Supplemental Information

Supplemental Figure Legends

Figure S1. Effects of PARPi in two consecutive cell cycles

(**A**) Examples showing how cells are classified into subpopulations of different cell-cycle phases. Left panel shows a sample taken directly after EdU pulse labeling, and right panel is from a sample taken 8 hours after EdU labeling. Gray dots represent EdU-negative cells and red dots are EdU-positive. (**B**) Effective inhibition of baseline PARylation by Olaparib in the first S phase. Cells were treated with DMSO, 2 μM Olaparib or 10 μM Olaparib (PARPi) in combination with or without 0.0005% methyl methanesulfonate (MMS) for 4 hours. Levels of PARylation were analyzed by western blot. MMS-induced PARylation serves a positive control for the detection of baseline PARylation in DMSO-treated cells. (**C**) An extension of Fig. 1A including additional time points in the first cell cycle and a lower concentration of Olaparib. U2OS cells were pulse-labeled with 2 μM EdU for 15 minutes and released in the presence or absence of 2 or 10 μM Olaparib. Cells were pre-extracted with detergent at indicated time points before immunofluorescence analysis of PCNA and EdU detection by click chemistry. Individual cells are plotted according to their DNA content (X axis), PCNA intensity (Y axis), and EdU positivity (colored in red). EdU-labeled cells were classified into G1, early-S, mid-S, late-S and G2 subpopulations, and the fractions of EdU-labeled cells in the subpopulations are shown in the stacked bar charts. (**D-E**) Effects of Veliparib and Talazoparib on two consecutive cell cycles. Samples were treated as in (C) but with indicated doses of (D) Veliparib or (E) Talazoparib. (**F**) Effects of EdU and PARPi on fork stability in the second S phase. Cells were labeled with EdU for 90 min and then given 26.5 hours to progress into the second S phase. During the last 4 hours of the time course, cells

were treated with either 10 μM Olaparib or DMSO. At the end of the time course, cells were analyzed by CldU/IdU labeling (20 minutes each) and DNA fiber assay. The longer/shorter ratios (CldU/IdU or IdU/CldU) of replication tracts in EdU^- and EdU^+ fibers were determined. More than 125 fibers for each condition were analyzed ($n > 125$). Significance was determined with a Mann-Whitney U test. **: p-value < 0.01, ***: p-value < 0.001.

Figure S2. PARPi induces a robust DNA damage response in the second S phase

(**A**) Quantification of western blots shown in Fig. 1B. The ratios of p-RPA32 S33 or p-Chk1 S317 level to total RPA32 or Chk1 level were first determined for each sample and then normalized to the 0-hour time point. Error bars: standard deviation of two independent experiments. (**B-D**) Representative microscopy images for the data presented in Fig. 1D-F. Scale bars: 10 μm. (**E**) The entry into the second S phase is requited for the robust induction of DNA damage by PARPi. Cells were pulse-labeled with 2 μM EdU for 15 minutes and then treated with DMSO, 10 μM Olaparib, 10 μM RO-3306, or Olaparib and RO-3306 for 24 hours. Mean γH2AX foci intensity of EdU-positive cells was quantified. Red bar: median intensity. More than 150 EdU-positive cells were analyzed in each sample ($n>150$). Significance was determined with a Mann-Whitney U test. ****: p-value < 0.0001. (**F**) Cell-cycle profiles of cells in Fig. 1G. Individual cells are plotted according to DNA content (X axis), PCNA intensity (Y axis), and EdU positivity (colored in red). The percentage of EdU-labeled cells in the second G1 was quantified in each sample.

Figure S3. Characterizations of PARPi-induced ssDNA gaps

(**A-F**) Effects of PrimPol and RECQ1 knockdown on the levels of ssDNA gaps in multiple experiments. (A, D) Western blot confirmation of siRNA-mediated PrimPol (A) and RECQ1 (D) knockdown. U2OS cells were transfected with indicated siRNA for 48 hours before processing for

