

Supplementary Information

TRIM23 mediates cGAS-induced autophagy in anti-HSV defense

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Supplemental Information

Supplementary Figure 1. TRIM23 restricts HSV-1 infection in mice and human cells.

Supplementary Figure 2. HSV-1 restriction by TRIM23 is IFN independent.

Supplementary Figure 3. Validation of pS39-TRIM23 antibody by IF analysis, and endogenous TRIM23 S39 phosphorylation in mouse cells.

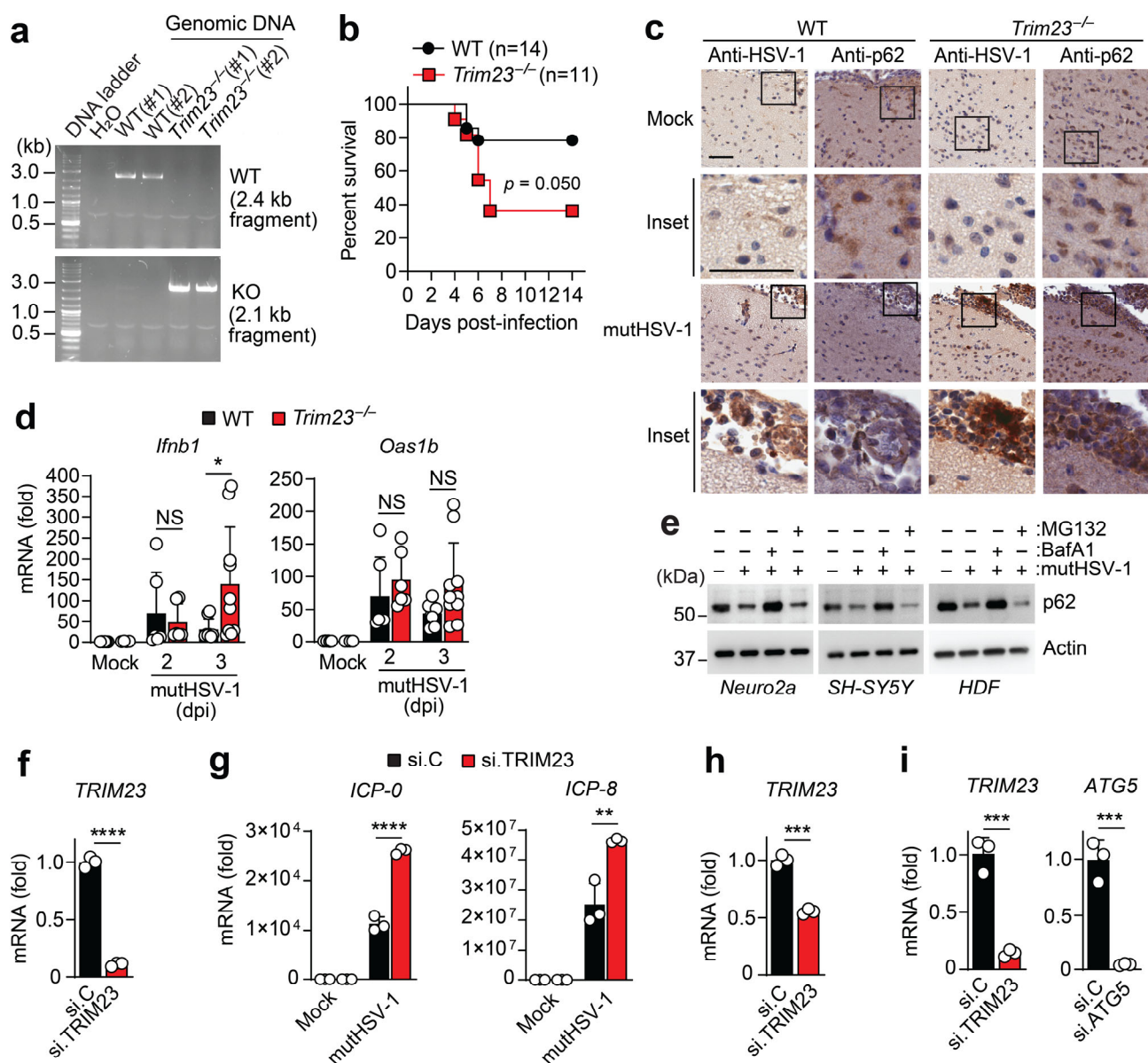
Supplementary Figure 4. TBK1 mediates TRIM23 S39 phosphorylation, thereby promoting TRIM23 ubiquitination.

