Cell Reports, Volume *32*

Supplemental Information

Tousled-Like Kinases Suppress Innate Immune Signaling Triggered by Alternative Lengthening of Telomeres

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Supplemental Figures

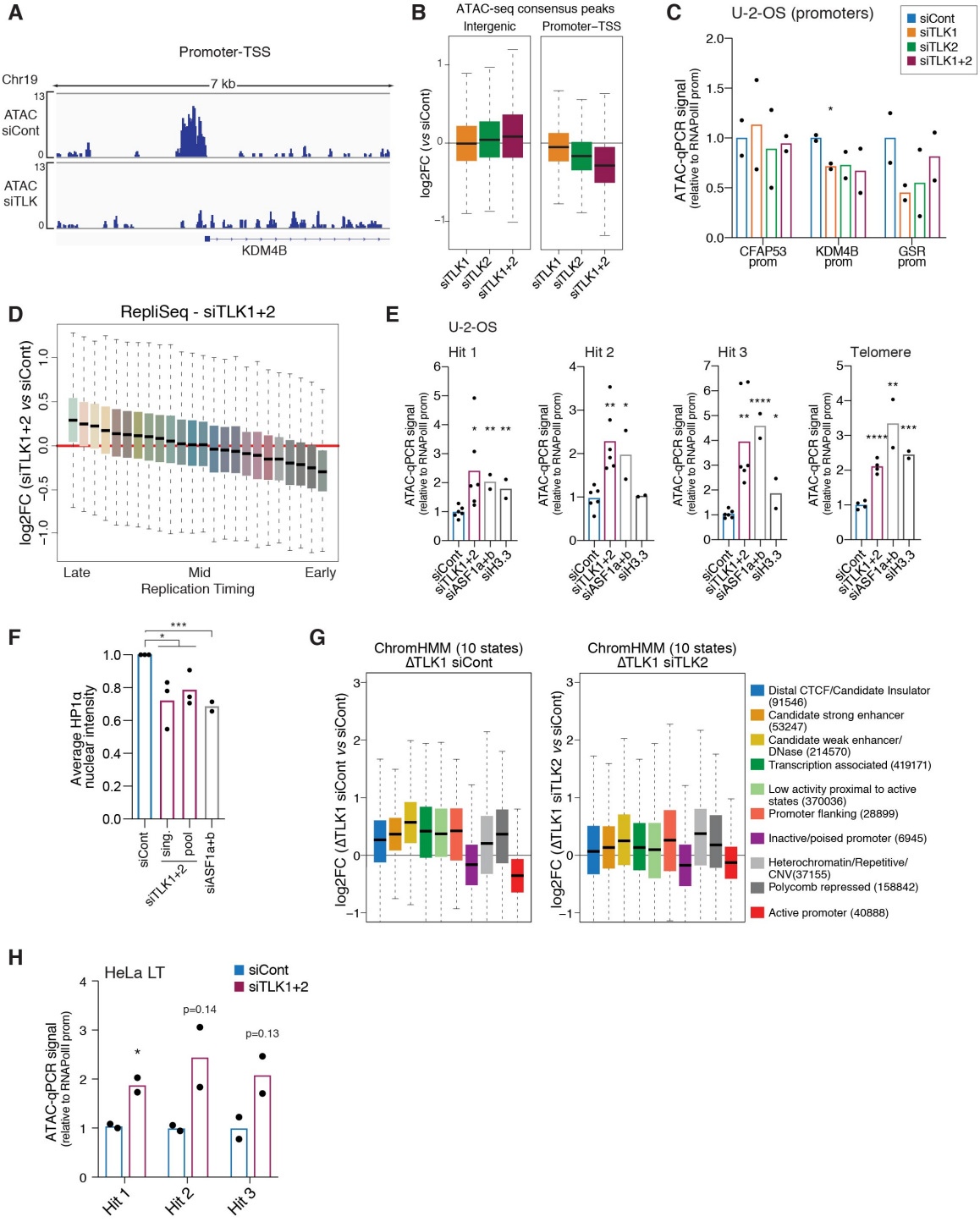

Figure S1. **Related to Figure 1. Loss of TLK activity compromises heterochromatin maintenance.** **(A)** Representative IGV track of ATAC-seq reads of a promoter-TSS region. **(B)** Boxplots of ATAC-seq FC (siTLK1, siTLK2, siTLK1+2 relative to siCont) computed in different genomic annotations, namely intergenic and promoter-TSS regions. **(C)** ATAC-qPCR at selected promoter regions in U-2-OS cells as in Figure 1G (n=2). We were unable to validate by ATAC-qPCR

