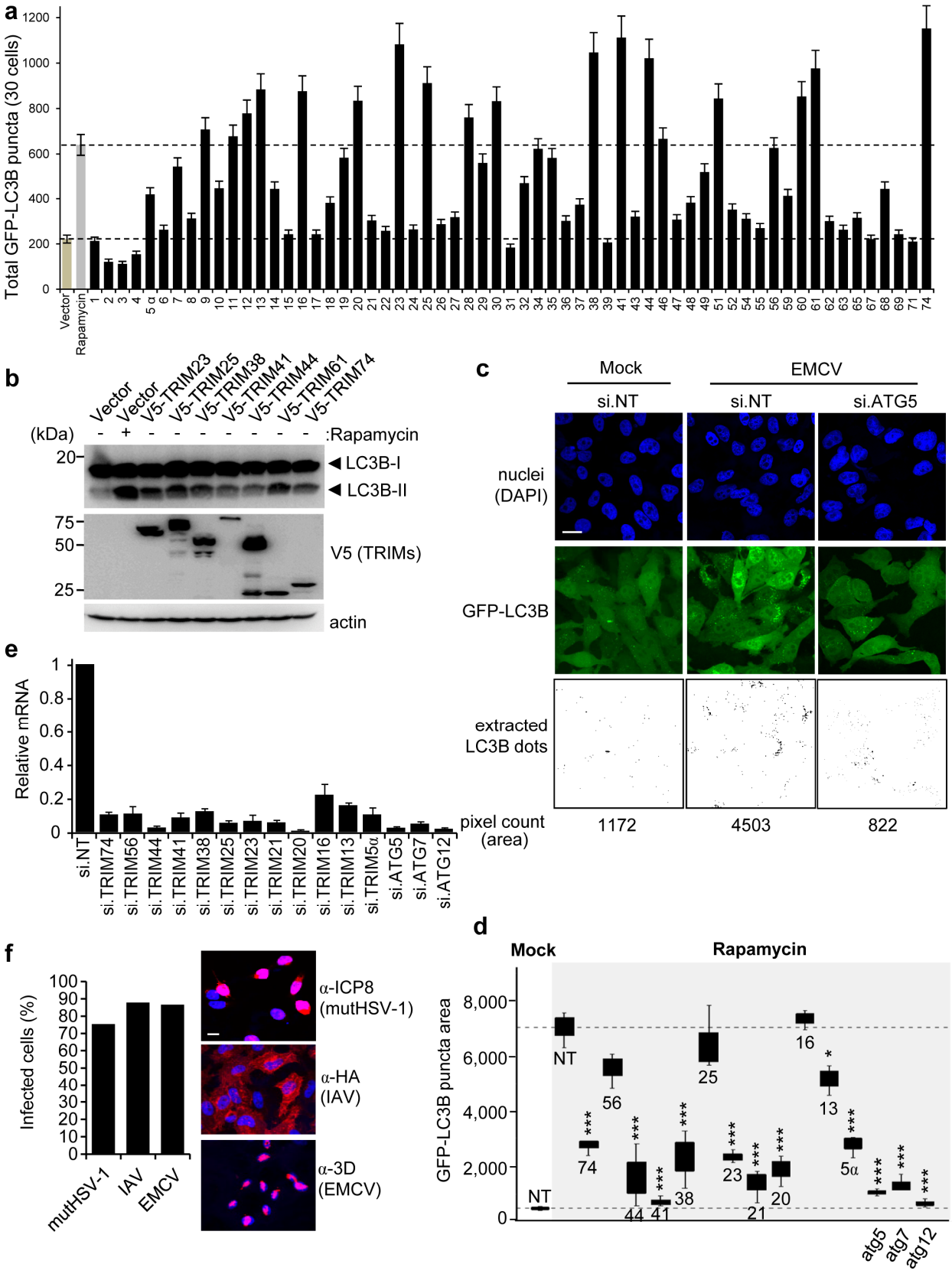
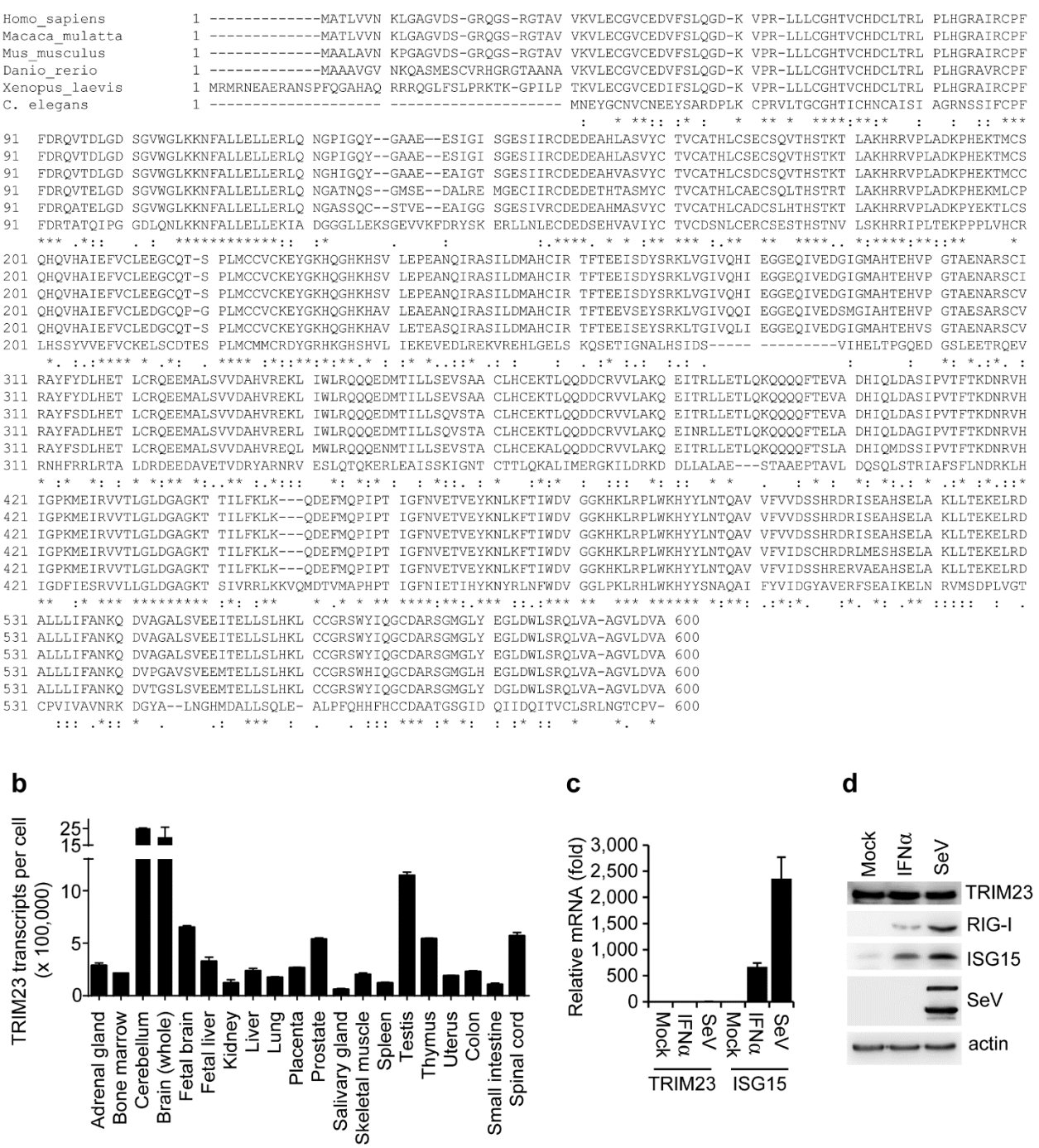
TRIM23 mediates virus-induced autophagy via activation of TBK1

Konstantin M.J. Sparrer, Sebastian Gableske, Matthew A. Zurenski, Zachary M. Parker, Florian Full, Gavin J. Baumgart, Jiro Kato, Gustavo Pacheco-Rodriguez, Chengyu Liang, Owen Pornillos, Joel Moss, Martha Vaughan, and Michaela U. Gack