Supplementary Figure 5. Autophagy induction by cGAS is mediated by TRIM23 and TBK1.

Supplementary Figure 6. S39 phosphorylation and TBK1 interaction of TRIM23 WT and mutants, and subcellular localization of activated TRIM23.

Supplementary Figure 7. Auto-phosphorylation and TRIM23 binding of TBK1 WT and mutants, impaired autophagy in *TBK1* mutant HSE fibroblasts, and working model for the cGAS-STING-TBK1-TRIM23 axis in autophagy.

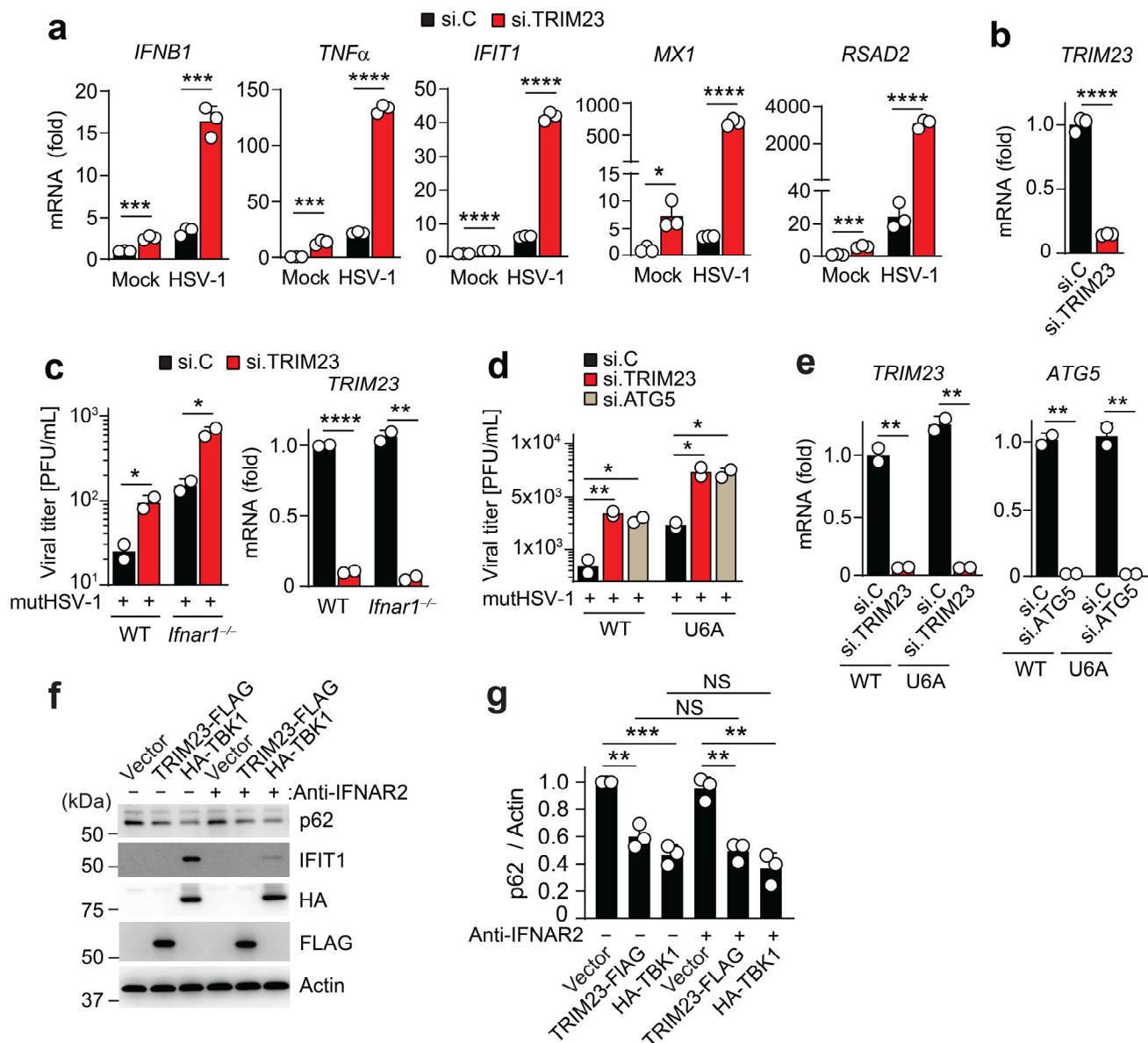
Supplementary Table 1. Primers and sgRNA sequences



Supplementary Fig. 1 TRIM23 restricts HSV-1 infection in mice and human cells.