the changes in promoter-TSS regions that became less accessible in ATAC-seq. We believe that this is the result of a technical artefact resulting from the broader distribution of reads to areas that became more accessible. **(D)** Boxplots of ATAC-seq FC (siTLK1+2 relative to siCont) through different replication timing chromatin regions from NHEK RepliSeq ENCODE dataset (NHEK was used an average track). **(E)** ATAC-qPCR at selected genomic regions in U-2-OS cells as in Figure 1G (n=6 for siCont/siTLK1+2 in Hit1-Hit3, n=4 for siCont/siTLK1+2 in Telomere, n=2 for siASF1a+b/siH3.3). **(F)** Average HTM quantification of chromatin-bound HP1α levels in U-2-OS cells, with each biological replicate having n>300 nuclei analyzed (n=3 for siCont/siTLK1+2, n=2 for siASF1a+b). **(G)** Boxplots of ATAC-seq FC in HeLa LT cells (ΔTLK1 siCont and ΔTLK1 siTLK2 relative to siCont) through different ChromHMM chromatin states. **(H)** ATAC-qPCR at selected genomic regions in HeLa LT cells as in Figure 1G (n=2). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, unpaired t test (Figure S1C, S1E, S1H), unpaired t test, one-tailed (Figure S1F). See Supplemental Tables S1, S2, S4 and S5 for additional data.