Supplementary Figures 1-9

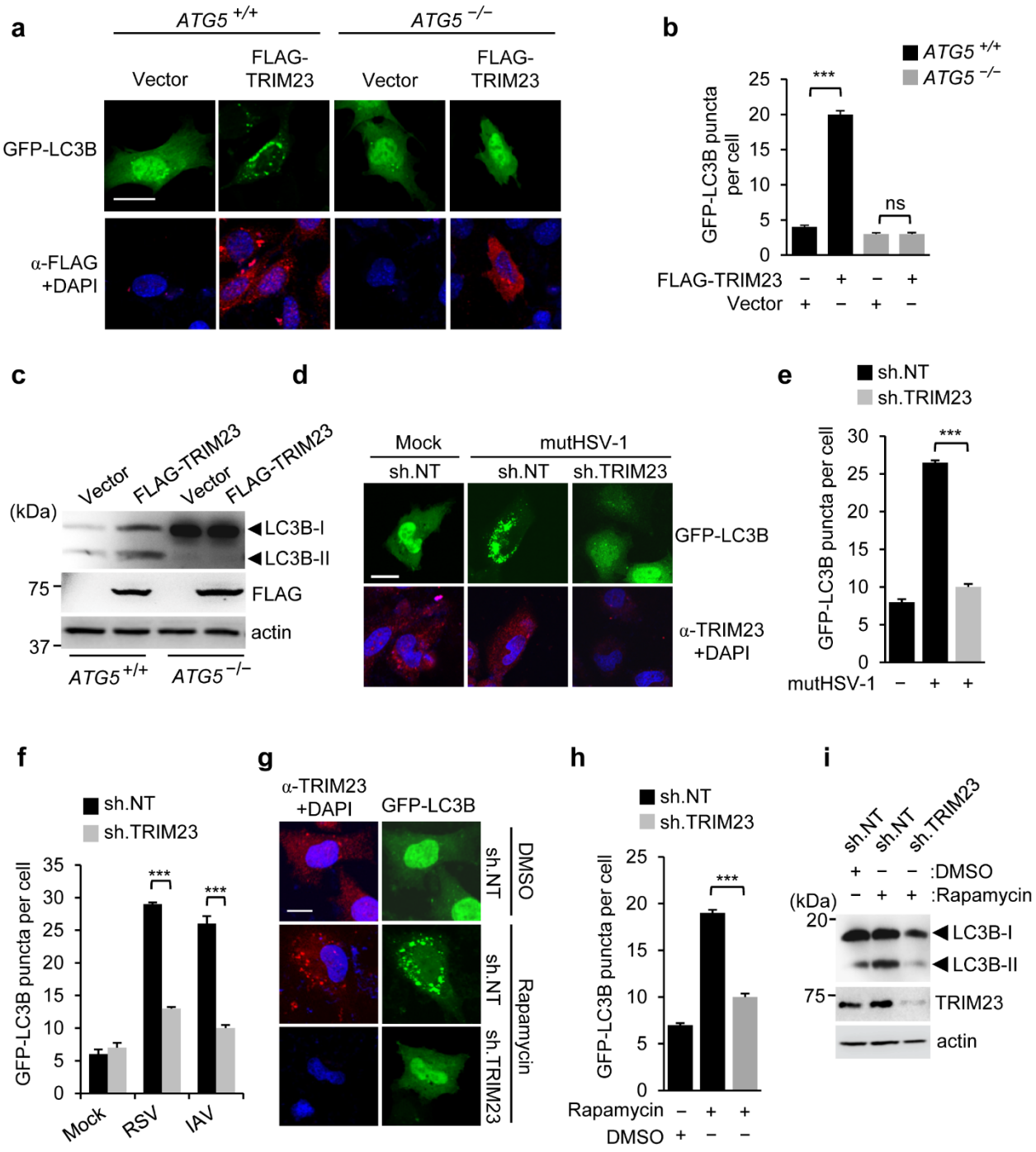

a
Total GFP-LC3B puncta (30 cells)
Vector
Rapamycin
b
Vector
Vector
V5-TRIM23
V5-TRIM25
V5-TRIM38
V5-TRIM41
V5-TRIM44
V5-TRIM61
V5-TRIM74
(kDa)
:Rapamycin
LC3B-I
LC3B-II
V5 (TRIMs)
actin
c
Mock
EMCV
si.NT
si.NT
si.ATG5
nuclei (DAPI)
GFP-LC3B
extracted LC3B dots
pixel count (area)
1172
4503
822
d
Mock
Rapamycin
GFP-LC3B puncta area
NT
NT
74
56
44
41
38
25
23
21
20
16
13
5α
atg5
atg7
atg12
e
Relative mRNA
si.NT
si.TRIM74
si.TRIM56
si.TRIM44
si.TRIM41
si.TRIM38
si.TRIM25
si.TRIM23
si.TRIM21
si.TRIM20
si.TRIM16
si.TRIM13
si.TRIM5α
si.ATG5
si.ATG7
si.ATG12
f
Infected cells (%)
mutHSV-1
IAV
EMCV
α-ICP8 (mutHSV-1)
α-HA (IAV)
α-3D (EMCV)

Supplementary Figure 1. TRIM proteins induce autophagy. a, cDNA screen of 61 TRIM proteins to test their ability to induce GFP-LC3B puncta formation in HeLa cells transiently transfected with GFP-LC3B and the indicated TRIM proteins. At 48 h post-transfection, cells were treated with 10 µg/mL E64D/pepstatin A for 3 h. GFP-LC3B puncta were analyzed by confocal microscopy, and for each sample, the GFP-LC3B puncta of 30 randomly selected cells were quantified. Transfection of empty vector as well as rapamycin treatment (for 2 h) served as negative and positive control, respectively. Results represent total GFP-LC3B puncta of 30 cells ± SD (n=5). Numbers indicate individual TRIM proteins. **b,** Conversion of endogenous LC3B-I to LC3B-II in HEK293T cells transiently transfected with empty vector or the seven TRIM proteins (all V5-tagged) that showed the highest autophagy-inducing activity in the cDNA screen (a). At 48 h post-transfection, cells were treated with 10 µg/mL E64D/pepstatin A for 2 h, and WCLs were analyzed by immunoblot (IB) with anti-LC3B, anti-V5, and anti-actin antibodies. Stimulation of cells with rapamycin served as positive control. **c,** Exemplary image analysis for the TRIM siRNA screen shown in Figures 1c-f. Example images show results from Figure 1e for uninfected (Mock) or EMCV-infected HeLa cells stably expressing GFP-LC3B (GFP-LC3B-HeLa) that had been transfected with non-targeting control siRNA (si.NT) or ATG5-specific siRNA (si.ATG5). Confocal microscopy images of GFP-LC3B puncta were automatically extracted using an ImageJ Macro and converted into binary images. The pixel counts of the area of GFP-LC3B puncta in a 500 x 500 pixels section were quantified. Scale bar, 20 µm. **d,** GFP-LC3B puncta formation in GFP-LC3B-HeLa cells that were transiently transfected with specific siRNAs targeting the indicated TRIM proteins and subsequently treated with 3 µM rapamycin and 10 µg/mL E64D/pepstatin A for 4 h. Cells transfected with non-targeting control siRNA (NT) served as control. GFP-LC3B puncta areas were quantified using ImageJ software as described above. Box and whisker plots show the distribution of the area of GFP-LC3B for 3 individual images each containing ~30 cells. Numbers indicate the individual TRIM proteins that were silenced using siRNA. *$p<0.05$, ***$p<0.0005$ (ANOVA test). **e,** Representative knockdown efficiency of the individual TRIM proteins in GFP-LC3B-HeLa cells for the screens shown in (d) and Figures 1c-f, determined by qPCR analysis. Relative knockdown efficiency is shown as compared to values for si.NT-transfected control cells, which were set to 1. Results represent the mean ± SD (n=3). **f,** Viral infection efficiency in GFP-LC3B-HeLa cells for the screens shown in Figures 1c-f, assessed by immunostaining of the indicated viral proteins (red) and immunofluorescence (IF) analysis. Nuclei were stained with DAPI (blue) to visualize individual cells. Results represent relative infection in % (n=75). Scale bar, 20 µm. ICP8, Infected cell protein 8. HA, Hemagglutinin. 3D, 3D polymerase. Data are representative of one screen (**a**), or at least two (**b,d,f**) or three (**c,e**) independent experiments.