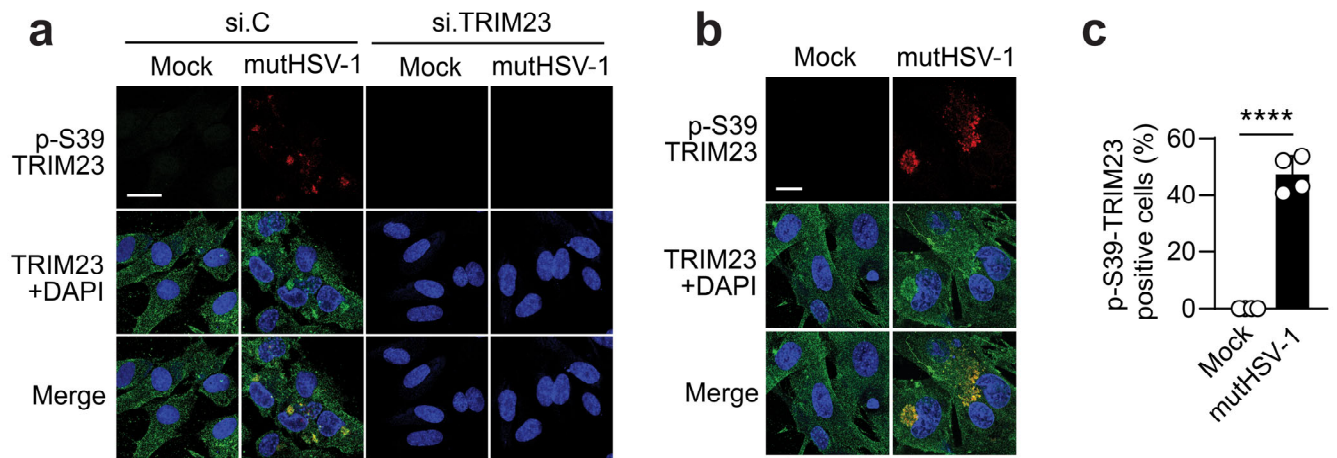
(a) Validation of *Trim23*^{-/-} and WT mice by PCR analysis of genomic DNA isolated from mouse tissue followed by agarose gel electrophoresis. The amplified 2.4 kb and 2.1 kb fragments confirm the WT and transgene allele, respectively. #1 and #2 represent two individual mice for each genotype. **(b)** Kaplan–Meier survival curves of *Trim23*^{-/-} mice and WT littermate controls of both sexes (8-week-old, sex-matched across treatments) that were infected *via* intracranial inoculation with 1 x 10⁴ PFU of WT HSV-1 (revertant). **(c)** p62 protein abundance and HSV-1 antigen expression in mid-sagittal brain sections of mock-treated or

mutHSV-1-infected (5×10^5 PFU) *Trim23*^{-/-} and WT mice, determined at day 3 post-infection by immunohistochemistry after staining with the indicated antibodies. Scale bars, 50 μ m. **(d)** *Ifnb1* and *Oas1b* transcripts in the whole brain lysates of *Trim23*^{-/-} and WT mice that were either mock-treated or infected with mutHSV-1 (5×10^5 PFU), determined by RT-qPCR at the indicated times post-infection. **(e)** Endogenous p62 protein abundance in the WCLs of the indicated cell lines that were either mock-treated or infected for 12 h with mutHSV-1 (MOI 10) and then either mock-treated or treated for 12 h with MG132 (20 μ mol) or BafA1 (50 nmol), determined by IB. **(f)** Representative knockdown efficiency of TRIM23 for the data shown in Figures 1f and 1g, determined by RT-qPCR at 48 h post-transfection. **(g)** Viral *ICP-0* and *ICP-8* transcripts in SH-SY5Y cells that were transfected for 30 h with the indicated siRNAs and then either mock-treated or infected with mutHSV-1 (MOI 0.1) for 24 h, determined by RT-qPCR. **(h)** Representative knockdown efficiency of TRIM23 for the data shown in Figures 1h, 1i and Supplementary Figure 1g, determined by RT-qPCR at 48 h post-transfection. **(i)** Representative knockdown efficiency of the indicated genes for the data shown in Figures 1j and 1k, determined by RT-qPCR at 48 h post-transfection. Data (b to i) are representative of at least two independent experiments (n = 14 mice for WT and n = 11 mice for *Trim23*^{-/-} [b], mean $\pm$ SD of n = 4 to 10 mice [d], n = 3 biological replicates [f to i]). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Mantel–Cox log rank test [b], two-tailed Student's t test [d, f to i]). NS, not significant. Source data are provided as a Source Data file.



