Supplemental Information

Tousled-like Kinases Modulate Reactivation of Gammaherpesviruses from Latency

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Figure S1. Viral reactivation is specific to TLK2 depletion, Related to Figure 1. Single TLK2 siRNAs. A) KSHV-293 cells were transfected with the GAPDH siRNA and the individual TLK2 siRNAs from the pool of four (TLK2 #7-10). Green and red fluorescence images of the cells were taken 48hrs post-transfection. B) Western blots were performed on the cell lysates harvested from this experiment for the indicated proteins to verify knockdown. A non-specific band is denoted by an asterisk "*".

Figure S2. Validation of siRNA-mediated knockdown of TLK2 leads to viral reactivation, Related to Figure 2. A) Second source TLK2 siRNA reactivates KSHV. KSHV-293 cells were reverse transfected with a non-targeting control or TLK2 siRNA (50nM). Brightfield, GFP, and RFP images were taken at 72hrs post-transfection. B) Western blots were performed for TLK2 to confirm knockdown and the loading control tubulin. C) KSHV does not affect TLK2 levels. Equal numbers of HEK-293 and KSHV-293 cells were collected and lysates were harvested. Western blots were performed to determine the level of TLK2 protein. D) Cell viability following TLK2 knockdown. 293 cells were reverse transfected with the non-targeting control siRNA or siRNAs against TLK2 or UBB. Images of the cells were taken at 72hrs post-transfection. E) TLK2 depletion does not lead to a significant decrease in cell proliferation. 293 cells were reverse transfected with siRNA targeting TLK1, TLK2, UBB, or the scrambled control. An MTS

cell proliferation assay was performed at the indicated timepoints. Error bars represent standard deviation from the mean.

Figure S3. Magnified view of the KSHV viral profiling array shown in Fig. 4A. This is a magnified view of the KSHV viral profiling array depicted in Fig. 4A.

Figure S4. TLK2 depletion does not alter total level of S10 phosphorylated histone H3, Related to Figure 5. A) pHistone H3 immunofluorescence assay. KSHV-293 cells were transfected with the scrambled control or TLK2 siRNA. An immunofluorescence assay for serine 10 phosphorylated histone H3 was performed 48 hours post-transfection. B) Overall histone levels are not changed following TLK2 depletion. KSHV-293 cells were reverse transfected with 50nM of siRNA targeting GAPDH, TLK1, or TLK2. Cell lysates were harvested 72 hours post-transfection and Western blots were performed for total histone H3, phospho-histone H3 (S10), TLK1, TLK2, GAPDH, and tubulin.

Figure S5. Comparison of EBV reactivation between TLK knockdown and IgG treatment, Related to Figure 6. Akata-BX1 (A) and Akata cells (B) were infected with lentivirus expressing either a scrambled control shRNA or shRNA targeting TLK1 or TLK2. A mock infected sample was treated with human IgG (10 μg/mL) 24 hours prior to harvest. Cells were harvested 120 hours after infection and Western blots were performed for the indicated proteins.

Supplemental Experimental Procedures

Cell Proliferation Assay

HEK-293 cells were reverse transfected with 50nM of the non-targeting control siRNA or a single siRNA against TLK1, TLK2, or UBB and plated in 96-well plates. Cells were incubated at 37°C and an MTS cell proliferation assay was performed at 24, 48, and 72 hours post-

transfection using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (cat# G3581; Promega) per manufacturer's instructions. Plates were read at an absorbance of 485nm on a FLUOstar Optima plate reader (BMG Labtech).