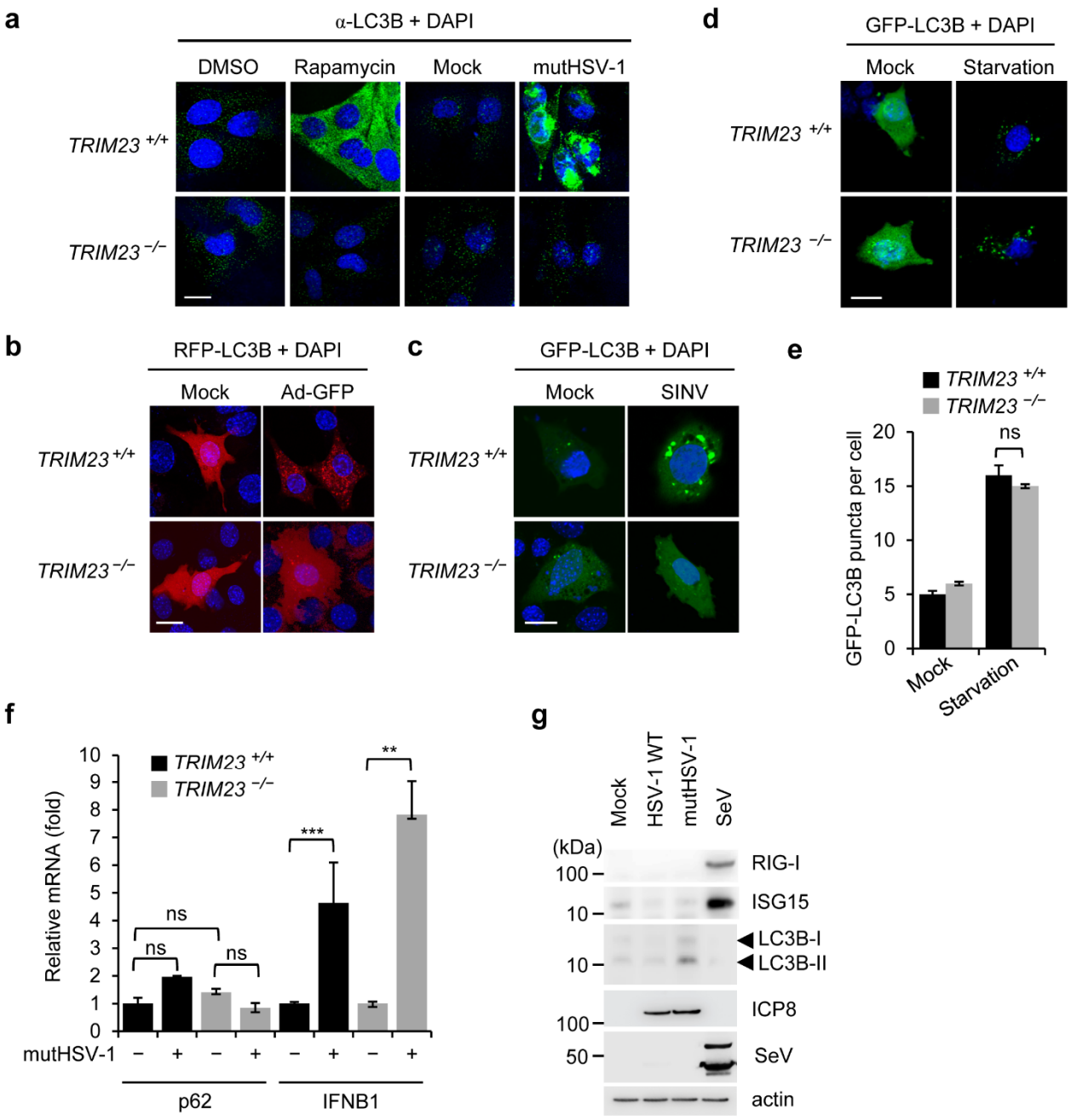
Supplementary Figure 2. TRIM23 expression in human tissues and cells. a, Alignment of TRIM23 amino acid sequences from the indicated species using Clustal Omega. Numbers indicate amino acids. Asterisks (*) indicate positions which have a single, fully conserved residue. Colons (:) indicate conservation between groups of strongly similar properties, and periods (.) indicate conservation between groups of weakly similar properties. **b,** Absolute copy number of TRIM23 transcripts in the indicated human tissues, determined by qRT-PCR analysis. Data represent the mean ± SD (n=3). **c,** Relative transcript levels of

TRIM23 and ISG15 (positive control) in HEK293T cells that were mock-treated, stimulated with 1,000 U/mL IFNα_2 for 18 h, or infected with 25 HA units per mL (HAU/mL) Sendai virus (SeV) for 18 h, determined by qRT-PCR analysis. Results are expressed as fold levels compared to mock-treated cells. Data represent the mean ± SD (n=3). **d,** Protein abundance of endogenous TRIM23 as well as RIG-I and ISG15, which are both IFN-stimulated genes (ISGs) and therefore served as positive controls, in HEK293T cells that were mock-treated, stimulated with 1,000 U/mL IFNα_2 for 18 h, or infected with 25 HAU/mL SeV for 48 h, determined by IB with anti-TRIM23, anti-RIG-I, and anti-ISG15 antibodies. WCLs were further immunoblotted with anti-SeV and anti-actin antibodies. Data are representative of one experiment (**a**), or at least two independent experiments (**b-d**).



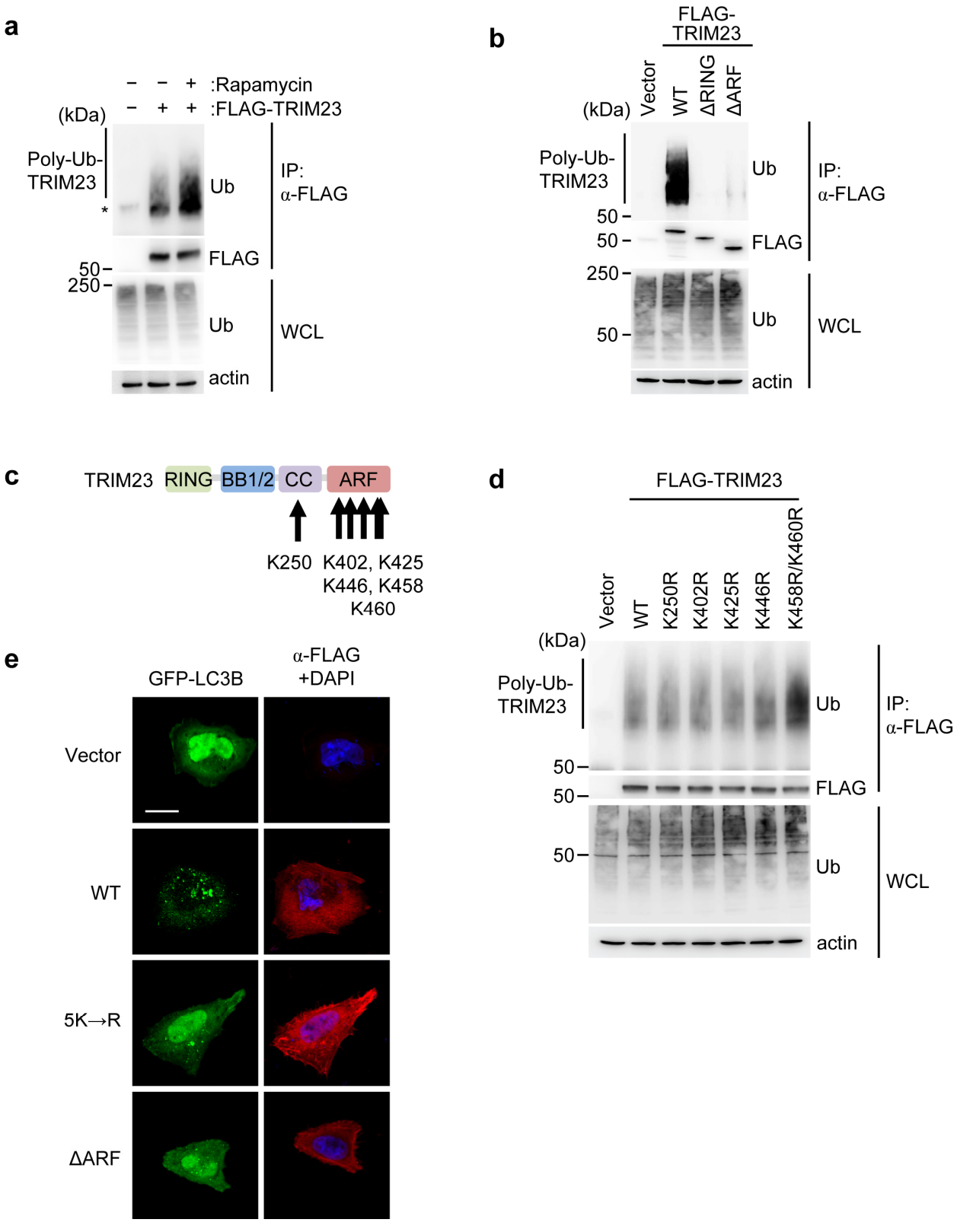
Supplementary Figure 3. TRIM23-mediated autophagy is dependent on ATG5. a, GFP-LC3B puncta formation in *ATG5* $^{+/+}$ and *ATG5* $^{-/-}$ MEFs transiently transfected with GFP-LC3B (green) together with empty vector or FLAG-tagged TRIM23 (red). At 48 h post-transfection, FLAG-TRIM23 was visualized by immunostaining with anti-FLAG antibody. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. **b,** Quantification of GFP-LC3B puncta for the experiment shown in (a). Results represent the mean GFP-LC3B puncta per cell ± SD (n=50). **c,** Endogenous LC3B-I to LC3B-II conversion in *ATG5* $^{+/+}$ and *ATG5* $^{-/-}$