western blot. (B, E) An extension of Fig 2C-D using two independent PrimPol and RECQ1 siRNAs. (C, F) Reproducibility of S1 nuclease assays in multiple experiments. The median values of the IdU/CldU ratios for each condition from or 3 or 4 repeats of Fig 2C-D are represented. Red bar: mean. Significance was calculated with two-tailed Student's T-tests. ****: p-value < 0.0001, n.s.: p-value > 0.05. (**G**) Quantifications of the western blots in Fig 2F. RAD51 iPOND signals were normalized to the inputs and presented as a ratio of the thymidine chase signal to the fork signal. Error bars represent the standard deviation from two independent experiments. Significance was calculated with two-tailed student's T-tests. *: p-value < 0.05. (**H**) PARPi induced RAD51 chromatin binding in the first and second S phases. Cells were treated with 10 μM PARPi for 4 or 24 hours and analyzed by chromatin fractionation. Relative levels of chromatin-bound RAD51 were normalized to histone H3. Significance was calculated with a two-tailed Student's T-test of 3 independent experiments. *: p-value < 0.05. Error bars: Standard deviation. (**I**) PARPi-induced ssDNA gaps persist into the next G1 phase. U2OS cells were treated as in Fig 2B except that cells were incubated for 24 hours in DMSO or 10 μM Olaparib in the presence or absence of 10 μM palbociclib before S1 nuclease digestion. Red bar: median IdU/CldU ratio. More than 125 CldU/IdU double-positive replication tracts were analyzed in each sample (n>125). Significance was determined with a Mann-Whitney U test. ****: p-value < 0.0001, n.s.: p-value > 0.05. (**J**) Compilation of the median values for the IdU/CldU ratios of three independent experiments shown in Fig 2I. Red bars: mean. Significance was calculated with two-tailed student's T-tests. **: p-value < 0.01.

Figure S4. Characterizations of PARPi-induced ssDNA formation

(**A**) Representative images for the data shown in Fig. 3A. Scale bar: 20 μm. (**B**) Cell-cycle profiles of cells in Fig. 3A with overlaid BrdU intensity. Cells were plotted according to mean PCNA

intensity (Y axis), DAPI intensity (X axis), and mean BrdU intensity (color gradient). (**C**) Effects of PrimPol knockdown on the cell cycle. U2OS cells were transfected with siCTRL or siPrimPol for 48 hours. Cells were then pulse labeled with 2 μM EdU for 15 minutes and released in the presence or absence of 10 μM Olaparib. Cells were pre-extracted with detergent at indicated time points before immunofluorescence analysis of PCNA and EdU detection by click chemistry. Individual cells are plotted according to DNA content (X axis), PCNA intensity (Y axis), and EdU positivity (colored in red). EdU-labeled cells were classified into G1, early-S, mid-S, late-S and G2 subpopulations, and the fractions of EdU-labeled cells in the subpopulations are shown in the stacked bar charts. (**D**) Cell-cycle profiles of cells in Fig. 3B with overlaid BrdU intensity. Cells were plotted according to mean PCNA intensity (Y axis), DAPI intensity (X axis), and mean BrdU intensity (color gradient). (**E**) PARPi-induced ssDNA formation in the second S phase requires CtIP. U2OS cells were transfected with siCTRL or siCtIP for 48 hours and then exposed to 10 μM Olaparib or DMSO for 24 hours. BrdU (20 μM) was added 24 hours after transfection and maintained upon exposure to Olaparib or DMSO. Two hours before the end of the experiment, BrdU was washed out and cells were incubated with fresh media with or without Olaparib. BrdU-labeled ssDNA and PCNA were analyzed by immunofluorescence under a non-denaturing condition. The BrdU intensity of S-phase cells ($PCNA^+$) was quantified. Red bar: median intensity. At least 160 S-phase cells were analyzed in each sample ($n \geq 160$). Significance was determined with a Mann-Whitney U test. ****: p-value < 0.0001.