Immunofluoresence Assay

KSHV-293 cells were reverse transfected with 50nM of the non-target control siRNA or a single siRNA against TLK2 and plated in chamber slides (cat# 177380; Thermo Scientific). Cells were incubated at 37°C for 48 hours and then stained for phosphorylated histone H3 by immunofluorescence assay. Samples were incubated in 4% paraformaldehyde pH 7.4 for 15 minutes at room temperature and washed twice in ice cold PBS. The cells were then incubated for 10 minutes with PBS containing 0.2% Triton X-100 (PBST) followed by three 5 minute washes with PBS. Then the cells were incubated with 10% normal goat serum in PBST for 30 minutes. The samples were then incubated with either rabbit anti phospho-histone H3 (Ser10) antibody (#3377; Cell Signaling) at 1:200 in 1% BSA in PBST or 1% BSA in PBST with no primary antibody overnight at 4°C. Three PBS washes were then performed and the cells were incubated with an Alexa Fluor 405 goat anti-rabbit secondary (cat# A31556; Invitrogen) at 4 μg/mL in 1% BSA for 1 hour at room temperature in the dark. The cells were then washed three times in the dark with PBS and a drop of Vectashield mounting media (cat# H-1000; Vector laboratories) was added with a coverslip. Images were acquired using a Nikon Eclipse Ti inverted microscope.

siRNATransfections

Cells were transfected with siRNAs using either Dharmacon DharmaFECT1 (HEK293, KSHV-293, and AGS-EBV cells) or Invitrogen Lipofectamine RNAiMAX (BCBL-1 cells). For the DharmaFECT reagents the following protocol was used. DharmaFECT was diluted in OptiMEM

(GIBCO) and incubated for 5 minutes at room temperature. Meanwhile, siRNAs were diluted in OptiMEM and dispensed into wells and the DharmaFECT/OptiMEM was then added to the wells. The final concentration of siRNA is indicated in the figure legends. The plate was gently agitated by hand and then incubated at room temperature for 20 minutes. Cells were then trypsinized, counted, and seeded in the wells containing the siRNA/DharmaFECT so that they were confluent at the end of the experiment. For transfection of BCBL-1 cells, siRNA (150nM final concentration) diluted in OptiMEM was added to the wells of a 12-well plate and then 2 μl of RNAiMAX was added to the siRNA/OptiMEM and mixed gently. It was incubated at room temperature for 15 minutes and cells were counted and 120,000 cells/well were plated in complete media lacking PS. Where indicated, fluorescent microscopy images were acquired using a Nikon Eclipse Ti inverted microscope.

Western Blots

At the indicated times post-transfection, cells were washed with cold PBS and lysed in RIPA buffer (150mM NaCl, 1% NP-40, 50mM Tris pH8, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Roche) for 30 minutes at 4°C. Samples were clarified by centrifugation for 10 minutes at 8.2 x g. Protein amounts were determined by a Bradford assay (BioRad) and equal amounts of protein were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked for 30 minutes at room temperature in either 5% nonfat dry milk (NFDM) or 5% BSA in a 1X TBS/0.1% Tween (TBST) solution. Membranes were then incubated with primary antibodies overnight at 4°C. Primary antibodies used in these experiments were against the following: TLK1 (#4125; Cell Signaling) at 1:2000, TLK2 (ab56816; Abcam) at 1:500, vIL-6 (#13-214-050; ABI) at 1:1000, K8.1A (#13-213-100; ABI) at 1:500, GAPDH (sc-25778; Santa Cruz) at 1:1000, EA-R p85 (sc-56979; Santa Cruz) at 1:250, EA-D (EBV-018-48180; Capricorn) at 1:500, STK38 (#H00011329-M01; Novus Biologicals) at 1:500, Zta (sc-53904; Santa Cruz) at 1:500, and α/β tubulin (#2148; Cell

Signaling) at 1:1000. After primary antibody incubation, membranes were washed 3X with 1X TBS/0.1% Tween. The blots were then probed with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) at 1:2000.

