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Supplemental information

Topoisomerase 1-dependent R-loop deficiency drives accelerated replication and genomic instability

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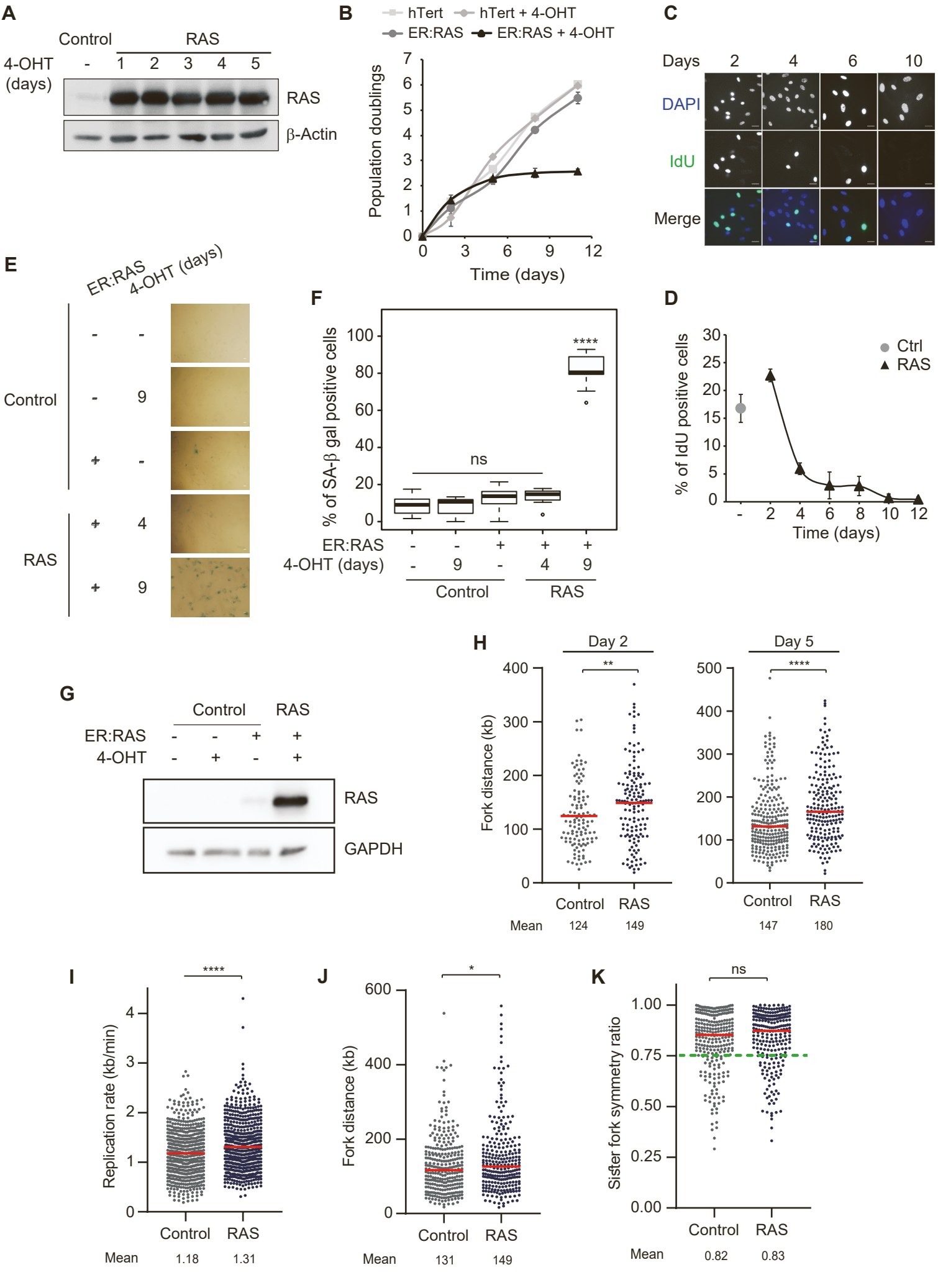
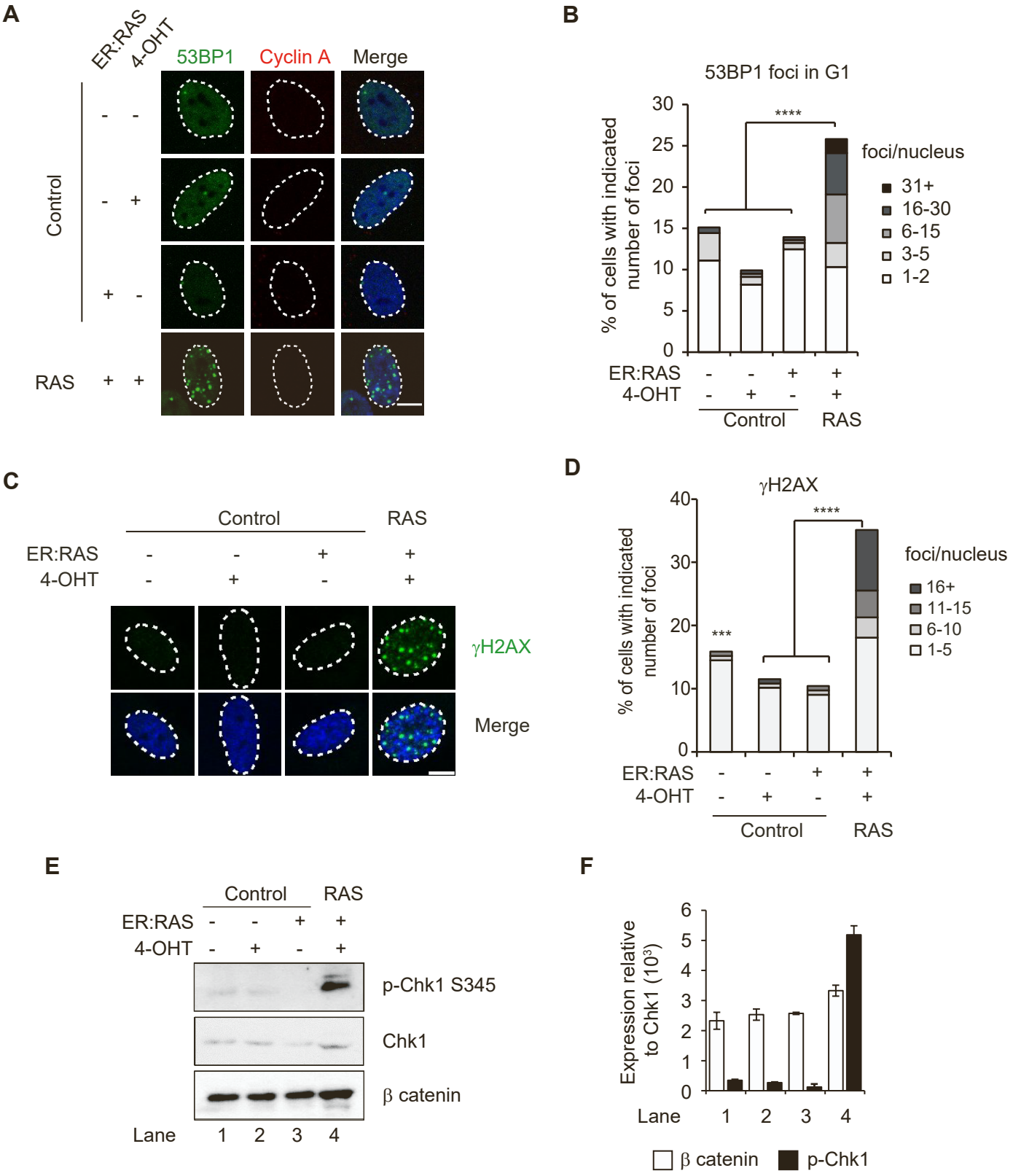
Supplementary Figure 1