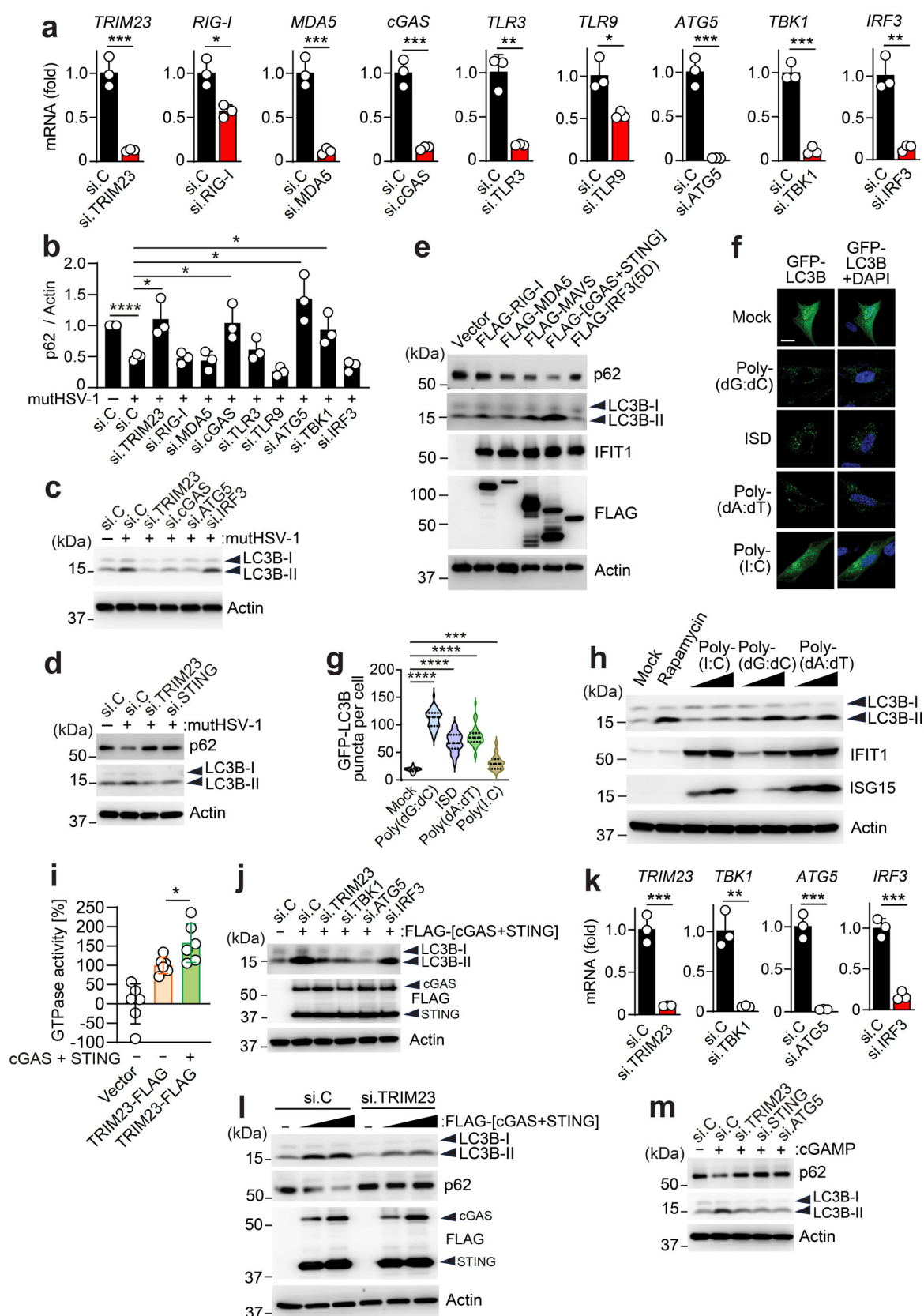
Supplementary Fig. 2 HSV-1 restriction by TRIM23 is IFN independent. (a) Antiviral gene transcripts in HDFs that were transfected for 30 h with the indicated siRNAs and then infected with WT HSV-1 (MOI 1) for 24 h, determined by RT-qPCR. (b) Knockdown efficiency of TRIM23 for the data shown in (a), determined by RT-qPCR. (c) Left: Viral titers in the supernatant of *Ifnar1*^{-/-} and WT MEFs that were transfected for 30 h with the indicated siRNAs and then infected with mutHSV-1 (MOI 0.1) for 24 h, determined by plaque assay. Right: Representative knockdown efficiency of TRIM23 for the data shown on the left, determined by RT-qPCR. (d) Viral titers in the supernatant of STAT2-deficient (U6A) and WT 2fTGH cells that