Quantitative and Reverse Transcriptase PCR Primers

qPCR primers used for transcript levels were vIL-6 (Forward: CTGTTACCGTACCGGCATCT; Reverse: AGAAGCTCCATGACGTCCAC), ORF57 (Forward: TGGACATTATGAAGGGCATCCTA; Reverse: CGGGTTCGGACAATTGCT), vGPCR (Forward: GTGCCTTACACGTGGAACGTT; Reverse: GGTGACCAATCCATTTCCAAGA), ORF50 (Forward: CGCAATGCGTTACGTTGTTG; Reverse: GCCCGGACTGTTGAATCG), BMRF1 (Forward: CAACACCGCACTGGAGAG; Reverse: GCCTGCTTCACTTTCTTGG), BCLF1 (Forward: TGCATGGCGGTCATTCC; Reverse: CATGGGCAAATACGCGG), BLLF1b (Forward: TGGGATGTAGACAAGTTACGCCT; Reverse: TGCTGACCCTTCTGCTGCT), and tubulin (Forward: CCCTTCCCACGCCTCC; Reverse: GGCTTCCACGGCTGGTG). Primers used for reverse transcriptase PCR were K1 (Forward: CCAAACGGACGAAATGAAAC; Reverse: TGTGTGGTTGCATCGCTATT) and ORF36 (Forward: ATCCAGACTGGTCCAAGCG; Reverse: ATTCTAGGATACACAATCG) while the primers for ORF50 and tubulin were the same as described above for qPCR.

Chromatin Immunoprecipitation (ChIP)

KSHV-293 cells were plated at 2 x 10^6 cells per 10-cm dish and transfected with 50nM of either a non-targeting control siRNA or a siRNA targeting TLK2 and cells were incubated for 96 hours post-transfection. The ChIP assay was performed using the EZ-ChIP kit (Millipore cat#17-371) per manufacturer's instructions. Methanol-free formaldehyde was added to the cells to a final concentration of 1% to crosslink the protein/DNA and incubated at room temperature for 10