MEFs transfected with empty vector or FLAG-tagged TRIM23, determined by IB with anti-LC3B antibody at 50 h post-transfection. **d,** Laser scanning confocal microscopy images of GFP-LC3B puncta formation in HeLa cells transiently transfected with GFP-LC3B (green) and either TRIM23-targeting shRNA (sh.TRIM23) or non-targeting control shRNA (sh.NT) for 38 h and subsequently infected with mutHSV-1 (MOI 4) for 12 h, or left uninfected (Mock). Knockdown of endogenous TRIM23 was confirmed by immunostaining with anti-TRIM23 antibody (red). Nuclei were stained with DAPI (blue). Scale bar, 20 μm. **e,** Quantification of GFP-LC3B puncta for the experiment shown in (d). Results represent the mean GFP-LC3B puncta per cell ± SD (n=50). **f**, Quantification of GFP-LC3B puncta in HeLa cells that were transiently transfected with GFP-LC3B together with sh.TRIM23 or sh.NT for 38 h and subsequently infected with RSV (MOI 0.5) or IAV (MOI 1) for 20 h, or left uninfected (Mock). Results represent the mean GFP-LC3B puncta per cell ± SD (n=50). **g,** Representative laser scanning confocal microscopy images of GFP-LC3B puncta formation in HeLa cells that were transiently transfected with GFP-LC3B (green) and sh.TRIM23 or sh.NT for 46 h and subsequently treated with either DMSO or rapamycin for 2 h. Knockdown of endogenous TRIM23 was confirmed by immunostaining with anti-TRIM23 antibody (red). Nuclei were stained with DAPI (blue). Scale bar, 20 μm. **h,** Quantification of GFP-LC3B puncta for the experiment shown in (g). Results represent the mean GFP-LC3B puncta per cell ± SD (n=50). **i,** Endogenous LC3B-I to LC3B-II conversion in HEK293T cells that were transfected with sh.NT or sh.TRIM23 for 48 h and subsequently treated with DMSO or rapamycin for 2 h, assessed by IB with anti-LC3B antibody. Knockdown of endogenous TRIM23 was confirmed by IB with anti-TRIM23 antibody. ns, statistically not significant, ***p <0.001 (Student's *t*-test). Data are representative of at least two independent experiments (**a-i**).

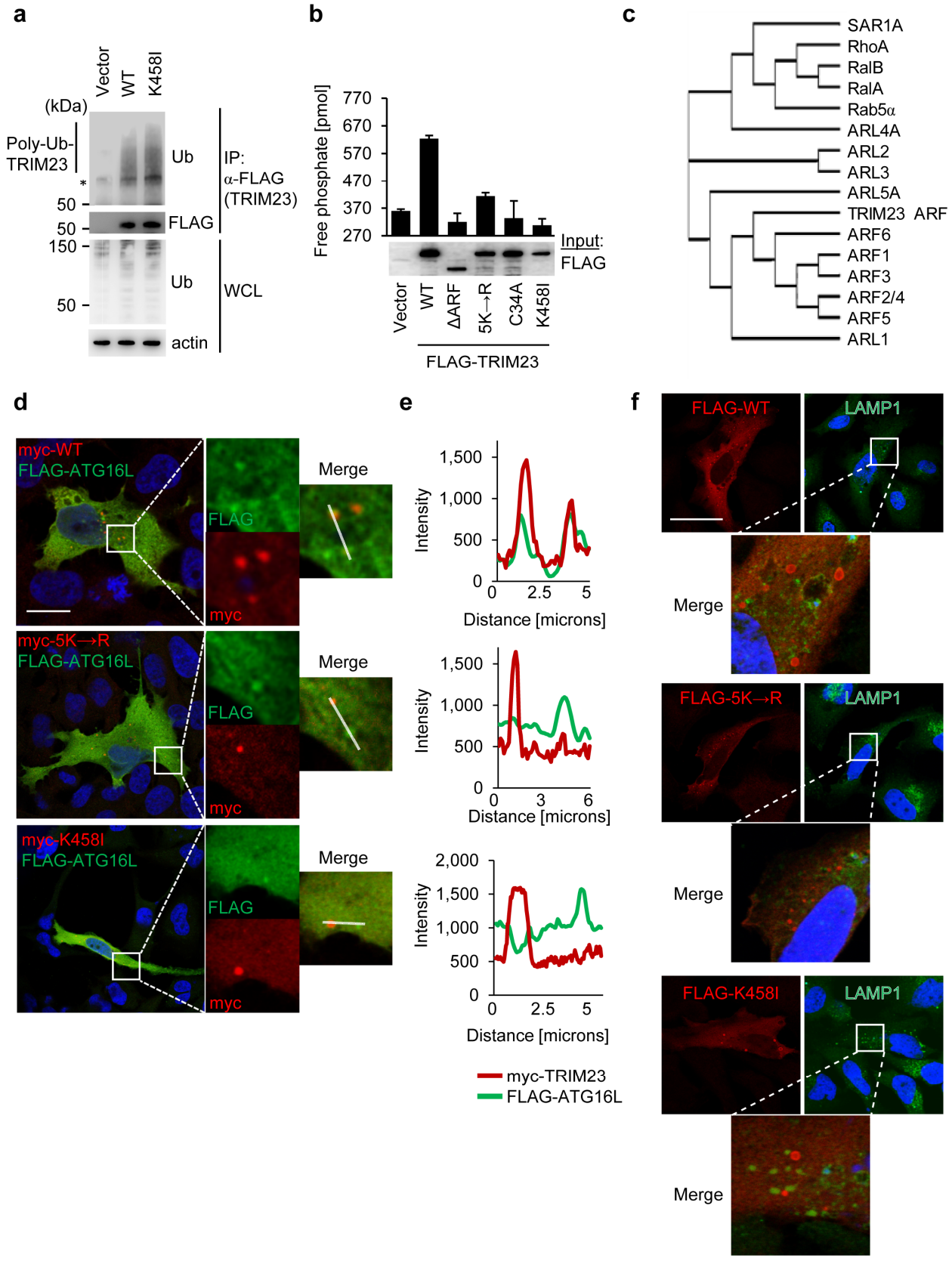


Supplementary Figure 4. Virus- and rapamycin-induced autophagy is abrogated in *TRIM23* $^{-/-}$ MEFs. **a,** Accumulation of endogenous LC3B (green) in *TRIM23* $^{+/+}$ and *TRIM23* $^{-/-}$ MEFs that were mock-treated, stimulated with rapamycin for 2 h, or infected with mutHSV-1 (MOI 4) for 12 h, determined by immunostaining using anti-LC3B antibody and confocal microscopy analysis. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. **b,** Representative laser scanning confocal microscopy images from the experiment shown in Figure 2d showing RFP-LC3B puncta formation in *TRIM23* $^{+/+}$ and *TRIM23* $^{-/-}$ MEFs that were transiently transfected with RFP-LC3B (red) and subsequently infected with Ad-GFP (MOI 100) for 48 h. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. **c,** Representative laser scanning confocal

microscopy images from the experiment shown in Figure 2e showing GFP-LC3B puncta formation in *TRIM23* $^{+/+}$ and *TRIM23* $^{-/-}$ MEFs that were transiently transfected with GFP-LC3B (green) and subsequently infected with SINV (MOI 5) for 24 h. Nuclei were stained with DAPI (blue). Scale bar, 20 µm. **d,** Representative confocal microcopy images of GFP-LC3B puncta formation in *TRIM23* $^{+/+}$ and *TRIM23* $^{-/-}$ MEFs that were transiently transfected with GFP-LC3B (green) and incubated in starvation medium (Hank's buffered salt solution) for 2 h. Nuclei were stained with DAPI (blue). Scale bar, 20 µm. **e**, Quantification of GFP-LC3B puncta from the experiment shown in (d). Results represent the mean GFP-LC3B puncta per cell ± SD (n=50). **f,** Relative transcript levels of *p62* in *TRIM23* $^{+/+}$ and *TRIM23* $^{-/-}$ MEFs that were either left uninfected or infected with mutHSV-1 (MOI 4) for 12 h, determined by qRT-PCR. Analysis of *IFNB1* transcripts served as control. Values were normalized to *GAPDH* mRNA levels, and are presented as fold induction relative to values for uninfected control samples, which were set to 1. **g,** Protein abundance of endogenous RIG-I, ISG15, LC3B-I and LC3B-II in HEK293T cells that were left uninfected (Mock), or infected with WT HSV-1 or mutHSV-1 (each MOI 4) for 16 h, or SeV (300 HAU/mL) for 48 h, determined by IB with the indicated antibodies. WCLs were further immunoblotted with anti-ICP8 (HSV-1) and anti-SeV antibodies to confirm efficient viral infection. ns, statistically not significant, **p <0.01, ***p <0.001 (Student's *t*-test). Data are representative of at least two independent experiments (**a-g**).