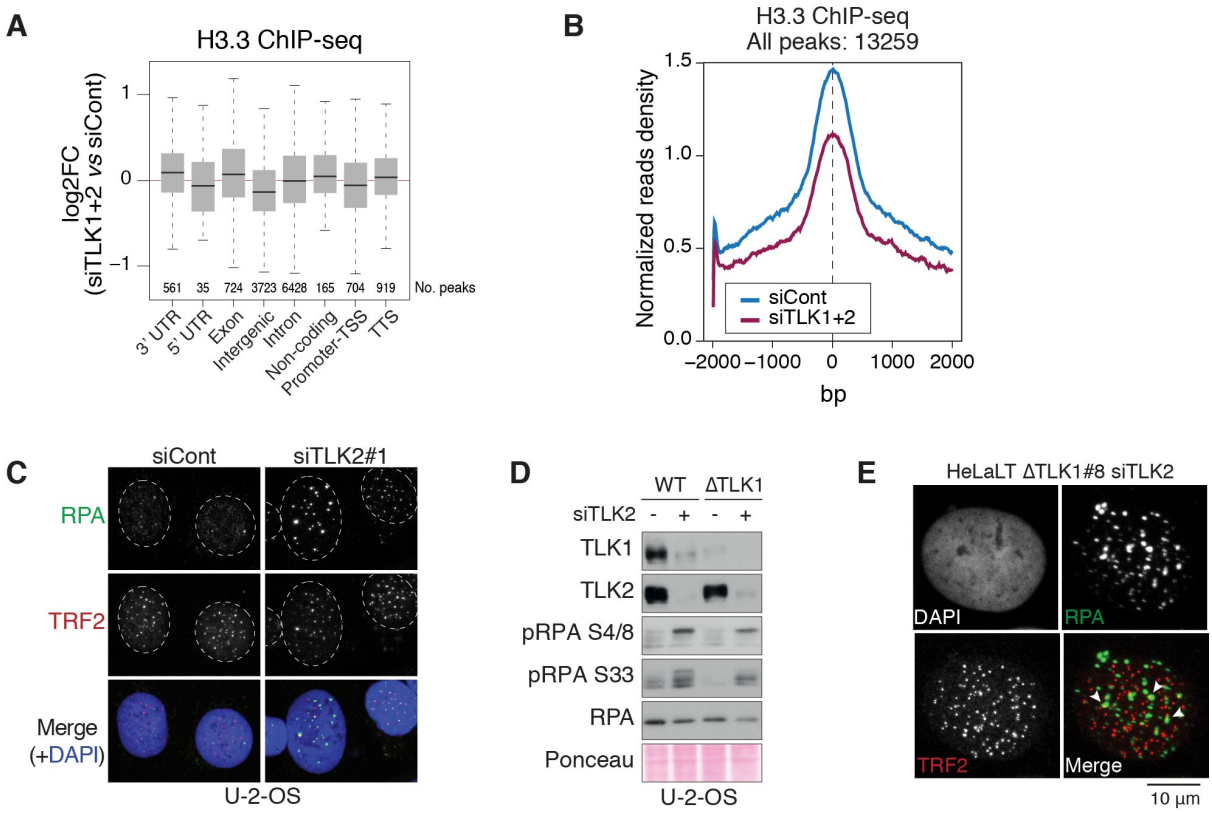

Figure S2. **Related to Figure 2. TLK activity suppresses telomeric recombination. (A)** Boxplots of H3.3 ChIP-seq FC (siTLK1+2 relative to siCont) through different genomic annotations in U-2-OS cells (n=2). **(B)** Normalized H3.3 ChIP-seq read density at all centered H3.3 peaks with +/- 2 kb performed in U-2-OS cells (n=2). See Supplemental Table S3 for additional data. **(C)** Representative IF of RPA-TRF2 staining in U-2-OS cells. **(D)** Western blot of replication stress signalling markers upon TLK loss in U-2-OS cells, parental (WT) and TLK1 CRISPR knockout clone (ΔTLK1). **(E)** Representative IF of RPA-TRF2 staining in HeLa LT ΔTLK1 siTLK2 cells. White arrowheads indicate colocalization of RPA-TRF2 foci.