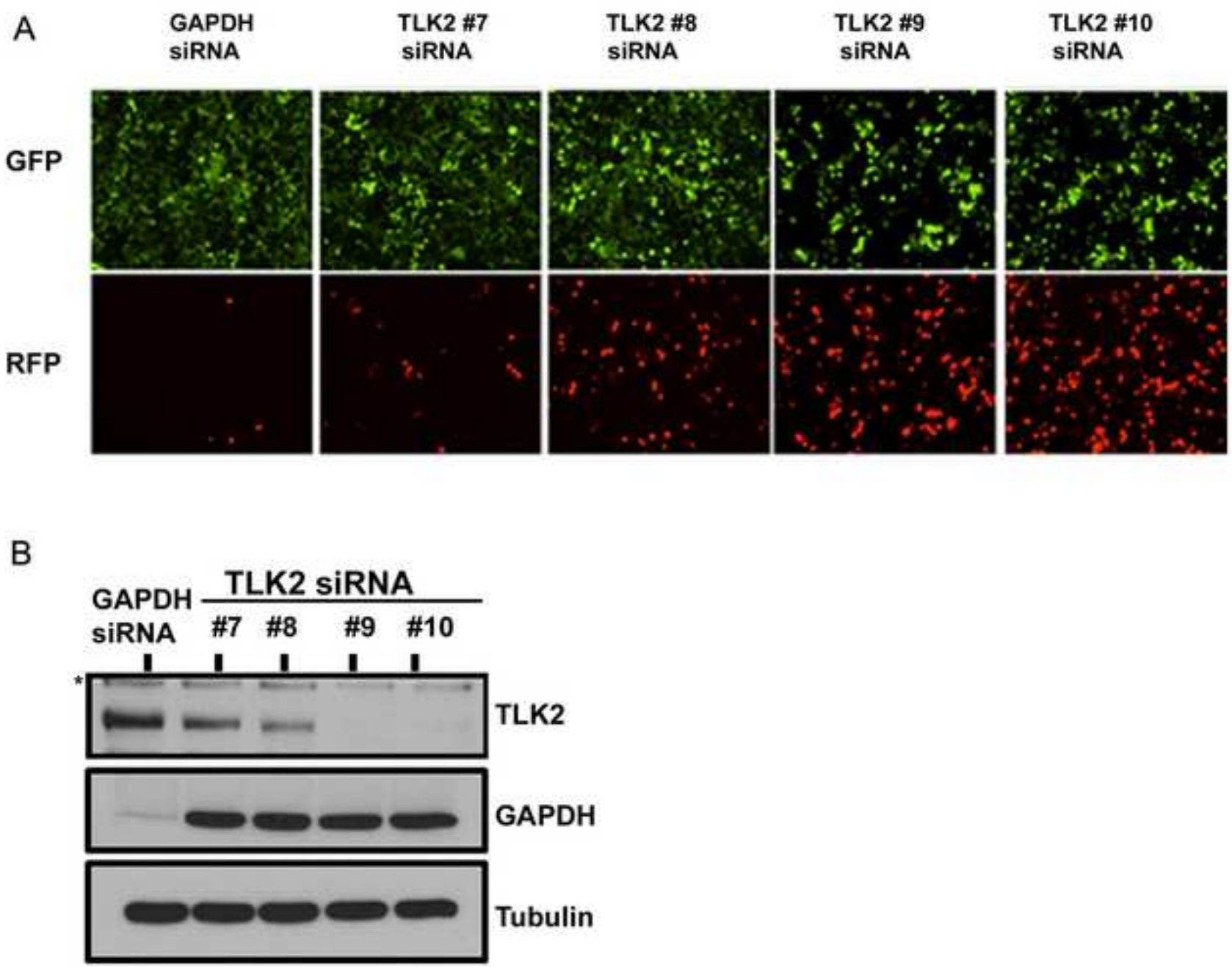
minutes. Unreacted formaldehyde was quenched by addition of 10X glycine to a final 1X concentration. Cells were washed 2X with cold PBS containing a protease inhibitor cocktail and cells were collected and pelleted. Cells were lysed in an SDS lysis buffer and sonicated on ice water using a Branson 450 digital sonicator for 4 cycles of 10 second pulses with 1 minute of rest between pulses with amplitude set at 20%. Samples were clarified by centrifugation at 12,000 x g for 10 minutes at 4°C. Each sample was pre-cleared using Protein G agarose beads for 1 hour at 4°C. Beads were removed by centrifugation and 1% of total sample volumes were used as input controls. Immunoprecipitations were performed by adding either 2 μg of normal mouse IgG or anti-phospho histone H3 (Ser10) (Millipore cat#CS200553) and incubating overnight at 4°C. The antibody/chromatin complexes were captured by addition of Protein G agarose beads for 1 hour at 4°C and washed once each in a low salt buffer, high salt buffer, lithium chloride buffer, and twice in TE buffer. Elution buffer (1% SDS and 0.1M $NaHCO_3$) was then added to the IP and input samples, incubated for 30 minutes at room temperature, and the Protein G beads were removed from the IP samples by centrifugation. Crosslinks were reversed by addition of 0.2M NaCl, incubation overnight at 65°C, then treatment with RNase A for 30 minutes at 37°C, followed by incubation with 10mM EDTA, 40mM Tris-HCl, and proteinase K for 2 hours at 45°C. Purified DNA was then recovered through use of spin filters and wash buffers supplied in the EZ-ChIP kit. The amount of serine 10 phosphorylated histone H3 bound to the ORF50 promoter was determined by qPCR using ORF50 promoter-specific primers (Fwd: GGTACCGAATGCCACAATCTGTGCCCT; Rev: TTGTGGCTGCCTGGACAGTATTCTCAC).

