Figures and figure supplements

Translesion polymerase kappa-dependent DNA synthesis underlies replication fork recovery

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Figure 1. PolK is required for replication fork restart due to nucleotide deprivation. (A) Schematic for measuring replication fork restart by DNA fiber analysis. Quantification of fork restart efficiency (% stalled forks) in HU-treated (2 mM) RPE-1 cells using two independent siRNAs against individual TLS.

B) Time course of fork restart in siPolK #1 and siPolK #2.

C) Effect of deoxynucleosides with no HU wash off.

D) Effect of HU wash off followed by deoxynucleosides with no HU wash off.

E) Effect of 5 mM APH or Gem.

F) Western blot analysis of phosphorylated CHK1, RPA32, and CHK2 after HU, APH, or Gem treatment.

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Figure 1 continued

Pols as indicated. Representative images of the DNA fiber tracts are shown. (B) Quantification of fork restart efficiency in RPE-1 cells comparing different HU (2 mM) treatment time-points in the presence or absence of PolK siRNA knockdown. (C) Quantification of fork restart efficiency in HU-treated RPE-1 cells with either a wash step with fresh media or with no wash (HU still present) supplemented with 250 μM deoxynucleosides (dNs) for recovery. (D) Western blot analysis of RPE-1 cells treated with 2 mM HU for 4 hr followed by either a wash step with fresh media or no wash (HU still present) supplemented with 250 μM deoxynucleosides (dNs) or 250 μM ribonucleosides (rNs) for 30 or 60 min chase. (E) Quantification of fork restart efficiency comparing fork-stalling agents, HU (2 mM) or APH (5 μM), in the presence or absence of PolK siRNA knockdown. (F) Western blot analysis of RPE-1 cells treated with either HU (2 mM), APH (5 μM), or Gemcitabine (Gem, 1 μM) for 4 hr, followed by a wash step and recovery in fresh media for 2 hr. Data for % stalled forks are represented by mean ± s.d. of three independent experiments and p-values calculated using t-test with Welch’s correction. n.s. = no significance, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

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Figure 1—figure supplement 1. siRNA knockdown efficiencies and complementation of CRISPR 293T sgPolK clonal cells.
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Figure 1—figure supplement 2. Gemcitabine-induced stalled forks are not amenable for fork restart assays.
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Figure 2. PolK functions in concert with the FA pathway to promote replication fork restart. (A) Quantification of fork restart efficiency in FANCD2-deficient patient cells (PD20) complemented with either vector only, FANCD2 WT, or K561R mutant in the presence or absence of PolK siRNA and treated as indicated. PD20 (vector only) cells were treated with 300 nM Chk1i (AZD7762) throughout the duration of HU and CldU time points as a positive control for the detection of elevated fork-stalling events. Western blot analysis showing siRNA knockdown efficiency in PD20 cells. (B) Quantification of fork restart efficiency in 293T CRISPR PolK (sgPolK) cells complemented with either empty vector or GFP-PolK WT in the presence or absence of FANCD2 siRNA and treated as indicated. CldU (red) tract length measurements of restarted forks determine the varying degree of individual fork restart events. Western blot analysis showing expression and siRNA knockdown efficiency in sgPolK 293 T cells. Data for % stalled forks are represented by mean ± s.d. of three independent experiments and p-values calculated using t-test with Welch’s correction. Data for tract length measurements are plotted from three independent experiments with mean ± s.e.m. and p-values calculated using Mann-Whitney t-test. n.s. = no significance, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