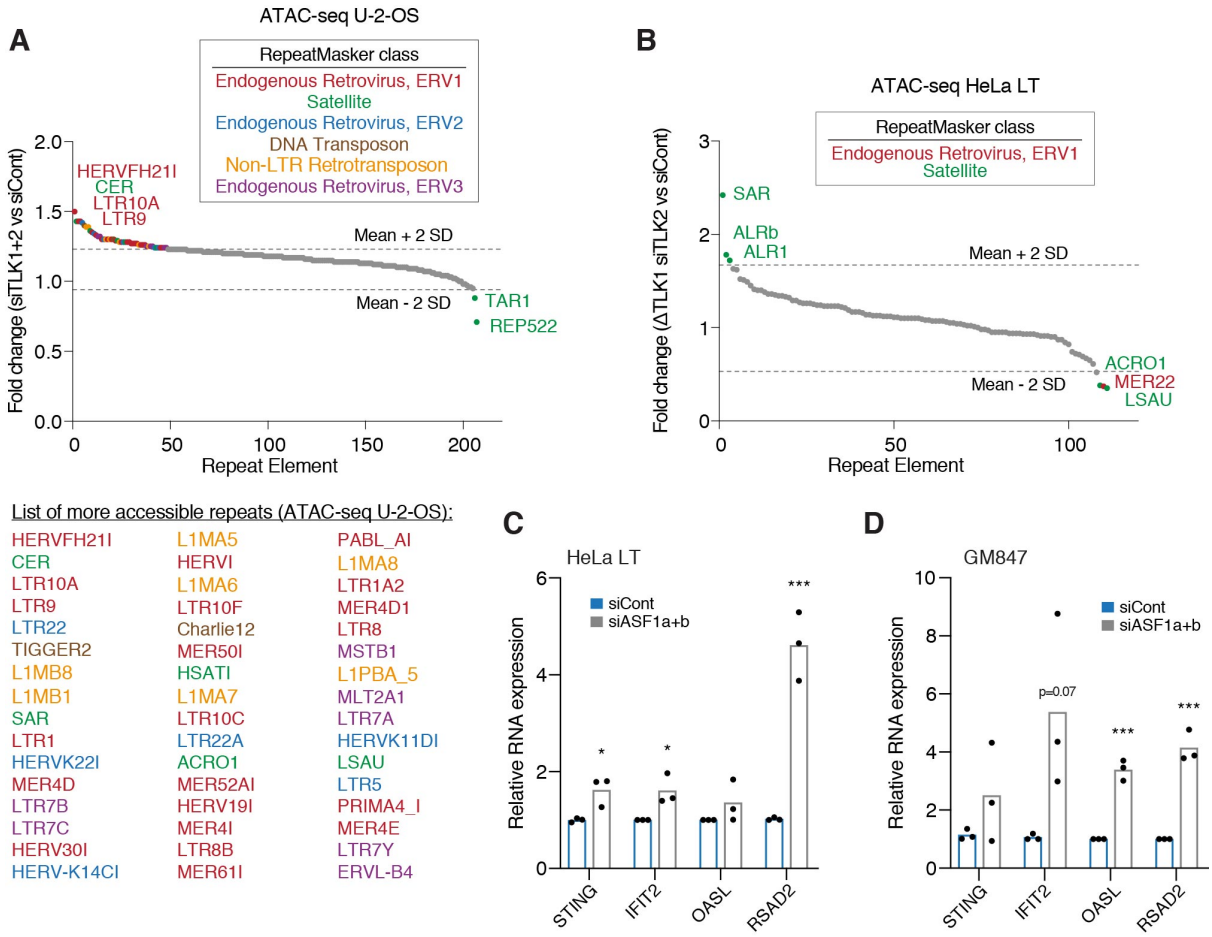

Figure S3. Related to Figure 3. TLK depletion activates innate immune signaling. (A, B) Analysis of repetitive element accessibility (ATAC-seq, n=2). Fold change from ATAC-seq (siTLK1+2 vs siCont in U-2-OS, panel A; ΔTLK1 siTLK2 vs siCont in HeLa LT, panel B) aligned to different repeat types. Data are plotted as a rank order from highest to lowest. The horizontal dotted line represents a cut-off of 2 SD from the mean. Repeats enriched more than 2 SD from the mean are labelled, colors represent the RepeatMasker broad repeat class to which that repeat type belongs. See Supplemental Tables S4 and S5 for additional data. **(C)** Expression levels of interferon response genes by RT-qPCR in HeLa LT cells. Data were normalized to unchanging expression gene levels (B-actin) and the signal obtained in siCont conditions was set to 1 (n=3). **(D)** Expression levels of interferon response genes by RT-qPCR in GM847 cells, data were analyzed as in (C) (n=3). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, unpaired t test (Figure S3C, S3D).