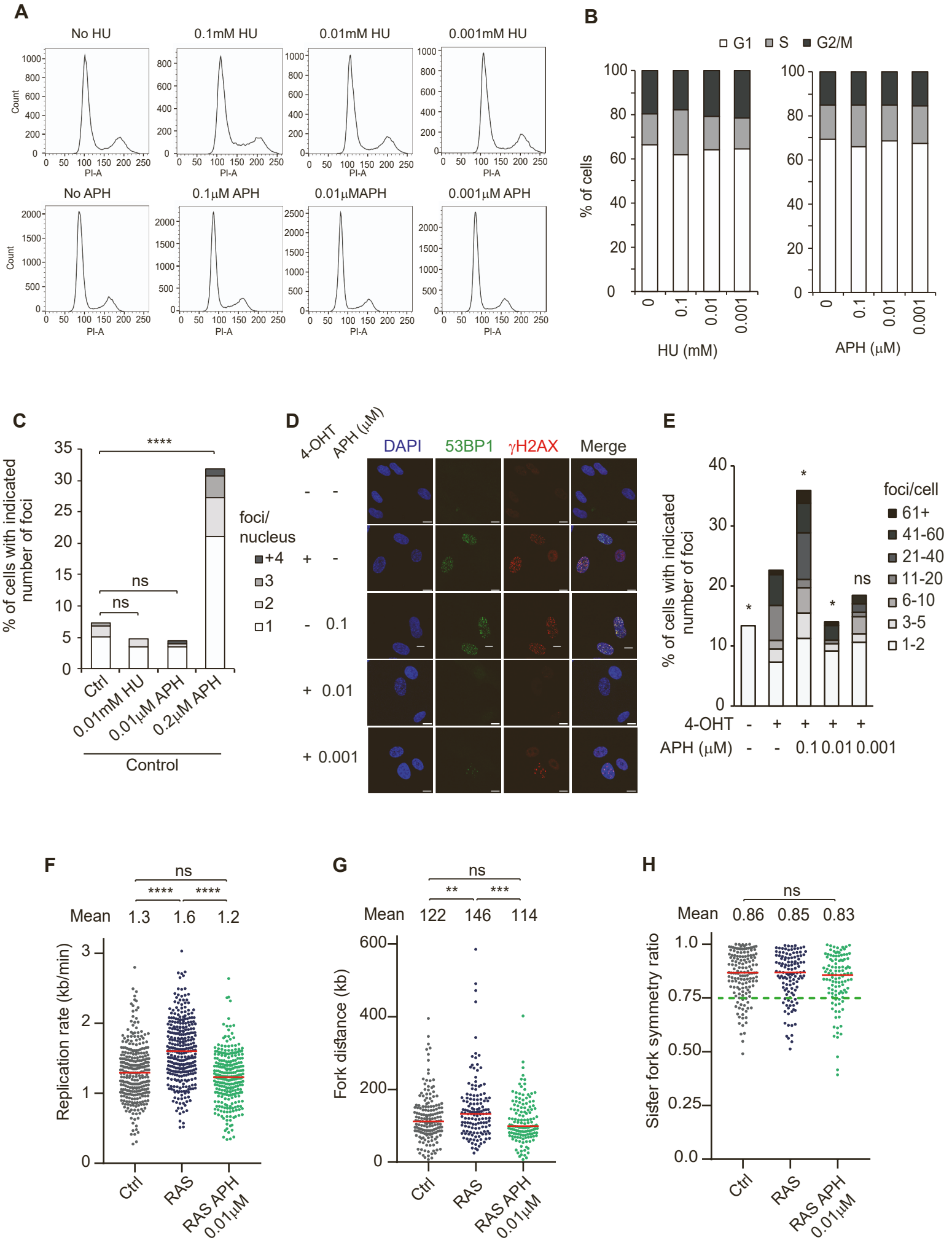
Supplementary Figure 1. Related to Figure 1. RAS expression increases replication rate and induces senescence. (**A**) Protein levels of HRAS and β-Actin in ER:RAS infected FSE-hTert cells with (RAS) or without (Control) 4-OHT treatment at the indicated time points
(days). (**B**) Quantification of population doublings at the indicated time points in: FSE-hTert cells (hTert, n = 4); FSE-hTert with 4-OHT treatment (hTert + 4-OHT, n = 2); FSE-hTert with ER:RAS infection, without 4-OHT treatment (ER:RAS, n = 4); FSE-hTert with ER:RAS infection and 4-OHT treatment (ER:RAS + 4-OHT, n = 4). n represents the number of independent experiments; data are mean ± SEM. (**C**) Representative images of IdU (green) and DAPI staining (blue) in RAS-expressing cells at the indicated time points. (**D**) Quantification of the percent of control (Ctrl) and RAS nuclei positive for IdU incorporation at the indicated time points (days post RAS activation). Data are mean ± SEM from two independent experiments. (**E**) Representative images of senescence associated β-gal activity; positive cells are stained blue. (**F**) Quantification of senescence associated β-gal activity in FSE-hTert cells with (+) or without (-) ER:RAS infection and 4-OHT treatment at the indicated time points (days). Data are the summary of two independent experiments. *P* values calculated compared to FSE-hTert cells without ER:RAS infection nor 4-OHT treatment. (**G**) Protein levels of HRAS and GAPDH in WI38-hTert cells with (+) or without (-) ER:RAS infection and 4-OHT treatment as indicated. (**H**) Fork distances (kb) in control and RAS-expressing ER:RAS FSE hTert cells for 2 or 5 days; at least 110 fibers per condition were analyzed. (**I**) Fork rates (kb/min) in WI38-hTert infected with ER:RAS without (Control) or with (RAS) 4-OHT treatment for 5 days; at least 600 fibers per condition were analyzed. (**J**) Fork distances (kb) in Control and RAS WI-38 cells; at least 260 fibers per condition were analyzed. (**K**) Sister fork symmetry in Control and RAS WI-38 cells; at least 230 fibers per condition were analyzed. Dashed green line indicate asymmetry ratio threshold. (**H-K)** Red lines indicate medians, means are indicated. (**I-K**) Data are the summary of two independent experiments, (**H**) Data for RAS day 2 are the summary of 2 independent experiments; data for RAS day 5 are the summary of 4 independent experiments. Mann-Whitney rank-sum test, ns - nonsignificant; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. Scale bars, 20 μm.