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Figure 3. PoI\(K\) interacts with K48-linked polyubiquitinated PCNA via its UBZ domains in a HU-dependent manner. (A) Western blot analysis of RPE-1 cells treated with the indicated siRNAs and HU (2 mM) time-points. (B) Western blot analysis of 293T sgPoI\(K\) cells complemented with either empty plasmid, plasmid encoding PoI\(K\) WT, or plasmid encoding PoI\(K\) K164R and treated with HU (2 mM) time-points. (C) Diagram showing the domains of PoI\(K\) and their interactions with PCNA. (D) Western blot analysis of 293T cells transfected with poI\(K\) siCtrl, poI\(K\) siPol\(K\), or poI\(K\) siFANCD2 and treated with HU (2 mM) time-points. (E) Diagram showing the fork restart assay protocol. (F) Western blot analysis of RPE-1 cells transfected with poI\(K\) siCtrl, poI\(K\) siPol\(K\), or poI\(K\) siFANCD2 and treated with HU (2 mM) time-points.
vector or GFP-PolK WT and pulsed with HU (2 mM) for 4 hr before wash step and recovery for the indicated time-points. (C) Schematic diagram showing domains of PolK. Formaldehyde-induced crosslinking of 293T sgPolK cells treated with HU (2 mM) for 4 hr as indicated. Extracts from cells complemented with either empty vector, GFP-PolK WT or a double ubiquitin-binding domain mutant (UBZ) of GFP-PolK were then subjected to anti-GFP pulldown, followed by Western blot analysis with the indicated antibodies. (D) Ubiquitin chain restriction digest analysis using similarly treated and immunoprecipitated (IP) samples as in (C) to enrich for polyubiquitinated PCNA that is bound by GFP-PolK and induced by HU. Samples on beads were then incubated with 900 ng of indicated recombinant DUBs for 1 hr at 37°C prior to Western blot analysis with the indicated antibodies (upper and lower panels). SARS PLpro catalytic mutant (C112A) was used for negative control as indicated (lower panel). (E) Quantification of fork restart efficiency in 293T sgPolK cells complemented with either empty vector, GFP-PolK WT, Catalytic-Dead (CD), or ubiquitin-binding mutant (UBZ). CldU (red) tract length measurements of restarted forks were determined for WT and the different PolK mutants. (F) Quantification of fork restart efficiency in U2OS cells treated with PCNA siRNA and complemented with siRNA-resistant HA-tagged PCNA-WT or ubiquitin site mutant HA-PCNA K164R. Western blot analysis showing exogenously expressed siRNA-resistant HA-PCNA in U2OS cells. Data for % stalled forks are represented by mean ± s.d. of three independent experiments and p-values calculated using t-test with Welch’s correction. Data for tract measurements are plotted from three independent experiments with mean ± s.e.m. and p-values calculated using Mann-Whitney t-test. n.s. = no significance, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

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**Figure 3—figure supplement 1.** PolK interacts with FANCD2 and RPA independently of its UBZ domain.

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**Figure 3—figure supplement 2.** HU-dependent PCNA polyubiquitination is susceptible to an in vitro K48-specific polyUb DUB cleavage reaction. DOI: https://doi.org/10.7554/eLife.41426.008

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Figure 4. PolK prevents MRE11-dependent nascent DNA degradation. (A) Schematic for measuring nascent DNA degradation (shortened CldU-labeled tracts) by DNA fiber analysis (A–C,E). Quantification of nascent DNA degradation (changes in CldU tract lengths) in 293 T cells treated with the

**Figure 4 continued on next page**
indicated siRNAs. (B) Quantification of nascent DNA degradation in 293T sgPolK cells that were complemented with either empty vector, GFP-PolK WT or the indicated GFP-PolK mutants. (C) Quantification of nascent DNA degradation in 293T sgPolK cells complemented with either empty vector or GFP-PolK WT were treated with or without Mre11 inhibitor, Mirin (50 μM), in the presence of HU as indicated. (D) Quantification of fork restart efficiency in RPE-1 cells with the indicated siRNAs and treated with or without Mirin (50 μM) in the presence of HU (2 mM) as indicated. (E) Quantification of nascent DNA degradation in parental 293T or 293T sgPolK cells treated with the indicated siRNAs. (F) Quantification of fork restart efficiency in parental 293T or 293T sgPolK cells treated with the indicated siRNAs. Data for % stalled forks are represented by mean ± s.d. of three independent experiments and p-values calculated using t-test with Welch’s correction. Data for tract length measurements are plotted from three independent experiments with mean ± s.e.m. and p-values calculated using Mann-Whitney t-test. n.s. = no significance, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

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Figure 4—figure supplement 1. RAD51 and BRCA2 depletion in RPE-1 cells has no effect on replication fork restart after HU treatment.