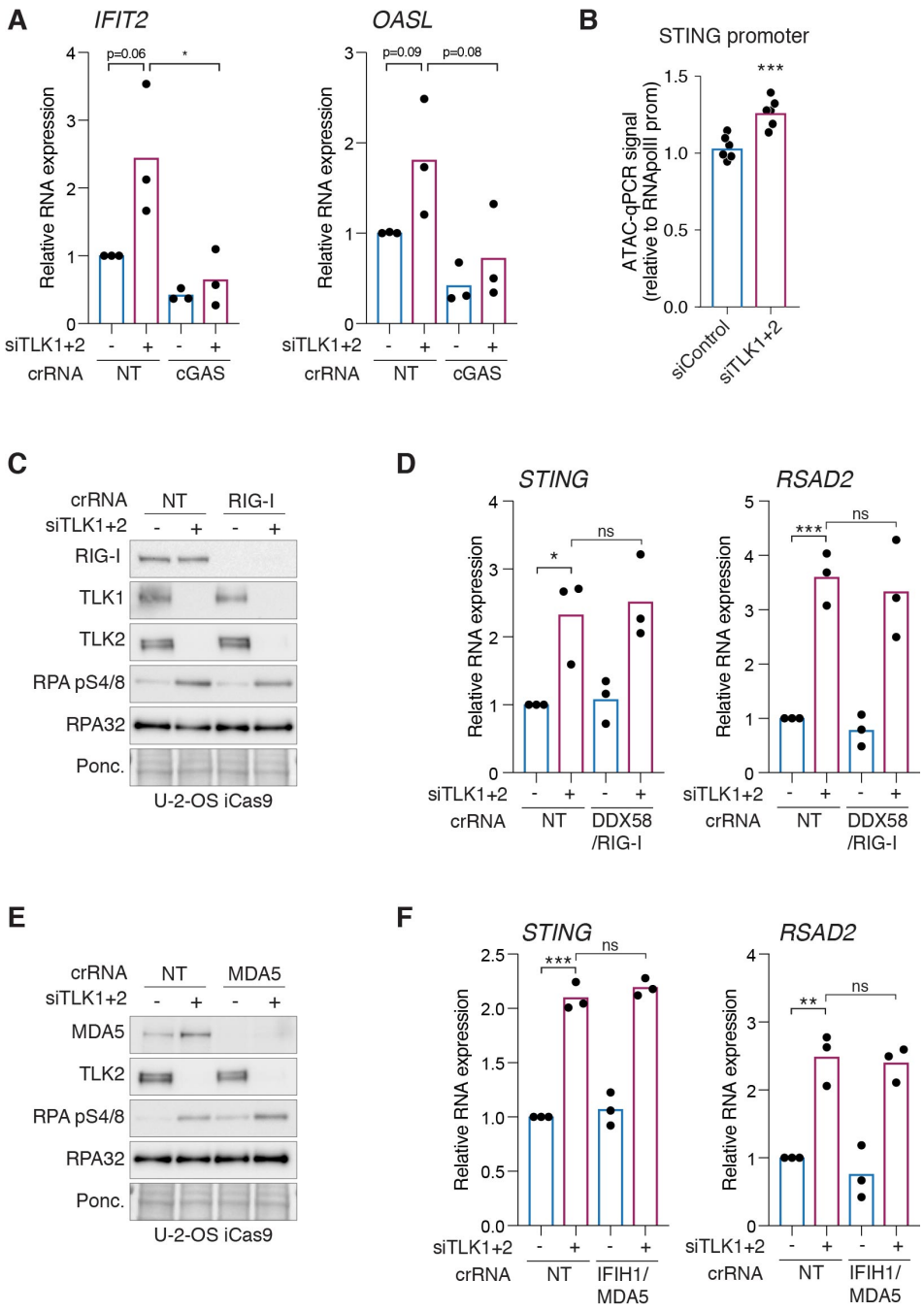

Figure S4. **Related to Figure 4. Innate immune activation is dependent on the cGAS-STING-TBK1 axis. (A)** Expression levels of IFIT2 and OASL by RT-qPCR in U-2-OS iCas9 cells 72 h after knockout induction and 48 h after being treated with the corresponding siRNAs. Data were normalized to unchanging expression gene levels (B-actin) and the signal obtained in siCont conditions was set to 1 (n=3). **(B)** ATAC-qPCR at STING promoter in U-2-OS cells as in Figure 1G (n=6). **(C)** Western blot showing RIG-I knockout and TLK depletion in U-2-OS iCas9 cells. Ponceau staining is shown as a loading control. **(D)** Expression levels of STING and RSAD2 by RT-qPCR in U-2-OS iCas9 cells 72 h after knockout induction and 48 h after being treated with the corresponding siRNAs. Data were

analyzed as in (A) (n=3). **(E)** Western blot showing MDA5 knockout and TLK depletion in U-2-OS iCas9 cells. Ponceau staining is shown as a loading control. **(F)** Expression levels of STING and RSAD2 by RT-qPCR in U-2-OS iCas9 cells 72 h after knockout induction and 48 h after being treated with the corresponding siRNAs. Data were analyzed as in (A) (n=3). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, unpaired t test (Figure S4A, S4B, S4D, S4F).