Supplementary Figure 2



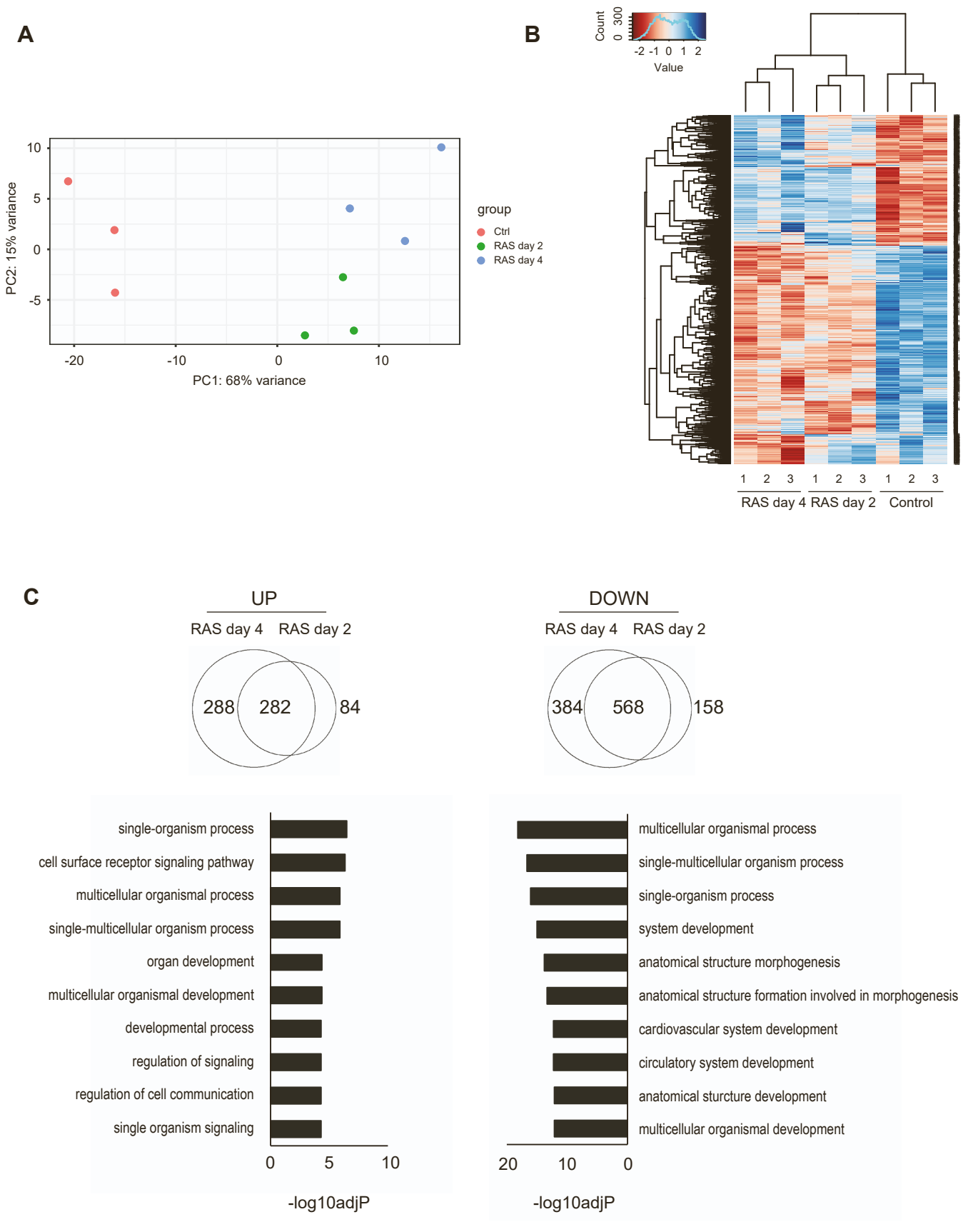
Supplementary Figure 2. Related to Figure 1. RAS expression leads to replication-induced DNA damage. (**A,B**) 53BP1 foci in G1-phase (cyclin A negative) in FSE-hTert cells with (+) or without (-) ER:RAS infection and 4-OHT treatment as indicated. Representative images of 53BP1 foci (green) in cyclin A (red) negative cells and DAPI staining (blue) (**A**). Percentage of cells with indicated number of foci per nucleus (**B**); at least 85 nuclei per condition were analyzed. Only foci smaller than 1 μm in diameter were scored. *P* values calculated compared to ER:RAS FSE-hTert cells treated with 4-OHT (RAS). Data are representative of three independent experiments with similar results. (**C,D**) γH2AX foci in FSE-hTert cells as indicated in **A**. Representative images of γH2AX foci (green) and DAPI staining (blue) (**C**). Percentage of cells with the indicated number of γH2AX foci (**D**); at least 90 nuclei per condition were analyzed. *P* values calculated compared to ER:RAS FSE-hTert cells treated with 4-OHT (RAS). Data are representative of two independent experiments with similar results. (**E**) Protein levels of phosphorylated Chk1 ser345, Chk1 and β-catenin in FSE-hTert cells with (+) or without (-) ER:RAS infection and 4-OHT treatment as indicated. (**F**) Quantification of protein levels presented in **E**, normalized to Chk1. Mann Whitney rank-sum test, *** $P < 0.001$; **** $P < 0.0001$. Scale bars, 10 μm.