were transfected for 30 h with the indicated siRNAs and then infected with mutHSV-1 (MOI 0.1) for 24 h, determined by plaque assay. **(e)** Representative knockdown efficiency of the indicated genes for the data shown in (d), determined by RT-qPCR. **(f)** Endogenous p62 protein abundance in the WCLs of HEK293T cells that were transfected with either empty vector or FLAG-tagged TRIM23 in the absence or presence of anti-IFNAR2 antibody (2 µg/mL), determined at 24 h post-transfection by IB. HA-tagged TBK1, which induces autophagic flux, was included as a control. WCLs were further immunoblotted for endogenous IFIT1, a representative ISG, to confirm efficient inhibition of IFNAR signaling by anti-IFNAR2. **(g)** Densitometric analysis of the p62 signal intensities for the data shown in (f), normalized to the respective actin protein abundances. Values for empty vector (no IFNAR blocking) samples were set to 1. All data (a to g) are representative of at least two independent experiments (mean ± SD of n = 3 [a, b] or n = 2 [c to e] biological replicates; mean ± SD of n = 3 blots [g]). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (two-tailed Student's t test). NS, not significant. Source data are provided as a Source Data file.



Supplementary Fig. 3 Validation of p-S39-TRIM23 antibody by IF analysis, and endogenous TRIM23 S39 phosphorylation in mouse cells. (a) Validation of endogenous TRIM23 S39 phosphorylation staining in HDFs that were transfected for 30 h with the indicated siRNAs and then either mock-treated or infected with mutHSV-1 (MOI 5) for 16 h, determined by immunostaining with anti-p-S39-TRIM23 (red) and anti-TRIM23 (green) and confocal microscopy analysis. DAPI, nuclei (blue). Scale bar, 20 μ m. **(b)** Phosphorylation of endogenous TRIM23 at S39 in MEFs that were either mock-treated or infected with mutHSV-1 (MOI 5) for 16 h, determined as in (a). DAPI, nuclei (blue). Scale bar, 20 μ m. **(c)** Quantification of p-S39-TRIM23-positive cells for the data shown in (b). All data (a to c) are representative of at least two independent experiments (mean $\pm$ SD of n = 4 biological replicates [c]). ****p < 0.0001 (two-tailed Student's t test). Source data are provided as a Source Data file.

Supplementary Fig. 4 TBK1 mediates TRIM23 S39 phosphorylation, thereby promoting TRIM23 ubiquitination. **(a)** Representative knockdown efficiency of the indicated genes for the experiment shown in Figure 4a, determined by RT-qPCR at 48 h post siRNA transfection. **(b)** Densitometric analysis of the p-S39-TRIM23 signal intensities, normalized to the respective TRIM23 protein abundances, for the data shown in Figure 4a. **(c)** Ubiquitination and S39 phosphorylation of FLAG-tagged TRIM23 in transiently transfected HEK293T cells that co-expressed either empty vector (-) or increasing amounts of HA-TBK1, determined at 48 h post-transfection by IP with anti-FLAG and IB with the indicated antibodies. **(d)** Validation of CRISPR *TBK1* KO HEK293T single cell clones (Cl-1 and Cl-2) by confirming the absence of TBK1 protein in the WCLs by IB with anti-TBK1 and anti-actin (loading control). **(e)** Validation of CRISPR *TBK1* KO A549 cells, determined as in (d). All data (a to e) are representative of at least two independent experiments (mean $\pm$ SD of n = 3 biological replicates [a], n = 2 blots [b]). **p < 0.01; ***p < 0.001; ****p < 0.0001 (two-tailed Student's t test). Source data are provided as a Source Data file.