a
− − + :Rapamycin
− + + :FLAG-TRIM23
(kDa)
Poly-Ub-TRIM23
*
Ub
IP: α-FLAG
FLAG
50
250
Ub
WCL
actin
b
FLAG-TRIM23
Vector
WT
ΔRING
ΔARF
(kDa)
Poly-Ub-TRIM23
Ub
IP: α-FLAG
50
50
FLAG
250
50
Ub
WCL
actin
c
TRIM23
RING
BB1/2
CC
ARF
K250
K402, K425
K446, K458
K460
d
FLAG-TRIM23
Vector
WT
K250R
K402R
K425R
K446R
K458R/K460R
(kDa)
Poly-Ub-TRIM23
Ub
IP: α-FLAG
50
50
FLAG
50
Ub
WCL
actin
e
GFP-LC3B
α-FLAG +DAPI
Vector
WT
5K→R
ΔARF

Supplementary Figure 5: Ubiquitination and autophagy-inducing abilities of TRIM23 WT and mutants. **a,** Ubiquitination of FLAG-TRIM23 in transiently transfected HEK293T cells that were either mock-treated or treated with rapamycin for 4 h, assessed by Immunoprecipitation (IP) with anti-FLAG and IB with anti-ubiquitin (Ub) at 52 h post-transfection. Asterisk indicates unspecific band. **b,** Ubiquitination of FLAG-tagged TRIM23 WT or mutants in transiently transfected HEK293T cells, determined by IP with anti-FLAG and IB with anti-Ub at 48 h post-transfection. **c,** Schematic representation of TRIM23 domain structure as well as the ubiquitinated lysine residues in TRIM23 identified by mass spectrometry analysis of affinity-purified FLAG-TRIM23 from HEK293T cells. **d,** Ubiquitination of FLAG-tagged TRIM23 WT or the indicated mutants in transiently transfected HEK293T cells, determined as in (b). **e,** Representative laser scanning confocal microscopy images of GFP-LC3B puncta formation in HeLa cells that were transiently transfected with GFP-LCB (green) together with empty vector or FLAG-tagged TRIM23 WT or mutants (red). At 48 h post-transfection, cells were stained with anti-FLAG antibody, followed by confocal microscopy analysis. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. Data are representative of one (**c**), or at least two (**a,b,d,e**) independent experiments.


a
Vector
WT
K458I
(kDa)
Poly-Ub-TRIM23
Ub
IP: α-FLAG (TRIM23)
50
50
FLAG
150
Ub
WCL
50
actin
b
Free phosphate [pmol]
770
670
570
470
370
270
Input: FLAG
Vector
WT
ΔARF
5K→R
C34A
K458I
FLAG-TRIM23
c
SAR1A
RhoA
RalB
RalA
Rab5α
ARL4A
ARL2
ARL3
ARL5A
TRIM23
ARF
ARF6
ARF1
ARF3
ARF2/4
ARF5
ARL1
d
myc-WT
FLAG-ATG16L
FLAG
myc
Merge
myc-5K→R
FLAG-ATG16L
FLAG
myc
Merge
myc-K458I
FLAG-ATG16L
FLAG
myc
Merge
e
Intensity
1,500
1,000
500
0
0
2.5
5
Distance [microns]
Intensity
1,500
1,000
500
0
0
3
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Distance [microns]
Intensity
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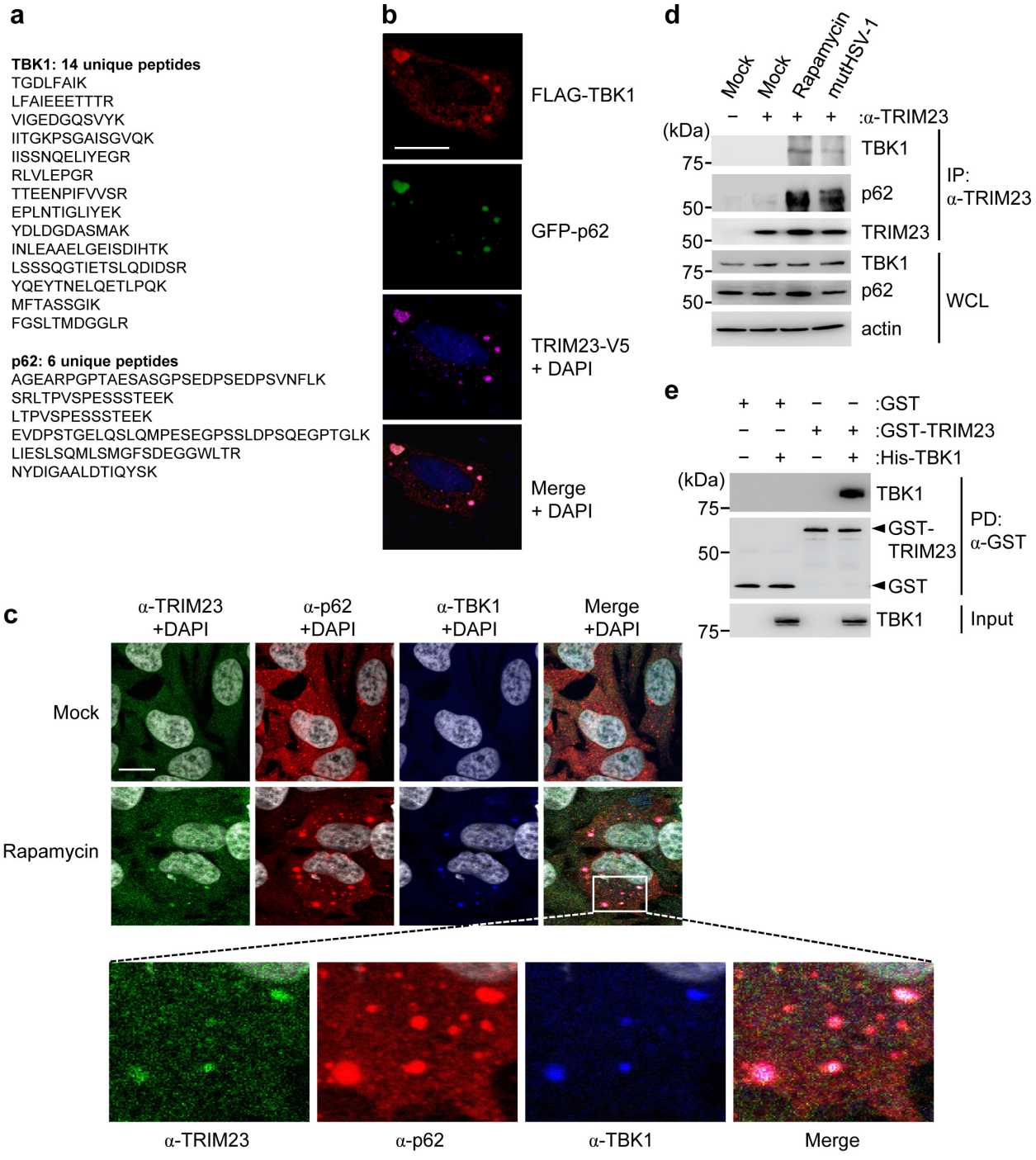
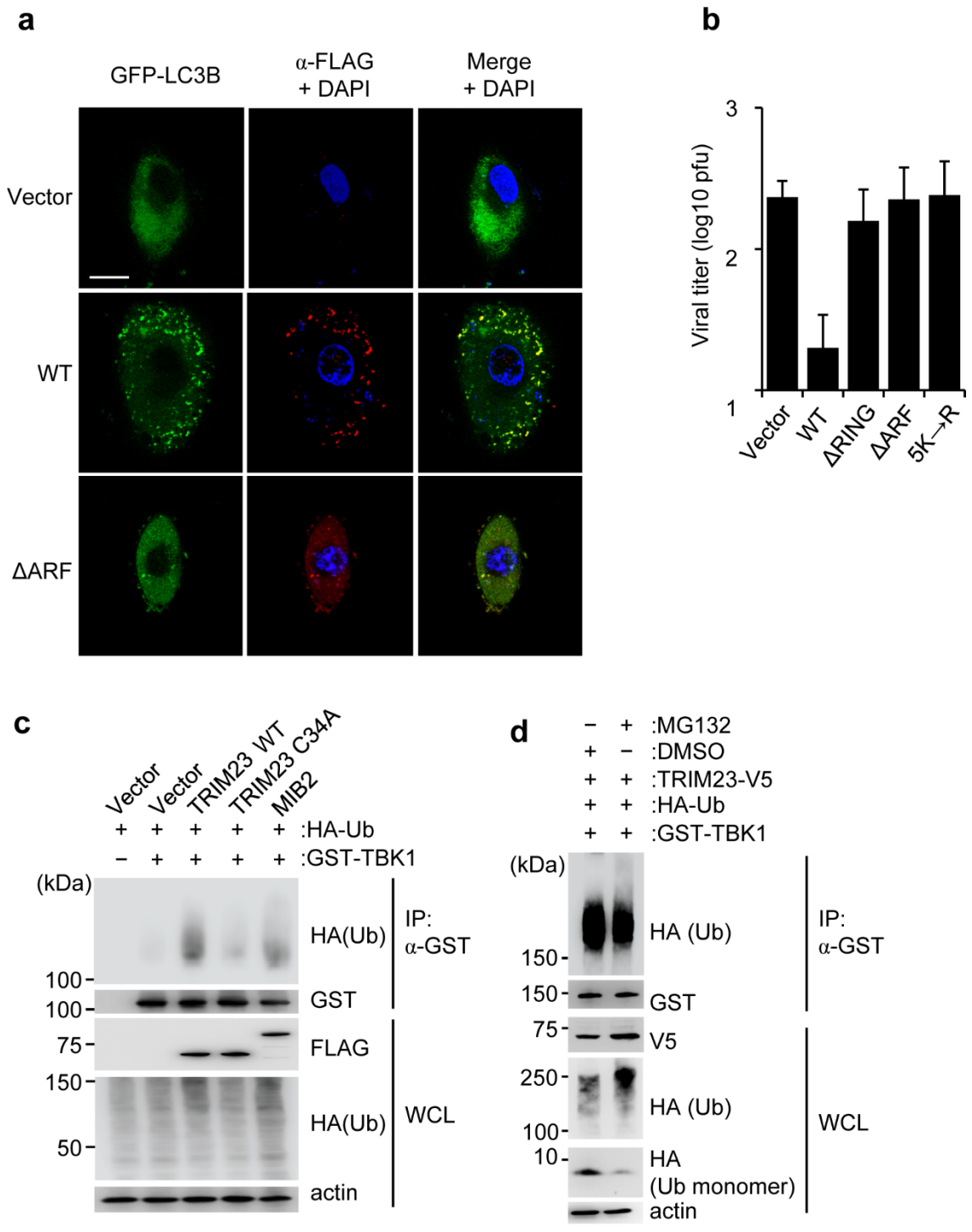
1,500
1,000
500
0
0
2.5
5
Distance [microns]
myc-TRIM23
FLAG-ATG16L
f
FLAG-WT
LAMP1
Merge
FLAG-5K→R
LAMP1
Merge
FLAG-K458I
LAMP1
Merge