Supplementary Figure 3



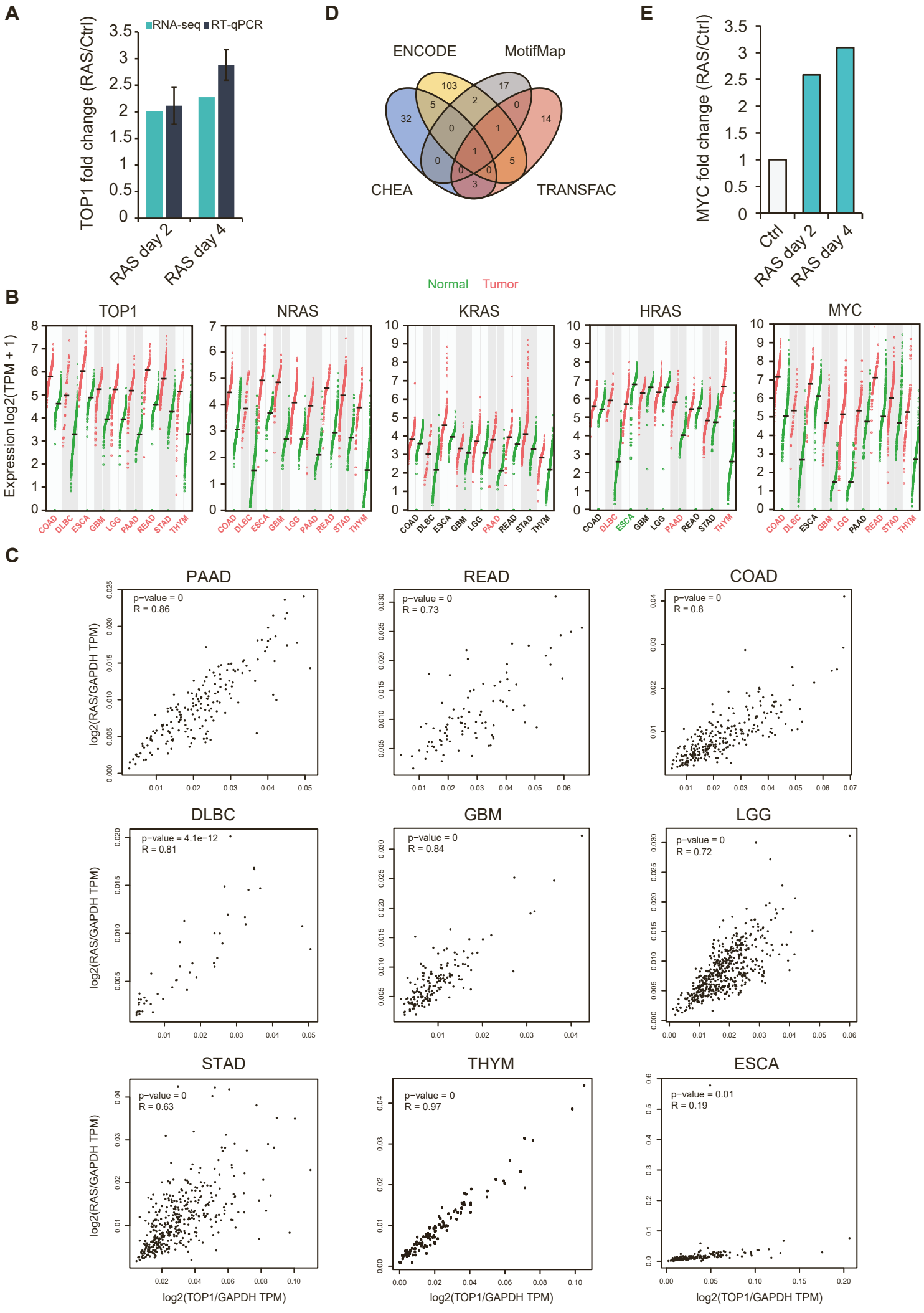
Supplementary Figure 3. Related to Figure 2. Mild replication inhibition does not affect cell cycle progression and restores normal replication dynamics. (**A**) Flow cytometry analysis of ER:RAS FSE-hTet cells treated with 4-OHT and HU or APH as indicated. (**B**) Quantification of data presented in **A**. Data are representative of two independent experiments. (**C**) Percentage of cells with the indicated number of co-localized γH2AX and 53BP1 foci in ER:RAS infected FSE-hTert cells without 4-OHT treatment (control), treated with HU and APH as indicated. Cells treated with 0.2μM APH as a control, using a high APH concentration that generates DNA damage. Data are the summary of two independent experiments. (**D,E**) Co-localization of γH2AX (red) and 53BP1 (green) foci in ER:RAS FSE-hTert cells with (+) or without (-) 4-OHT and APH treatment at the indicated concentrations. Representative images (**D**), percentage of cells with indicated number of foci (**E**); at least 140 nuclei per condition were analyzed. Data are representative of two independent experiments with similar results. *P* values calculated compared to ER:RAS FSE-hTert cells treated with 4-OHT (+) but without APH (-) (RAS). (**F-H**) DNA combing analysis of ER:RAS FSE-hTert cells with (+) or without (-) 4-OHT and APH treatments as indicated. (**F**) Fork rates (kb/min); at least 270 fibers per condition were analyzed. (**G**) Fork distances (kb); at least 130 forks per condition were analyzed. (**H**) Sister fork symmetry ratios; at least 110 forks per condition were analyzed. Red lines indicate medians. (**H**) Dashed green line indicates asymmetry ratio threshold. Data are the summary of two independent experiments. *P* values calculated by one-way ANOVA (**F-H**) and by Mann Whitney rank-sum test (**E**), ns - nonsignificant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Scale bars, 10 μm.