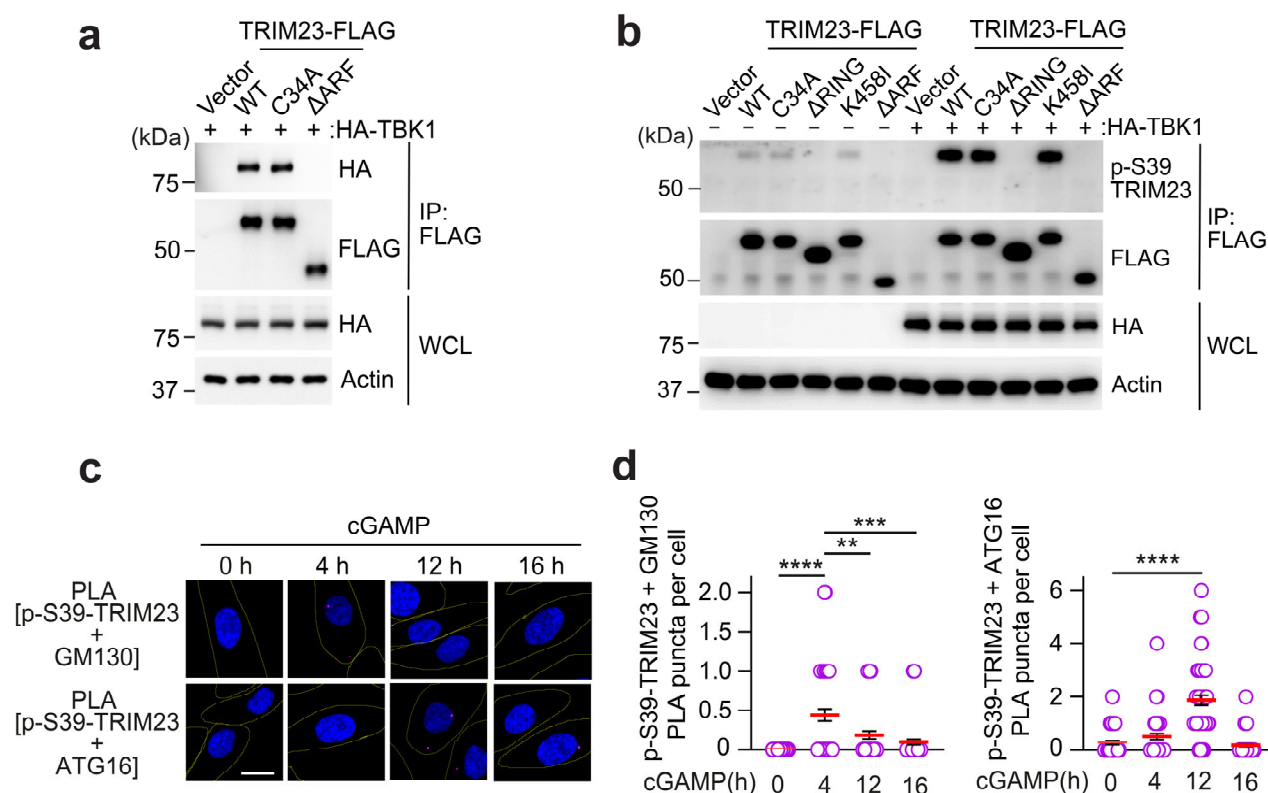


Supplementary Fig. 5 Autophagy induction by cGAS is mediated by TRIM23 and TBK1.