Figure S5. Effects of BRCA1 on PARPi-induced RAD51 loading and ssDNA formation

(**A**) BRCA1 is required for RAD51 loading onto post-replicative DNA. HEK293T cells were treated DMSO or 10 μM Olaparib for 60 minutes, and nascent DNA was labeled with 10 μM EdU during the last 20 minutes. Cells were either processed for iPOND to capture proteins at

progressing forks (Fork, F), or were chased with thymidine (T-chase, T) for 45 minutes to capture proteins behind replication forks. Levels of the indicated proteins in input cell extracts and iPOND samples analyzed by western blot (**B**) Representative images for the data shown in Fig. 4C. Scale bars: 20 μm. (**C**) BRCA1 foci in two consecutive cell cycles. Cells were transfected with siBRCA1 for 72 hours. Cells were either untreated or treated with 10 μM Olaparib during the last 4 or 24 hours. S-phase cells were labeled by adding 10 μM EdU in the last 15 minutes. The total intensity of BRCA1 foci in at least 100 individual S-phase cells (n > 100) was quantified. Significance was determined with a Mann-Whitney U test. *: p-value < 0.05, ****: p-value < 0.0001. (**D**) Representative images for the data shown in Fig. 4D. Scale bars: 20 μm. (**E-G**) BRCA1-deficient cells are defective for PARPi-induced ssDNA formation in the second S phase. U2OS cells were transfected with control or BRCA1 siRNA for 24 hours. Cells were then labeled with 20 μM BrdU for 48 hours. The last 24 hours of BrdU labeling was done in DMSO or 10 μM Olaparib. After BrdU labeling, cells were released into BrdU-free media for 2 hours before immunofluorescence analysis for BrdU-labeled ssDNA and PCNA. (E) Representative images are shown. (F) Cell-cycle profiles of the cells shown in E with overlaid BrdU intensity. Cells were plotted according to mean PCNA intensity (Y axis), DAPI intensity (X axis), and mean BrdU intensity (color gradient) (G) The BrdU intensity in S-phase cells (PCNA-positive) was quantified. Red bar: median intensity. More than 200 cells were analyzed in each sample (n>200). Significance was determined with a Mann-Whitney U test. **** p-value < 0.0001. (**H**) Western blot confirmation of siRNA-mediated RAD51 knockdown. U2OS cells were transfected with indicated siRNA for 48 hours before processing for Western blot.

Figure S6. Characterizations of PARP1-dependent PARPi effects and CPT-induced suppression of DNA synthesis

(**A**) Western blot confirmation of siRNA-mediated PARP1 knockdown. U2OS cells were transfected with indicated siRNA for 48 hours before processing for western blot. (**B**) U2OS cells were transfected with indicated siRNA and treated with DMSO or 10 μM Olaparib for 24 hours and S-phase cells were labeled with 10 μM EdU during the last 15 minutes. Samples were processed for immunofluorescence analysis of γH2AX foci intensity in EdU+ S-phase cells. More than 80 cells were analyzed in each sample (n > 80). Significance was determined with a Mann-Whitney U test. ****: p-value < 0.0001, n.s.: p-value > 0.05. (**C**) BRCA1-deficient cells do not suppress DNA synthesis upon CPT treatment. Cells were treated with DMSO or 0.05 μM CPT for 4 hours, and analyzed as in Fig. 4C-E.

Figure S7. Effects of BRCA1 on PARPi-induced replication changes and effects of CDC7i on ATRi-induced DNA damage in CPT-treated cells

(**A**) PARPi did not reduce origin firing in BRCA1 knockdown cells. Cells treated with siCTRL or siBRCA1 were exposed to 10 μM PARPi for the indicated durations and analyzed by CldU/IdU labeling (20 minutes each) and DNA fiber assay. The proportion of newly fired origins in all labeled replication tracts was quantified. Significance was calculated with a two-tailed Student's T-Test of three independent experiments. *: p-value < 0.05, ***: p-value < 0.001. (**B**) PARPi does not increase fork speed in BRCA1-deficient cells. DNA fibers from A were analyzed for fork speed using the IdU-labeled tracks. Red bar: median fork speed. More than 125 IdU-positive replication tracts were analyzed in each sample (n>125). Significance was determined with a Mann-Whitney U test. ****: p-value < 0.0001, n.s.: p-value > 0.05. **(C)** U2OS cells were treated with DMSO or 0.05 μM CPT for 4 hours, incubated with or without 10 μM VE-821 (ATRi) and/or 5 μM XL-413 (CDC7i), and labeled with 10 μM EdU during the last 15 minutes. The mean intensity of γH2AX in S-phase EdU+ cells was quantified. Red bar: median intensity. More than 200 S-phase cells

were analyzed in each sample (n>200). Significance was determined with a Mann-Whitney U test.

**** p-value < 0.0001.