}-Ubi and His\textsubscript{6}-UbiNOK](image)

G

![Western blot images for Cut5, Ctf4, Mcm10, RecQ4, PCNA, Mcm2, Mcm5, Mcm7, Cdc45, Psf2](image)

H

![Graph showing track length versus time with and without Benzonase](image)
### Figure 4

#### Panel A

![Graph A](image1.png)

#### Panel B

![Graph B](image2.png)

#### Panel C

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mcm7</th>
<th>Cdc45</th>
<th>Psf2</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td><img src="image3.png" alt="Mcm7" /></td>
<td><img src="image4.png" alt="Cdc45" /></td>
<td><img src="image5.png" alt="Psf2" /></td>
</tr>
<tr>
<td>1</td>
<td><img src="image6.png" alt="Mcm7" /></td>
<td><img src="image7.png" alt="Cdc45" /></td>
<td><img src="image8.png" alt="Psf2" /></td>
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<td>2</td>
<td><img src="image9.png" alt="Mcm7" /></td>
<td><img src="image10.png" alt="Cdc45" /></td>
<td><img src="image11.png" alt="Psf2" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image12.png" alt="Mcm7" /></td>
<td><img src="image13.png" alt="Cdc45" /></td>
<td><img src="image14.png" alt="Psf2" /></td>
</tr>
<tr>
<td>4</td>
<td><img src="image15.png" alt="Mcm7" /></td>
<td><img src="image16.png" alt="Cdc45" /></td>
<td><img src="image17.png" alt="Psf2" /></td>
</tr>
</tbody>
</table>

*Note: Images represent the expression of Mcm7, Cdc45, and Psf2 over time.*
A

<table>
<thead>
<tr>
<th>extract + DNA</th>
<th>extract - DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mcm7-Ubi</td>
<td>Mcm7-UbiNOK</td>
</tr>
<tr>
<td>Mcm7</td>
<td>Mcm7</td>
</tr>
<tr>
<td>Orc2</td>
<td>Orc2</td>
</tr>
<tr>
<td>PCNA</td>
<td>PCNA</td>
</tr>
<tr>
<td>Cdc45</td>
<td>Cdc45</td>
</tr>
<tr>
<td>Psf2</td>
<td>Psf2</td>
</tr>
<tr>
<td>histones</td>
<td>histones</td>
</tr>
</tbody>
</table>

B

Fold increase over control

+ ICRF193

PCNA  | Psf2

0  | 2  | 4  | 6  | 8  | 10 | 12

Priego Moreno et al Supp Fig 6
Priego Moreno et al Supp Fig 7

A

Mcm7 (long)

B

Mcm7 (short)
Mcm3
p97
Cdc45
Psf1

input
IgG
Mcm3

IP

S phase + bortp27(KIP1)
S phase - bort
S phase

S phase + bort
S phase

S phase

input

His-pulldown

C

buffer
buffer
p97mut
p97mut

Cdt1
Mcm2

D

control
p97mut

Mcm7-Ubi
Mcm7
PCNA
Psf2
histones
References