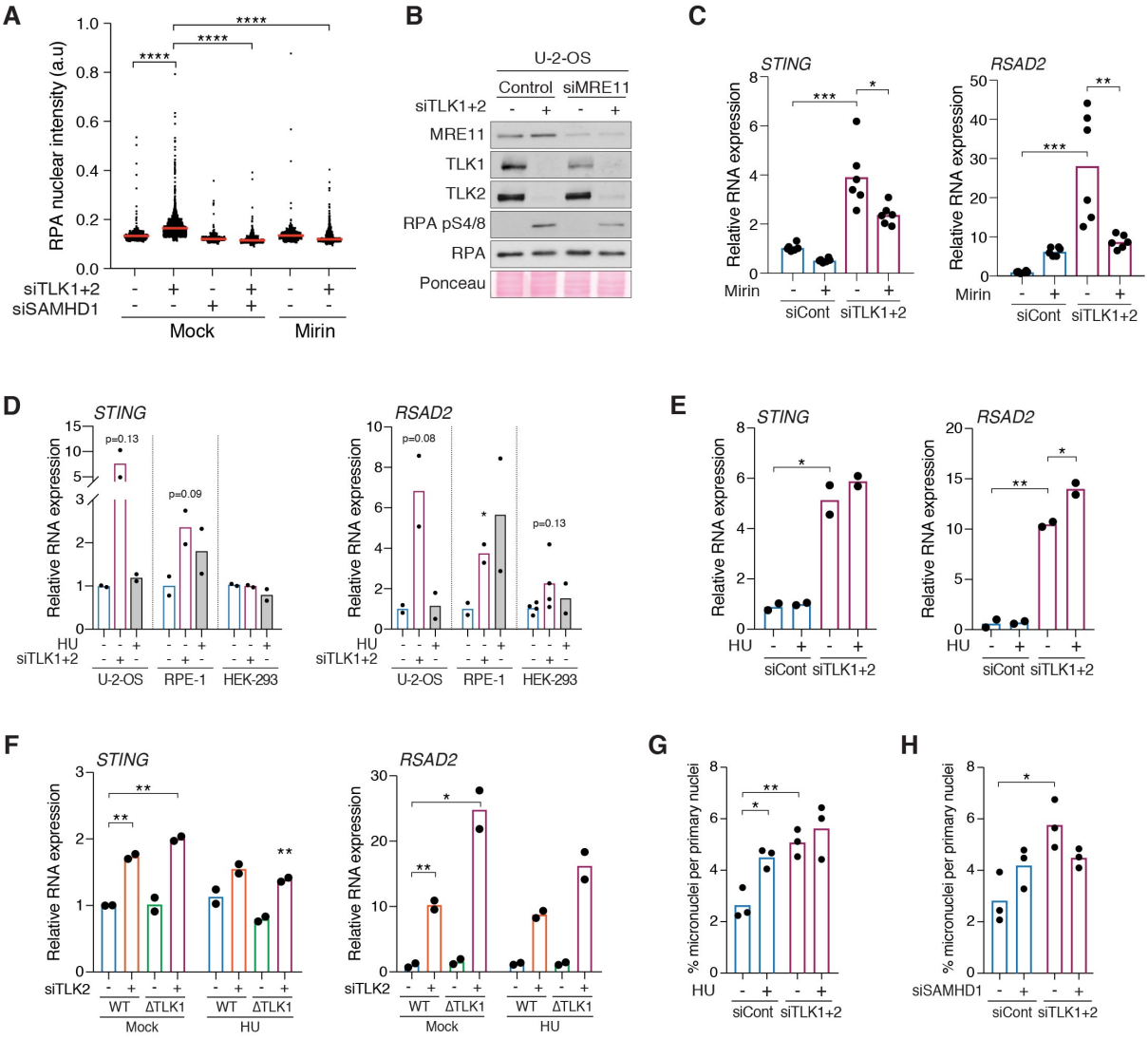

Figure S5. Related to Figure 5. Innate immune activation is independent of replication stress.

(A) HTM quantification of the nuclear intensity of chromatin-bound RPA in U-2-OS cells 48 h after siRNA transfection, mock treated or treated with 50 μM Mirin for 5 h prior to harvesting. Red bars indicate the median. **(B)** Western blot showing single or double depletion of TLKs and MRE11 in U-2-OS cells harvested 48 h after siRNA transfection. Ponceau staining is shown as a loading control. **(C)** Expression levels of *STING* and *RSAD2* by RT-qPCR in U-2-OS cells mock treated or treated with 50 μM Mirin for 5 h. Data were normalized to unchanging expression gene levels (B-actin) and the signal obtained in siCont conditions was set to 1 (n=6). **(D)** Expression levels of *STING* and *RSAD2* by RT-qPCR in U-2-OS, RPE-1 and HEK-293 cells 48 h after siRNA treatment, mock treated or treated with 2 mM HU for 2 h. Data were analyzed as in (C) (n≥2). **(E)** Expression levels of *STING* and *RSAD2* by RT-qPCR in U-2-OS cells 48 h after siRNA treatment, mock treated or treated with 2 mM HU for 2 h. Data were analyzed as in (C) (n=2). **(F)** Expression levels of *STING* and *RSAD2* by RT-qPCR in

HeLa LT cells 48 h after siRNA treatment, mock treated or treated with 2 mM HU for 2 h. Data were analyzed as in (C) (n=2). **(G)** Percentage of micronuclei in U-2-OS cells 48 h after siRNA treatment, mock treated or treated with 0.5 mM HU for 2 h. For each biological replicate a minimum of 260 cells were analysed (n=3). **(H)** Percentage of micronuclei in U-2-OS cells 48 h after siRNA treatment. For each biological replicate a minimum of 330 cells were analysed (n=3). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, unpaired t test with Welch's correction (Figure S5A), unpaired t test (Figures S5C-S5H).