Supplemental Table S1

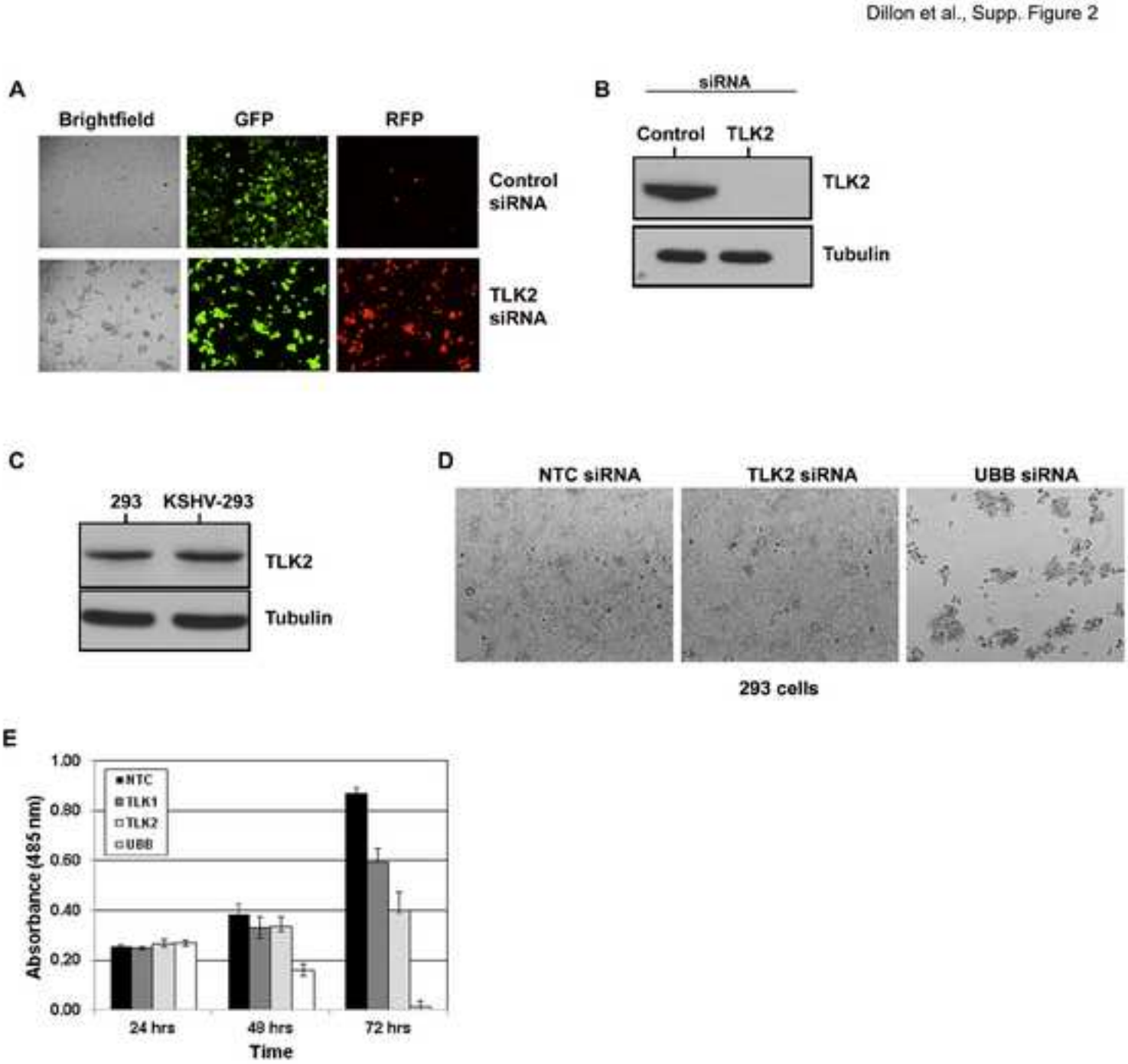
Supplemental Table S1: siRNA and shRNA sequences related to the Experimental Procedures	
siRNA	**Sequence**
Dharmacon	
UBB-06	5'-GUAUGCAGAUCUUCGUGAA-3'
TLK1-11	5'-GAGUAUGCAAGAUCGAUUA-3'
TLK1-12	5'-GAAGCUCGGUCUAUUGUAA-3'
TLK1-13	5'-GCAAUGACUUGGAUUUCUA-3'
TLK1-14	5'-GUUCAAAGAUCACCCAACA-3'
TLK2-07	5'-AAGAUGGCGUGUAGAGAUA-3'
TLK2-08	5'-UUACAAGGCAUUUGAUCUA-3'
TLK2-09	5'-GAUAGAAAGACAACGGAAA-3'
TLK2-10	5'-CCUCAAACCAGGUAAUAUU-3'
Sigma-Aldrich	
TLK2 (siRNA5)	5'-GAAGAAATCTTCAAACTCA-3'
shRNA	**Sequence**
TLK1	5'-CCGGCGGAGAAGAAACAATCGGAATCTCG AGATTCCGATTGTTTCTTCTCCGTTTTT-3'
TLK2	5'-GATAGAAAGACAACGGAAACTCG AGTTTCCGTTGTCTTTCTATC-3'