(a) Representative knockdown efficiency of the indicated genes for the experiment shown in

Figure 5a, determined by RT-qPCR at 48 h post siRNA transfection. **(b)** Densitometric analysis of the p62 signal intensities, normalized to the respective actin protein abundances, for the data shown in Figure 5a. **(c)** Endogenous LC3B-I-to-II conversion in HDFs that were transfected for 30 h with the indicated siRNAs and then either mock-treated (-) or infected with mutHSV1 (MOI 5) for 16 h, determined in the WCLs by IB. **(d)** Endogenous p62 protein abundance and LC3B-I-to-II conversion in HDFs that were transfected for 30 h with the indicated siRNAs and then either mock-treated (-) or infected with mutHSV-1 (MOI 10) for 16 h, determined in the WCLs by IB. **(e)** Endogenous p62 abundance and LC3B-I-to-II conversion in HEK293T cells that were transfected for 24 h with either empty vector or plasmids expressing the indicated proteins, determined as in (d). WCLs were further immunoblotted for endogenous IFIT1, an ISG, to confirm activation of downstream signaling by the transfected innate immune proteins. **(f)** GFP-LC3B puncta formation in HDFs that were transfected for 12 h with GFP-LC3B (green) and then either mock-treated or stimulated with poly(dG:dC)-LyoVec (3 μ g/mL), ISD-LyoVec (5 μ g/mL), poly(dA:dT)-LyoVec (1.5 μ g/mL), or poly(I:C)-LyoVec (1.5 μ g/mL) for 20 h, determined by confocal microscopy analysis. DAPI, nuclei (blue). Scale bar, 20 μ m. **(g)** Quantification of GFP-LC3B puncta for the data shown in (f). **(h)** Endogenous LC3B-I-to-II conversion in HDFs that were mock-treated, stimulated with poly(I:C)-LyoVec (0.5 and 1.5 μ g/mL), poly(dG:dC)-LyoVec (1 and 3 μ g/mL), or poly(dA:dT)-LyoVec (0.5 and 1.5 μ g/mL) for 20 h, or treated with rapamycin (3 μ M; positive control) for 4 h, determined in the WCLs by IB with the indicated antibodies. WCLs were further immunoblotted for endogenous IFIT1 and ISG15, which are both ISGs, to confirm activation of innate immune signaling by the administered stimuli. **(i)** GTPase activity of TRIM23-FLAG with or without ectopic co-expression of cGAS and STING after affinity purification from mammalian cells using anti-FLAG, determined by GTPase Glo assay. Empty vector expression served as a control.

Values for WT TRIM23 were set to 100%. **(j)** LC3B-I-to-II conversion in HEK293T cells that were transfected for 30 h with the indicated siRNAs and then co-transfected for 20 h with either empty vector or plasmids expressing FLAG-tagged cGAS and STING, determined in the WCLs by IB with the indicated antibodies. **(k)** Representative knockdown efficiency of the indicated genes for the experiment shown in (j and l), determined by RT-qPCR at 48 h post siRNA transfection. **(l)** LC3B-I-to-II conversion and p62 abundance in HEK293T cells that were transfected for 30 h with the indicated siRNAs and then co-transfected for 20 h with either empty vector or plasmids expressing FLAG-tagged cGAS and STING, determined in the WCLs by IB with the indicated antibodies. **(m)** Endogenous p62 protein abundance and LC3B-I-to-II conversion in HDFs that were transfected for 30 h with the indicated siRNAs and then treated with cGAMP (50 μ g/mL) for 12 h, determined in the WCLs by IB. All data (a to m) are representative of at least two independent experiments (mean $\pm$ SD of $n = 3$ biological replicates [a, k], $n = 3$ blots [b]; mean $\pm$ SEM of $n = 43$ cells [g], $n = 6$ biological replicates [i]). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (two-tailed Student's t test [a, b, k], two-tailed Student's t test with Welch's correction [g, i]). Source data are provided as a Source Data file.



Supplementary Fig. 6 S39 phosphorylation and TBK1 interaction of TRIM23 WT and mutants, and subcellular localization of activated TRIM23. **(a)** Binding of HA-tagged TBK1 to FLAG-tagged TRIM23 WT and mutants that were transiently expressed in HEK293T cells, determined at 48 h post-transfection by IP with anti-FLAG and IB with anti-HA. **(b)** S39 phosphorylation of FLAG-tagged TRIM23 WT or mutants in HEK293T cells that were co-transfected with either empty vector (-) or HA-TBK1, determined at 40 h post-transfection by IP with anti-FLAG and IB with anti-p-S39-TRIM23. **(c)** Representative confocal microscopy images showing the colocalization of p-S39-TRIM23 with GM130 (top panel) or ATG16 (bottom panel) in HDFs that were either mock-treated or stimulated with cGAMP (25 μ g/mL) for the indicated times, determined by PLA. PLA signal (magenta); nuclei (DAPI, blue). Scale bar, 20 μ m. **(d)** Quantification of the PLA signals for the data shown in (c). All data (a to d) are representative of at least two independent experiments (mean $\pm$ SEM of $n = 51$ -83 cells [d]). ** p