Supplementary Figure 4



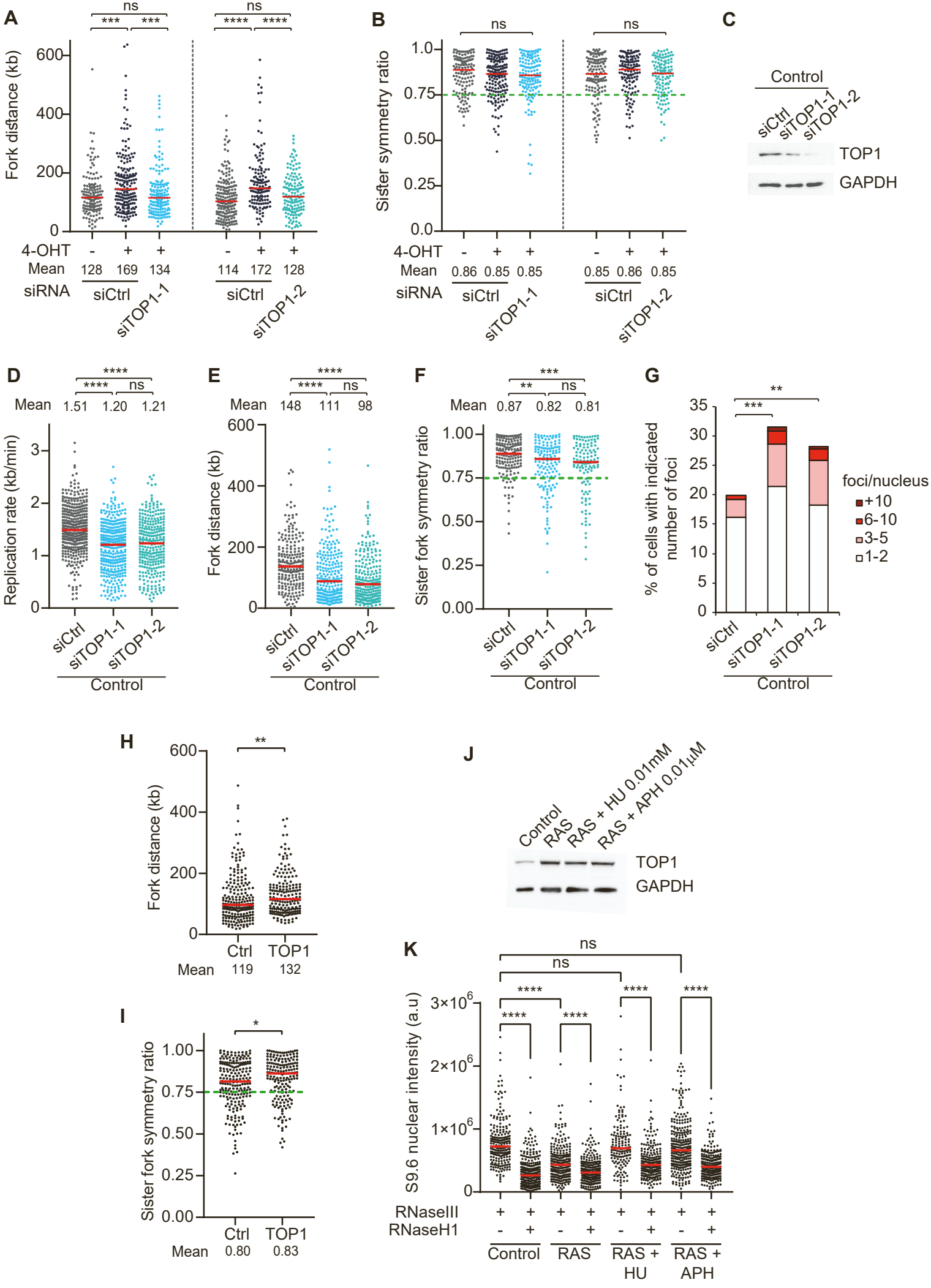
Supplementary Figure 4. Related to Figure 3. RNA-seq analysis of RAS-expressing cells. (**A**) Principal component analysis (PCA) plot of gene expression data showing the 1st and 2nd principal components for control (red), RAS day 2 (green) and RAS day 4 (blue). (**B**) Hierarchical clustering heat map for differentially expressed genes (DEGs) between RAS-expressing cells for 2 or 4 days (RAS day 2 and RAS day 4, respectively) and control cells. The Z-score centered log2-transformed gene in each sample is presented using a color scale. Three independent biological replicates are presented (1-3). (**C**) Top: Venn diagrams of upregulated (UP) or downregulated (DOWN) DEGs after RAS activation, showing overlap between RAS day 2 and RAS day 4. Bottom: Gene ontology (GO) term analyses of DEGs shared by RAS day 2 and RAS day 4. The top 10 enriched biological processes are shown.

Supplementary Figure 5



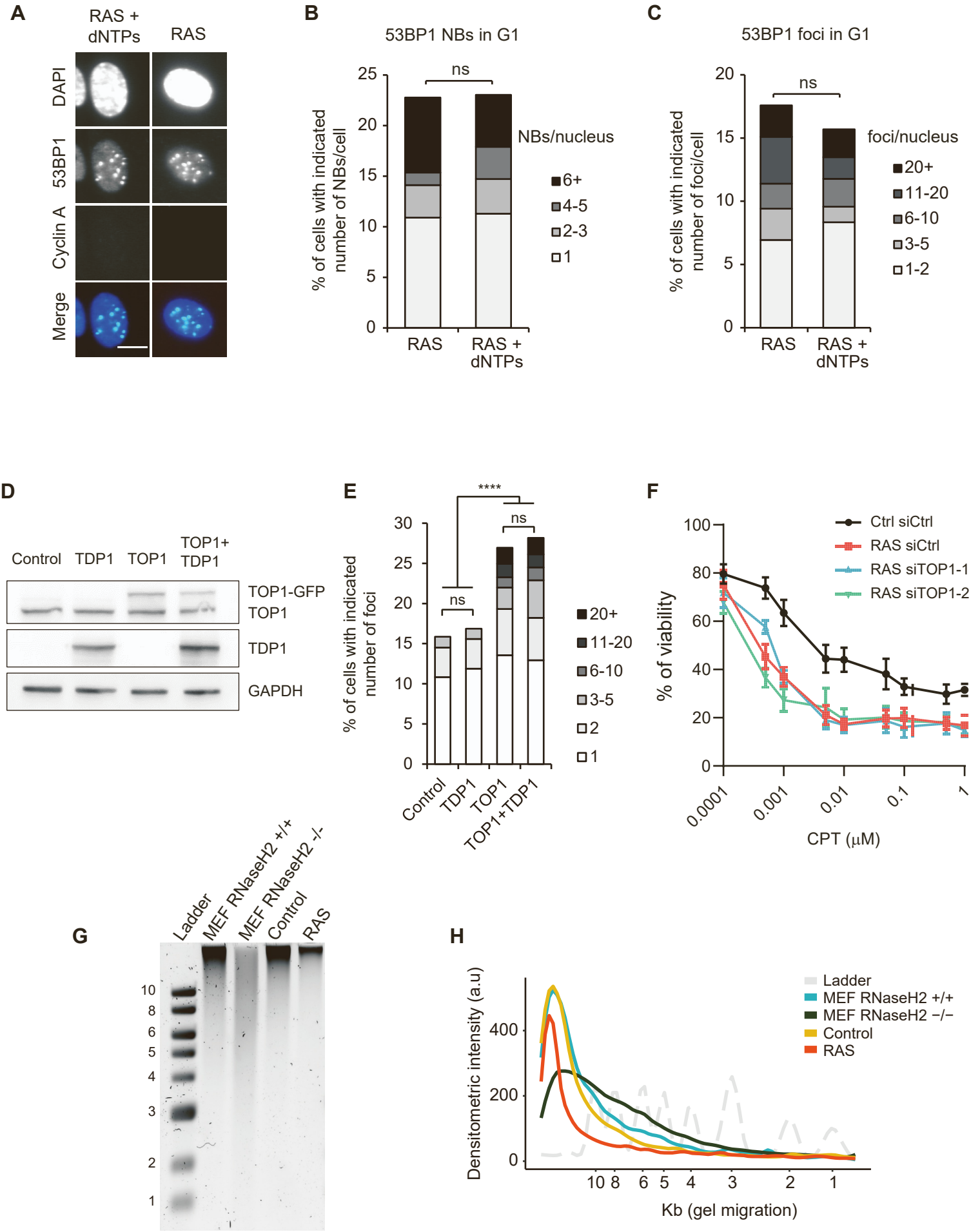
Supplementary Figure 5. Related to Figure 3. Elevated expression of TOP1 and RAS correlate in cancers. (**A**) RNA-seq and RT-qPCR analyses of TOP1 in ER:RAS FSE-hTert cells treated with 4-OHT for 2 and 4 days (RAS day 2 and RAS day 4, respectively) relative to non-treated cells. For the RT-qPCR analysis, the values were normalized to those of RNA polymerase II and are the average fold change (mean ± SEM, n = 3). (**B**) Expression levels log2(TPM + 1) of TOP1, NRAS, KRAS, HRAS and MYC in tumor samples (red dots) and tissue matching normal samples (green dots) from 9 cancers, as indicated. Color coding differential expression in tumors types compared with normal samples: red – significantly upregulated; green – significantly downregulated; black – no significant change. ANOVA p-value cutoff < 0.01. (**C**) Scatter plots of expression (TPM) correlation between TOP1 and NRAS normalized to GAPDH in tumor samples, as indicated. Pearson's correlation coefficient is presented. (**D**) Venn diagram of transcription factors binding the promoter of TOP1. (**E**) RNA-seq analysis of MYC fold change in cells as indicated in **A**.