Supplemental Figure S1

Dillon et al., Supp. Fig. 1



Supplemental Figure S2



Supplmental Fig. S3

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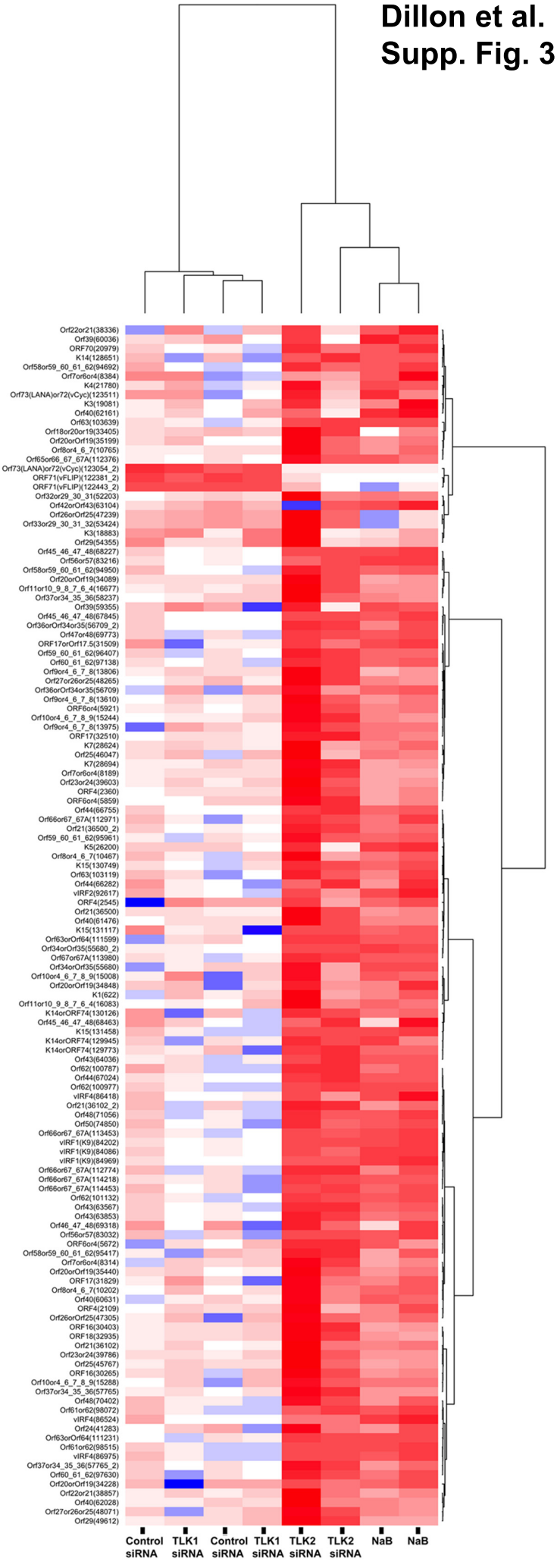
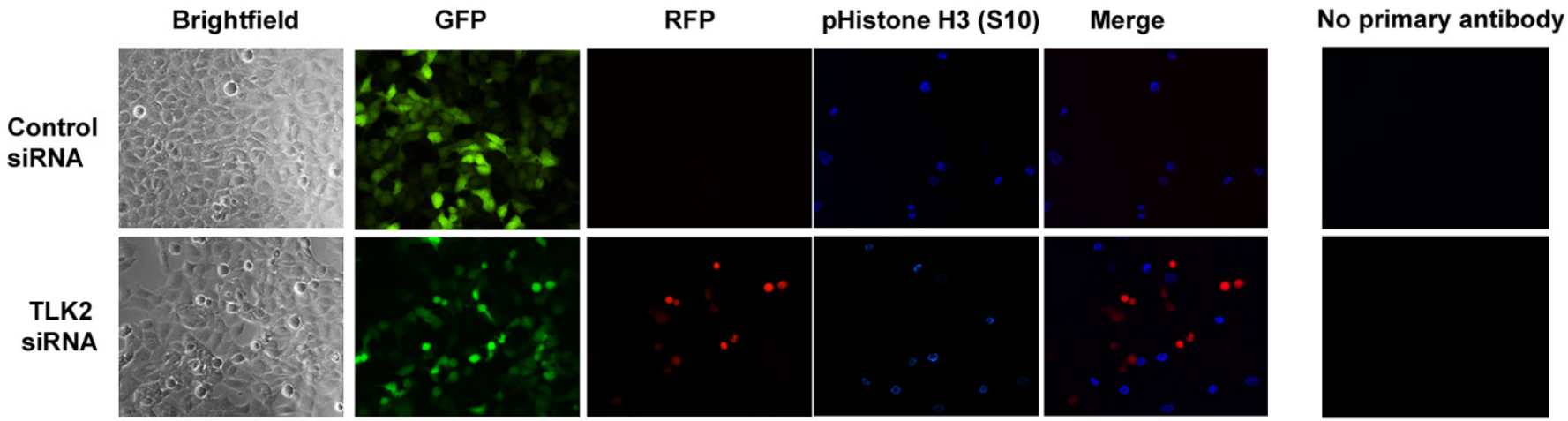
Supp. Fig. 3