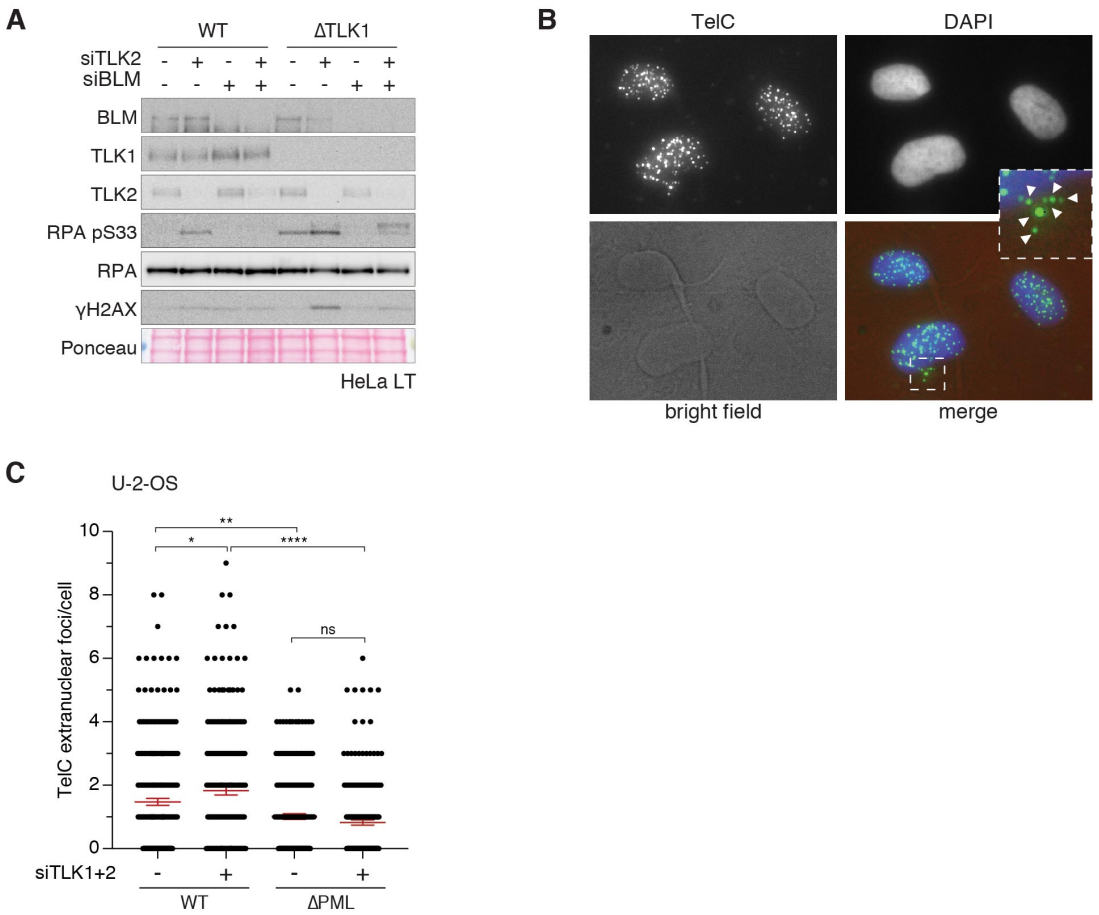

Figure S6. **Related to Figure 6. ALT induction contributes to innate immune induction following TLK1/2 depletion. (A)** Western blot of HeLa LT parental (WT) and TLK1 knockout clones (ΔTLK1) 48 h after being treated with the corresponding siRNAs. Ponceau staining is shown as a loading control. **(B)** Representative IF image of extranuclear TelC FISH signal in U-2-OS cells treated with siTLK1+2. **(C)** Number of extranuclear TelC foci per cell in parental U-2-OS cells or U-2-OS ΔPML cells 48 h after siRNA treatment. For each biological replicate, a minimum of 100 cells were analysed (n=2). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, unpaired t test with Welch's correction (Figure S6C)