Supplementary Figure 6



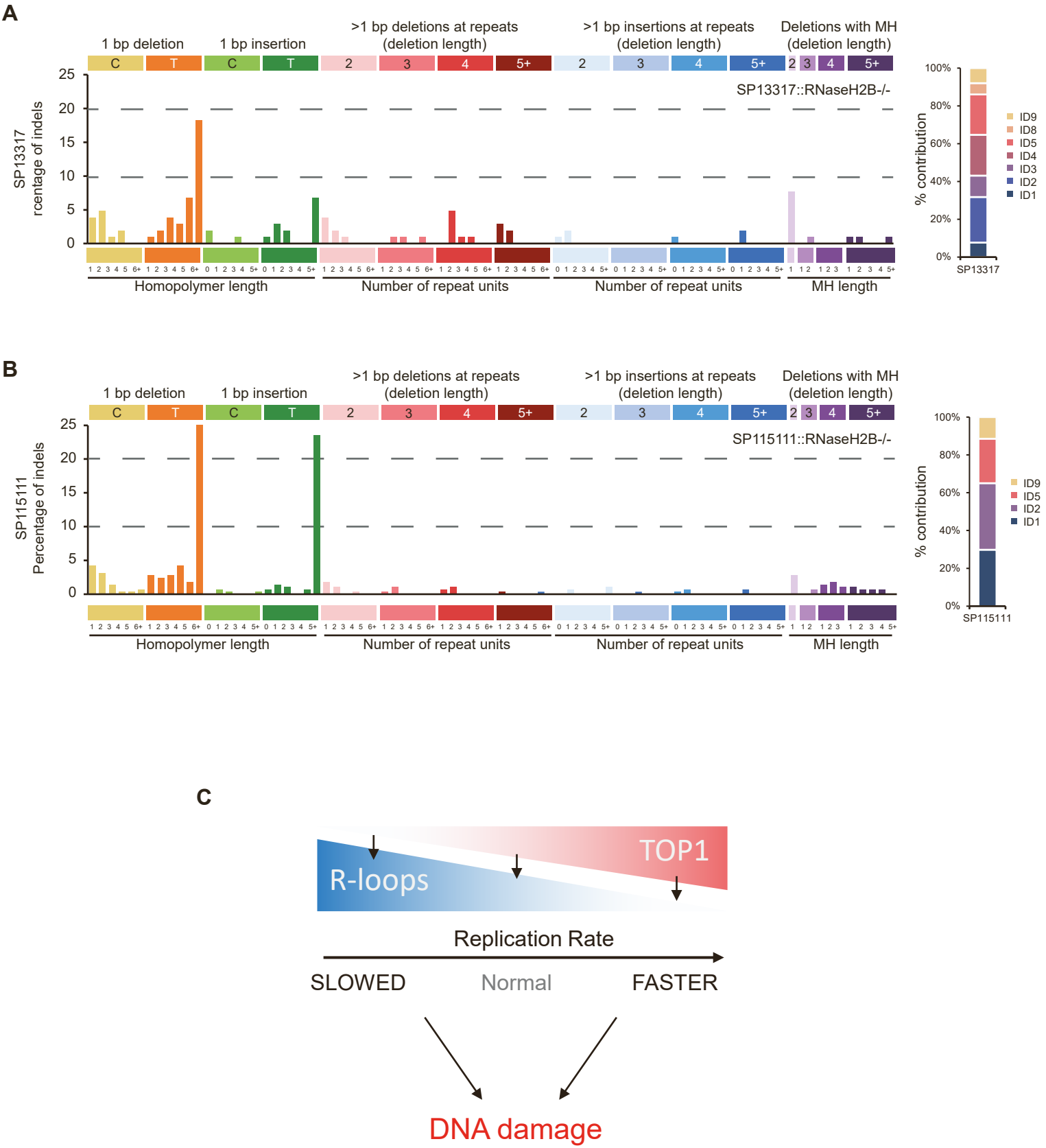
Supplementary Figure 6. Related to Figure 3. Downregulation or elevated TOP1 promotes aberrant replication dynamics. (**A,B**) DNA combing analysis of ER:RAS FSE hTert cells with (+) or without (-) 4-OHT treatment and siRNA against TOP1, as indicated. (**A**) Fork distances (kb); at least 140 forks per condition were analyzed. (**B**) Sister fork symmetry ratios; at least 125 forks per condition were analyzed. (**A,B**) Means are indicated; Red lines indicate medians. *P* values calculated by one-way ANOVA. Data are the summary of two independent experiments. (**C**) Protein levels of TOP1 and GAPDH in ER:RAS FSE-hTert cells without 4-OHT treatment (Control), treated with two independent siRNAs against TOP1 (siTOP1-1 and siTOP1-2) and non-targeting siRNA (siCtrl), as indicated. (**D-F**) DNA combing analysis of ER:RAS FSE-hTert cells without 4-OHT (control) as indicated in **C**. (**D**) Fork rate (kb/min); at least 280 fibers per condition were analyzed. (**E**) Fork distances (kb); at least 220 forks per condition were analyzed. (**F**) Sister fork symmetry ratios; at least 120 forks per condition were analyzed. Dashed green line indicates asymmetry ratio threshold (**F**). Red lines indicate medians. Data are the summary of two independent experiments. (**G**) Percentage of cells with indicated number of co-localized γH2AX and 53BP1 foci in ER:RAS FSE-hTert cells without 4-OHT treatment (Control) as indicated in **C**. At least 400 nuclei per condition were analyzed. Data are the summary of two independent experiments. *P* values calculated by one-way ANOVA. (**H,I**) DNA combing analysis of HEK-293 cells transfected with a control GFP vector (Control) or with TOP1-GFP (TOP1). (**H**) Fork distances (kb); at least 180 forks per condition were analyzed**.** (**I**) Sister fork symmetry ratios; at least 180 forks per condition were analyzed, dashed green line indicate asymmetry ratio. Means are indicated; Red lines indicate medians. *P* values calculated by Mann Whitney rank-sum test. Data are the summary of two independent experiments. (**J**) Protein levels of TOP1 and GAPDH in ER:RAS FSE-hTert cells with (RAS) or without (Control) 4-OHT treatment, HU and APH treatments as indicated. (**K**) Mean nuclear fluorescence intensity of RNA-DNA hybrid antibody (S9.6) in individual nuclei in ER:RAS FSE hTert cells with (RAS) or without (Control) 4-OHT treatment, HU/APH, RNaseIII and RNaseH1 treatments as indicated. ns – non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Supplementary Figure 7