Supplemental Fig. S4

Dillon et al., Supp Fig. 4

A



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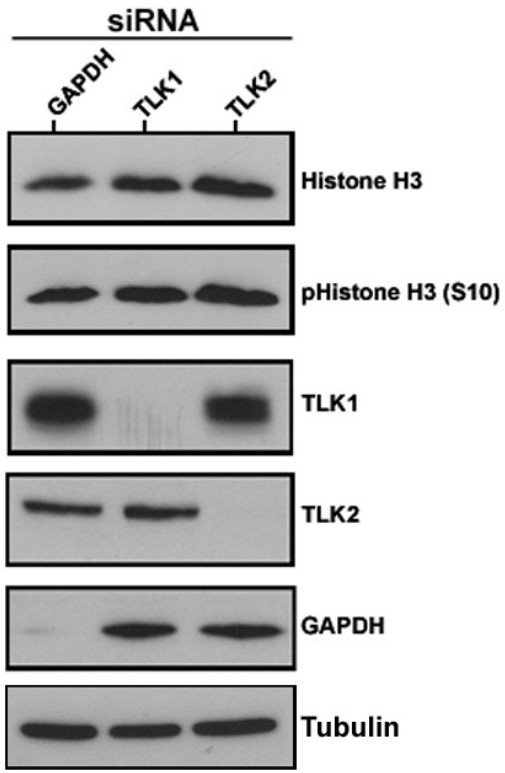

Supplemental Figure S5

Dillon et al., Supp Fig. 5