< 0.01, ***p < 0.001 ****p < 0.0001 (Student's t test with Welch correction [d]). Source data are provided as a Source Data file.

treated (-) or infected with mutHSV1 (MOI 1) for 16 h, determined in the WCLs by IB with anti-LC3B and anti-actin (loading control). Bottom: Densitometric quantification of the LC3B-II signal intensity, normalized to the respective actin protein abundances, for the data shown on top. **(c)** Binding of FLAG-tagged TRIM23 to HA-TBK1 WT and mutants that were transiently expressed in *TBK1* KO HEK293T cells, determined at 48 h post-transfection by IP with anti-FLAG and IB with anti-HA. **(d)** Proposed model of the cGAS-STING-TBK1-TRIM23 axis in autophagy regulation during HSV-1 infection. See Discussion section for details. Data (a to c) are representative of at least two independent experiments (mean $\pm$ SD of n = 3 blots [b]). ***p < 0.0001 (two-tailed Student's t test). Source data are provided as a Source Data file.

Supplementary Table 1. Primers and sgRNA sequences

A. Mouse genotyping primers	
F1-G	5'-TGCTCTGTTCTCCACCTGCG-3' (forward)
R1-G	5'-CTTTTTGTGTTAAGCATAGCTATGG-3' (forward)
R-NEO	5'-GGTGGGCTCTATGGCTTCTG-3' (reverse)
B. Primers for TRIM23 and TBK1 mutagenesis	
TRIM23-S39D	5'-GGAGACAAAGTTCCCCGTCTTTTGCTTTG-3' (forward) 5'-TTGCAAGTCAAAGACATCTTCACAACTCC-3' (reverse)
TRIM23-S39E	5'-GGAGACAAAGTTCCCCGTCTTTTGCTTTG-3' (forward) 5'-TTGCAATTCAAAGACATCTTCACAACTCC-3' (reverse)
TRIM23-S39A	5'-GGAGACAAAGTTCCCCGTCTTTTGCTTTG-3' (forward) 5'-TTGCAAGGCAAAGACATCTTCACAAAC-3' (reverse)
TBK1-K38A	5'-ACTGGTGATTTATTTGCTATCGCTGTATTTAATAACATAAGCTTCCTTC-3' (forward) 5'-GCTTATGTTATTAATAACAGCGATAGCAAATAAATCACCAGTTTTCTTATGT-3' (reverse)
TBK1-G159A	5'-ACTCACAGATTTTGCTGCAGCTAGAGAATTAGAAG-3' (forward) 5'-TTCTCTAGCTGCAGCAAAATCTGTGAGTTTGTACAC-3' (reverse)
TBK1-S172A	5'-ATGATGAGCAGTTTGTGCTCTGTATGGCAC-3' (forward) 5'-GTGCCATACAGAGCAACAACTGCTCATCAT-3' (reverse)
C. Small guide RNA oligonucleotides	
TBK1 gRNA 1	5'-GCTACTGCAAATGTCTTTCG-3'
TBK1 gRNA 2	5'-GAGGAAAACAGATTGGTT-3'
Non-targeting gRNA	5'-ACGGAGGCTAAGCGTCGCAA-3'
D. RT-qPCR primers and probes	
HSV-1 <i>ICP-0</i>	5'-CGCCCCCGCAACTGC-3' (forward) 5'-GGACACGGAAGTTCGA-3' (reverse) 5'-CCCCATCCACGCCCTG-3' (probe)
HSV-1 <i>ICP-8</i>	5'-CGACAGTAACGCCAGAAGCTC-3' (forward) 5'-GGAGACAAAGCCCAAGACGG-3' (reverse) 5'-ACGCGCGAGCGTACACGTAT-3' (probe)
HSV-1 <i>gB</i>	5'-CCCTTGGCGTTGATCTTG-3' (forward) 5'-CACCGCTACTCCCAGTTTATGG-3' (reverse) 5'-TCGATCACCTCCTCGAAGGG-3' (probe)
HSV-1 <i>UL36</i>	5'-GTGTCGCCGTCCCTGAA-3' (forward) 5'-CCCGCACACACGTTCTT-3' (reverse) 5'-TCGTACACGCTCCTAACCATTGGC-3' (probe)