Supplementary Figure 7. Related to Figure 7. DNA damage is induced by accelerated replication. (**A**) Representative images of 53BP1 NBs and foci (green) in cyclin A (red) negative cells and DAPI staining (blue) in FSE-hTert cells infected with ER:RAS and treated with 4-OHT for 5 days with (RAS + dNTPs) or without nucleoside supplementation (RAS). (**B,C**) Percentage of cells with indicated number of 53BP1 NBs (> 1 μm in diameter) (**B**) or foci (< 1 μm in diameter) **(C**) per nucleus in cyclin A negative RAS (n = 293) and RAS + dNTPs (n = 273) cells. Results are the summary of two independent experiments. Mann-Whitney rank-sum test. (**D**) Protein levels of TOP1, TDP1 and GADPH in HEK-293 cells transfected with a control GFP plasmid (Control), TDP1-GFP, TOP1-GFP, as indicated. (**E**) Percentage of cells with indicated number of 53BP1 foci in HEK-293 cells as indicated in **D**. At least 1000 nuclei per condition were analyzed. Data are the summary of two independent experiments. *P* values calculated by one-way ANOVA. (**F**) Cell viability assay for ER:RAS FSE hTert cells with (RAS) or without (Ctrl) 4-OHT treatment and siRNA treatment as indicated. Cells were treated with increasing concentrations of camptothecin (CPT), a TOP1 inhibitor. (**G,H**) Alkaline gel electrophoresis of RNaseH2 treated genomic DNA, (**G**) shows an increase in fragmentation for the RNaseH2$^{-/-}$ MEF clone compared with the RNaseH2$^{+/+}$ control MEF clone. No significant change is observed between ER:RAS FSE hTert cells with (RAS) or without (Control) 4-OHT treatment. (**H**) Quantification of the alkaline gel presented in **G**. ns – nonsignificant; **** $P < 0.0001$. Scale bar, 10 μm.

Supplementary Figure 8



Supplementary Figure 8. Related to Figure 7. Indel spectra of RNaseH2B null. (**A,B**) Indel mutational spectrum of RNaseH2B$^{-/-}$ tumor samples. ID4 signature contributes to SP13317 (**A**), but no to SP115111 (**B**). (**C**) A model of TOP1 regulating R-loop levels promoting replication stress. Either excess or shortage of TOP1 affects R-loop homeostasis, replication dynamics and genome stability. Excess TOP1 reduces R-loop levels leading to accelerated replication and induces DNA damage; and shortage of TOP1 increases R-loop levels, which slows replication rate and induces DNA damage.