Supplementary Figure 6. Characterization of GTPase- or K27-polyubiquitin-deficient mutants of TRIM23. a, Ubiquitination of FLAG-tagged TRIM23 WT or the mutant K458I in transiently transfected HEK293T cells, assessed by IP with anti-FLAG and IB with anti-Ub at 48 h post-transfection. Transfection of empty vector served as control. Asterisk indicates unspecific band. **b,** *In vitro* GTPase activity of purified FLAG-TRIM23 WT or the indicated mutants. Purified proteins were incubated *in vitro* with GTP for 2 h, followed by measuring the generation of free phosphate using a colorimetric reaction. Results are expressed as mean ± SD (n=2). **c,** Cladogram showing the relation of the TRIM23 ARF domain to other small GTPases: Sar1/Arf family: SAR1A, ARF1-6 and ARL1-5; Ras family: RalB, RalA; Rho/Rac family: RhoA; Rab family: Rab5α. **d,** Co-localization of myc-tagged TRIM23 WT or mutants (red) with FLAG-tagged ATG16L (green) in transiently transfected HeLa cells. At 48 h post-transfection, cells were immunostained with anti-myc and anti-ATG16L antibodies. DAPI, nuclei (blue). Scale bar, 20 µm. **e,** Color-coded intensity profiles of the merged confocal images from (d) (white lines). **f,** Co-localization analysis of FLAG-tagged TRIM23 WT or mutants (red) and endogenous LAMP1 (green) in transiently transfected HeLa cells. At 48 h post-transfection, cells were immunostained using anti-FLAG and anti-LAMP1 antibodies. DAPI, nuclei (blue). Scale bar, 20 µm. Data are representative of one (**c**), or at least two (**a**, **b**, **d-f**) independent experiments.



Supplementary Figure 7. Ternary complex formation of TRIM23, TBK1 and p62. a, Amino acid sequence of unique peptides of TBK1 and p62 identified by mass spectrometry analysis of affinity-purified FLAG-TRIM23 from HEK293T cells that were infected with mutHSV-1 (MOI 1) for 12 h. **b,** Co-localization of FLAG-TBK1 (red), GFP-p62 (green), and TRIM23-V5 (magenta) in transiently transfected HeLa cells. At 48 h post-transfection, cells were stained with anti-FLAG and anti-V5 antibodies, followed by confocal

microscopy analysis. DAPI, nuclei (blue). Scale bar, 20 µm. **c,** Co-localization of endogenous TRIM23 (green), endogenous p62 (red), and endogenous TBK1 (blue) in HeLa cells that were either treated with DMSO (Mock) or rapamycin for 8 h, assessed by immunostaining with anti-TRIM23, anti-p62, and anti-TBK1 antibodies and confocal microscopy. DAPI-stained nuclei are depicted in white. Scale bar, 20 µm. **d,** Binding of endogenous p62 and TBK1 to endogenous TRIM23 in HEK293T cells that were either mock-treated, stimulated with rapamycin for 4 h, or infected with mutHSV-1 (MOI 1) for 24 h, determined by IP with anti-TRIM23 antibody, followed by IB with anti-p62 and anti-TBK1 antibodies. **e,** *In vitro* binding of recombinant His-tagged TBK1 and purified recombinant GST or GST-TRIM23, determined by pulldown (PD) assay using GST-agarose, followed by IB with anti-TBK1. Data are representative of one (**a**) or at least two (**b-e**) independent experiments.



Supplementary Figure 8. Effect of TRIM23 WT and mutants on viral growth and TBK1 ubiquitination. **a,** GFP-LC3B puncta formation as well as localization of TRIM23 WT and ΔARF mutant in *TRIM23* $^{-/-}$ MEF cells transiently transfected with GFP-LC3B (green) together with empty vector, or FLAG-tagged TRIM23

WT or ΔARF mutant. TRIM23 WT and mutant proteins were immunostained using anti-FLAG antibody (red). Nuclei were stained with DAPI (blue). Scale bar, 20 μm. **b,** Replication of mutHSV-1 in TRIM23 $^{-/-}$ MEFs that were transiently transfected with either empty vector, or TRIM23 WT or mutants, and subsequently infected with mutHSV1 (MOI 0.1) for 48 h. Viral titers in the cell supernatant were determined by plaque assay. Results are expressed as mean ± SD (n=2). **c,** Ubiquitination of GST-TBK1 in transiently transfected HEK293T cells that co-expressed HA-tagged Ub and either empty vector or FLAG-tagged TRIM23 WT or C34A, or MIB2 (positive control), determined by IP with anti-GST and IB with anti-HA at 48 h post-transfection. **d,** Ubiquitination of GST-TBK1 in transiently transfected HEK293T cells that co-expressed V5-tagged TRIM23 and HA-Ub. At 24 h post-transfection, cells were treated with MG132 or DMSO for 4 h, followed by IP with anti-GST and IB with anti-HA. Data are representative of at least two independent experiments (**a-d**).