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Figure 5. PolK-dependent DNA synthesis in the presence of HU. (A) Schematic for measuring replication fork speed (CldU tract length) under HU treatment for 4 hr. To ensure that the CldU-labeled DNA was under constant high dose HU treatment, cells were pre-treated with 2 mM HU for 40 min.
prior to the addition of CldU in the presence of HU for 4 hr. Quantification of fork speed in RPE-1 cells treated with the indicated siRNAs. (B) Schematic of a modified iPOND assay to measure proteins associated with the replisome under nucleotide starvation conditions. Similar to (A), 293 T cells were either left untreated or pretreated with HU (2 mM) for 40 min prior to the addition of EdU (10 μM) in the presence of HU for 4 hr. Cells were then either collected immediately (EdU samples) or chased with Thymidine (10 μM) for 1 hr (EdU + chase) in the presence or absence of HU. Samples without HU were treated for only 10 min with EdU or chased with Thymidine for 1 hr. Western blot analysis showing the biotin-streptavidin pulldown after click-reaction in parental 293 T cells and probed with the indicated antibodies. (C) Schematic for measuring EdU incorporation intensity by direct fluorescence measurements in HU-treated cells. U2OS sgPolK cells were complemented with either empty vector, GFP-PolK WT, GFP-PolK mutant constructs, or different Y-family TLS Pols, GFP-Pol eta or GFP-Pol iota. Cells were pretreated with HU (2 mM) for 1 hr, prior to the addition of EdU (10 μM) in the presence of HU for 4 hr. Mean EdU intensity per nucleus measured by ImageJ were plotted from three independent experiments. (D) Single-molecule localization imaging of EdU signal distribution per foci or nuclei. RPE-1 cells were treated with the indicated siRNAs prior to pretreatment with HU (2 mM) for 1 hr, followed by the addition of EdU in the presence of HU for 4 hr. Representative super-resolution images of nuclei with EdU signal in magenta are shown. Quantification of EdU foci counts per nuclei and amount of EdU counts per foci are plotted from three independent experiments. (E) U2OS sgPolK cells complemented with either GFP-PolK WT or GFP-PolK CD were pulse-labeled with EdU and treated with HU (2 mM) or not (NT). Treatment conditions and quantification of EdU foci per nuclei by super-resolution imaging techniques were done as in (D). (F) Quantification of fork restart efficiency in RPE-1 cells treated with the indicated siRNAs whereby ‘restarted’ forks are measured as previously elongating forks (IdU tracts) that become converted to CldU tracts in the presence of HU (2 mM). Cells were pretreated with HU for 40 min prior to the addition of CldU for 4 hr to ensure that CldU pulse-labeled cells were already under constant high-dose HU treatment. (G) Quantification of fork restart efficiency in RPE-1 cells treated with the indicated siRNAs whereby IdU pulse-labeled forks under constant HU treatment are measured to determine whether they can be ‘restarted’ after HU wash off (CldU pulse-label). Data for % stalled forks and quantification of EdU foci counts by single-molecule localization imaging are represented by mean ± s.d. of three independent experiments and p-values calculated using t-test with Welch’s correction and indicated above the plots. Data for tract length measurements are plotted from three independent experiments with mean ± s.e.m. and p-values calculated using Mann-Whitney t-test. n.s. = no significance, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

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**Figure 5—figure supplement 1.** Mirin treatment does not affect replication fork speed in either PolK- or FANCD2-depleted RPE-1 cells.
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Figure 5—figure supplement 2. PolK-dependent DNA synthesis under HU is unaffected by DRB treatment.
DOI: https://doi.org/10.7554/eLife.41426.013
Figure 5—figure supplement 3. Single-molecule localization image of EdU signal, PCNA, and GFP-PolK in U2OS cells.
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Figure 6. Loss of PolK leads to a p53-dependent cell cycle delay and 53BP1 nuclear body accumulation following HU pulse treatment. (A) Schematic for measuring cell cycle progression after recovery from HU pulse treatment (A–C). RPE-1 cells treated with the indicated siRNAs were initially pulsed-

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labeled with EdU (10 μM) for 1 hr to label untreated S-phase cells, followed by a wash step, an HU (2 mM) pulse treatment for 4 hr, another wash step, and recovery (chase) with fresh media for the indicated time. Recovery of EdU-positive, HU pulse-treated cells were tracked by FACS analysis and the proportion of cells in different cell cycle phases were determined by DAPI DNA content (FlowJo). Data represented from three independent experiments with mean ± s.d (A). (B) RPE-1 cells treated with the indicated siRNAs were treated with HU (4 hr), followed by a wash step, and chase for 24 hr with fresh media. Cells were then fixed and stained for SA-β-Gal activity. Data represented from three independent experiments with mean ± s.d., p-value calculated using t-test with Welch’s correction. (C) RPE-1 cells treated with the indicated siRNAs were treated with HU (4 hr), followed by a wash step, and chase for 18 hr with fresh media. Cells were then fixed and co-stained for Cyclin A and 53BP1. Only Cyclin A-negative RPE-1 cells (G1 phase) were quantified for 53BP1 nuclear bodies. Data represented from three independent experiments with a minimum of 300 Cyclin A-negative cells per experiment; mean ± s.d. was plotted and p-value calculated using t-test with Welch’s correction. (D) A model depicting how PolK promotes replication stress recovery and genome stability in an FA pathway-dependent manner in response to conditions of nucleotide starvation. n.s. = no significance, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.
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Figure 6—figure supplement 1. Mirin treatment does not rescue genome instability in HU pulse-treated PolK- or FANCD2-deficient cells.

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