Figure 2f:

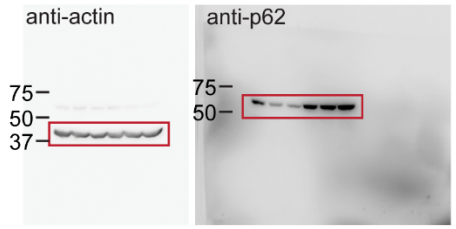


Figure 2j:

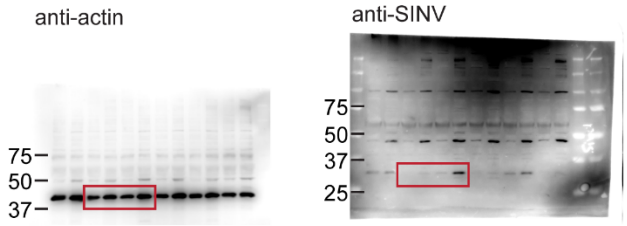


Figure 3a:

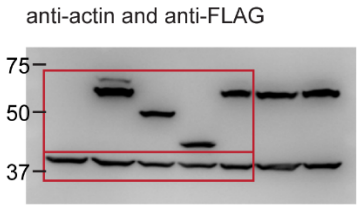


Figure 3d:

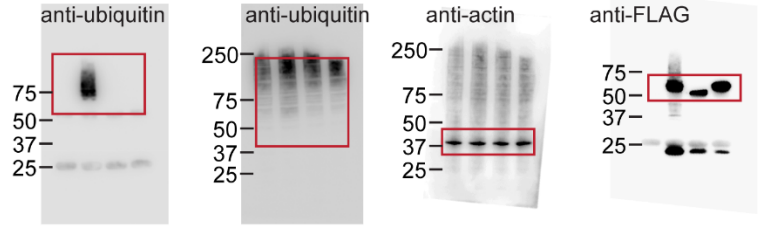


Figure 3e:

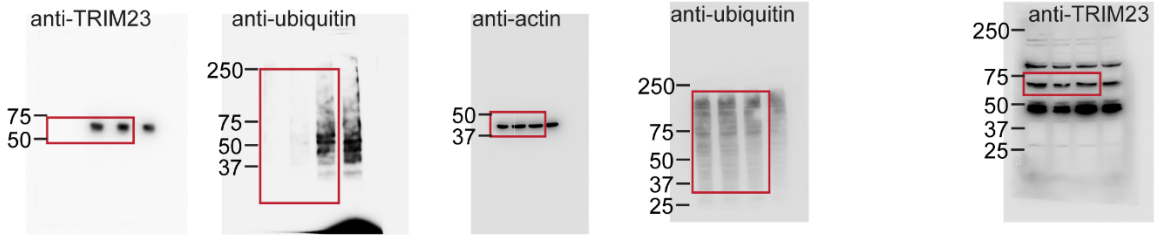
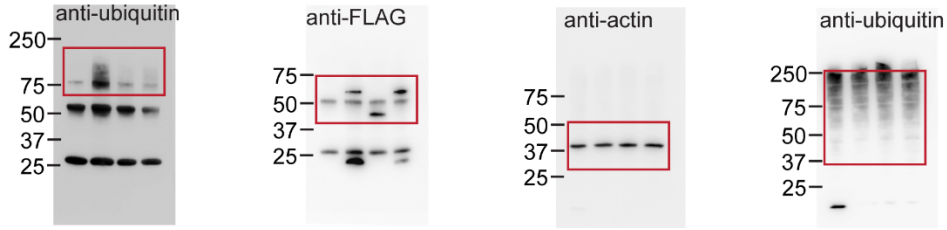


Figure 3f:



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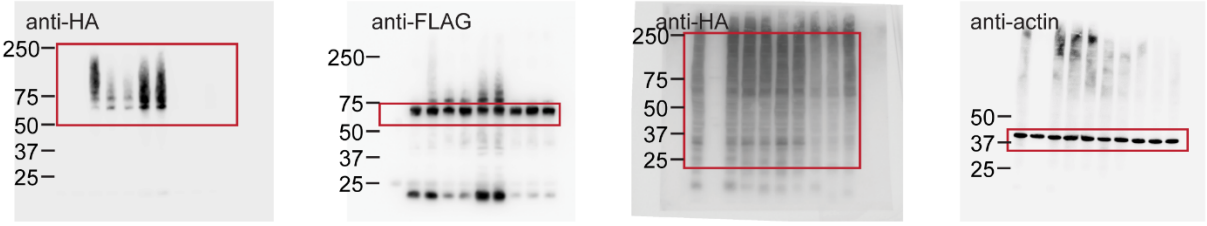
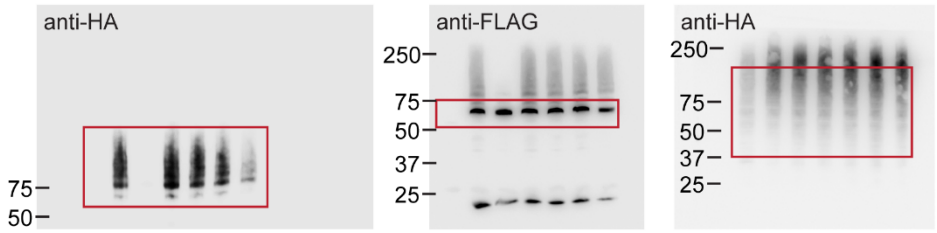


Figure 3i:



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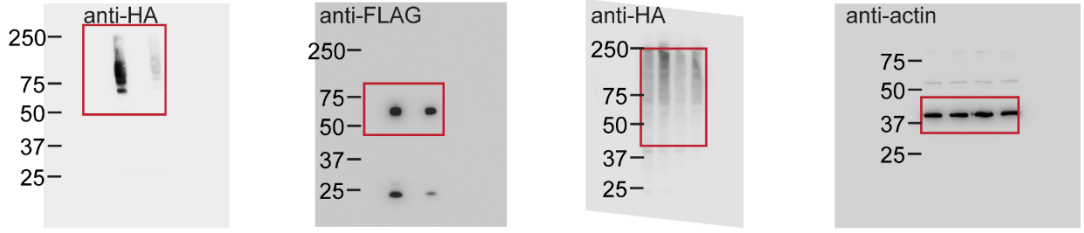


Figure 4c:

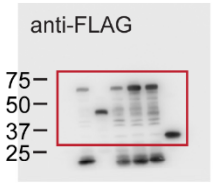


Figure 4d:

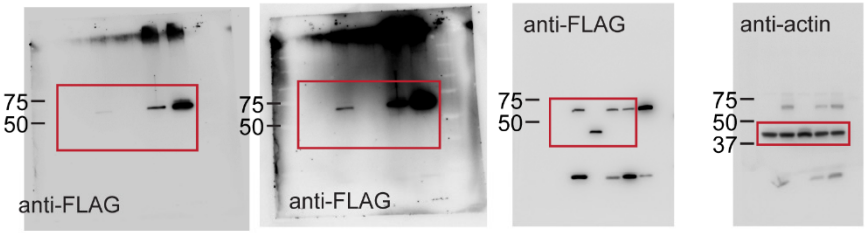


Figure 5a:

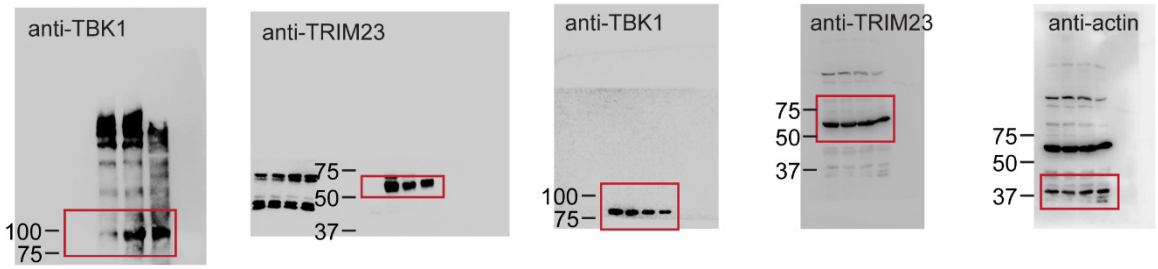


Figure 5b:

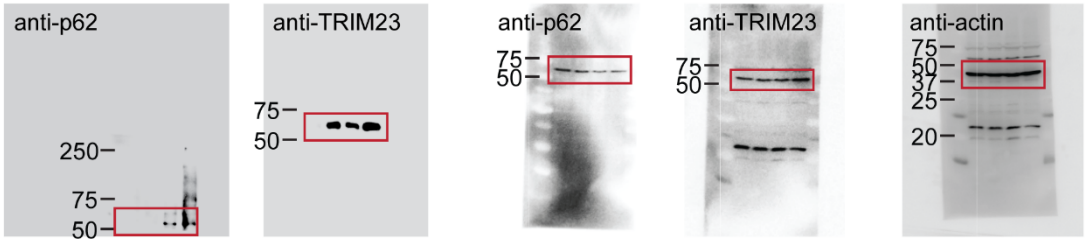


Figure 5c:

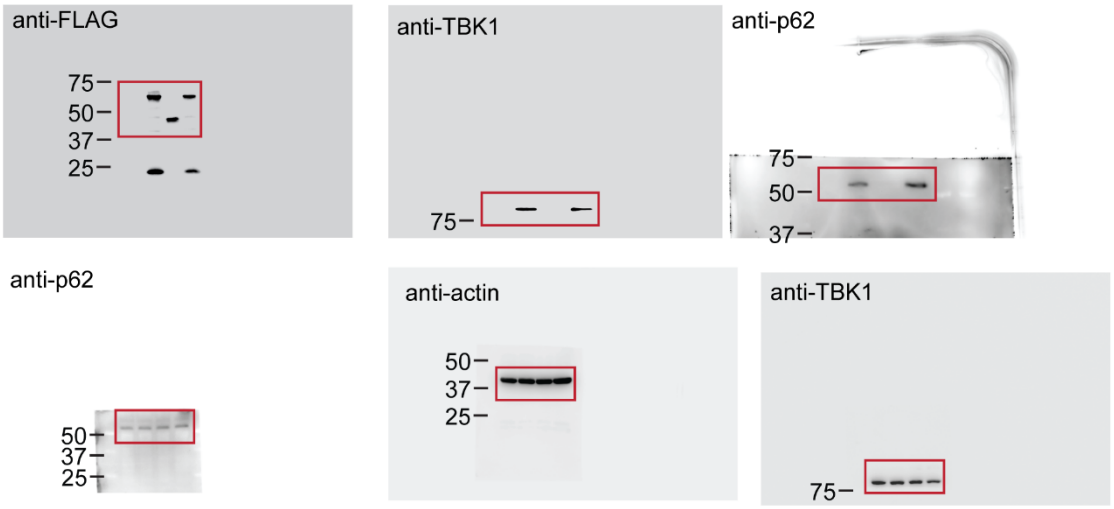


Figure 5e:

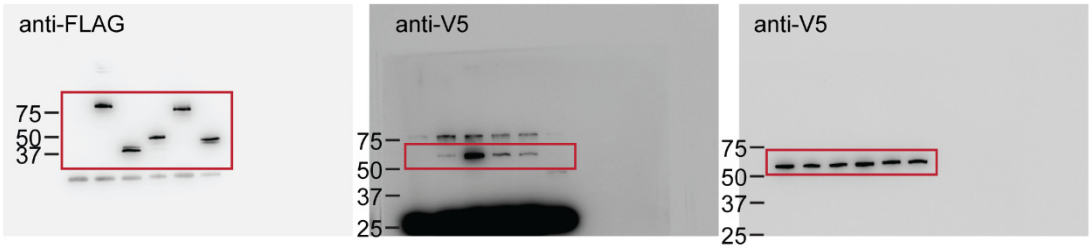


Figure 5h:

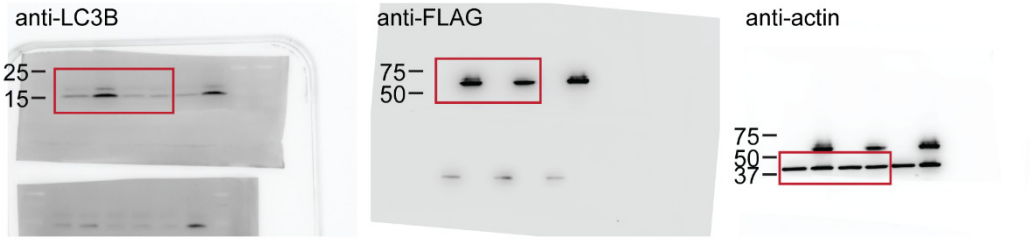


Figure 6a:

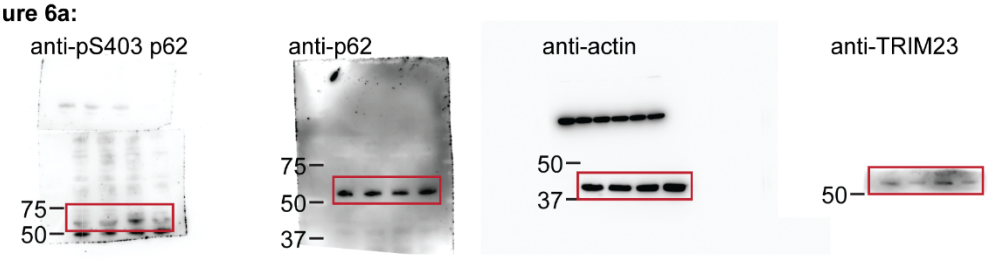


Figure 6b:

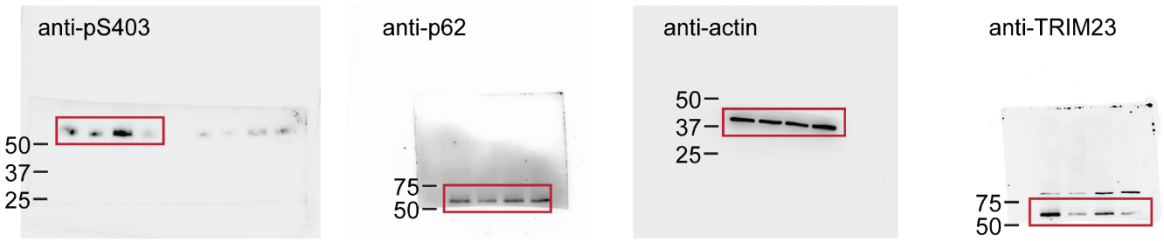


Figure 6c:

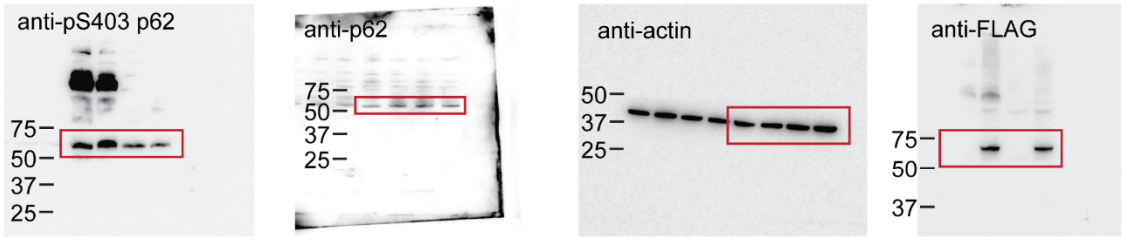


Figure 6d:

Figure 6f:

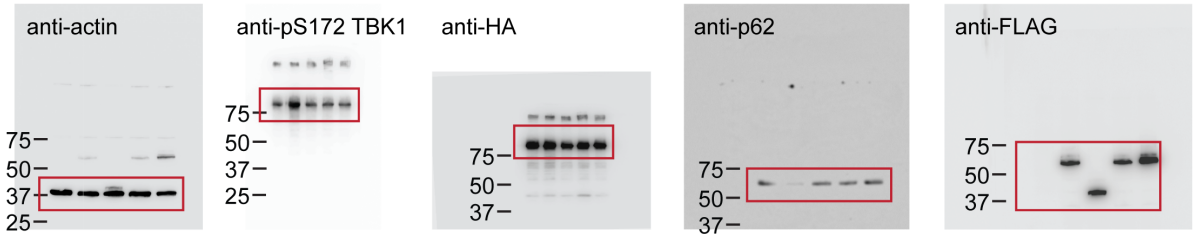
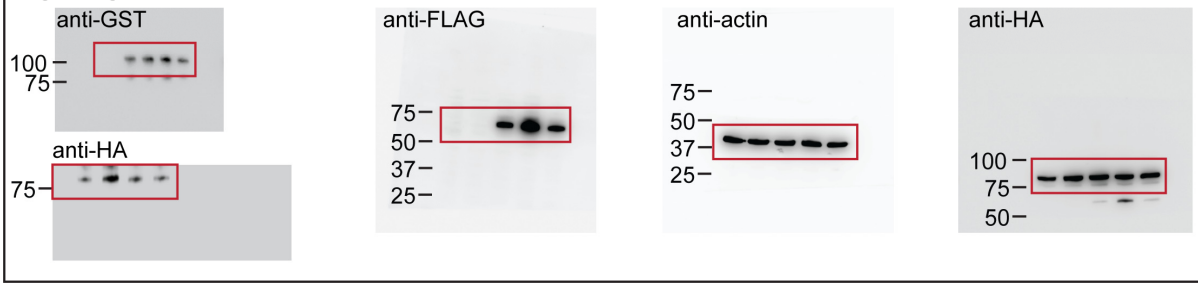
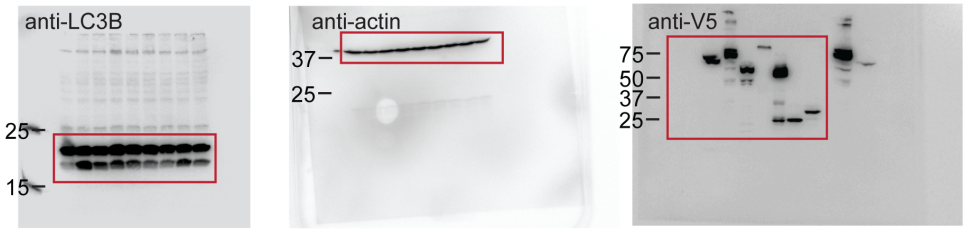


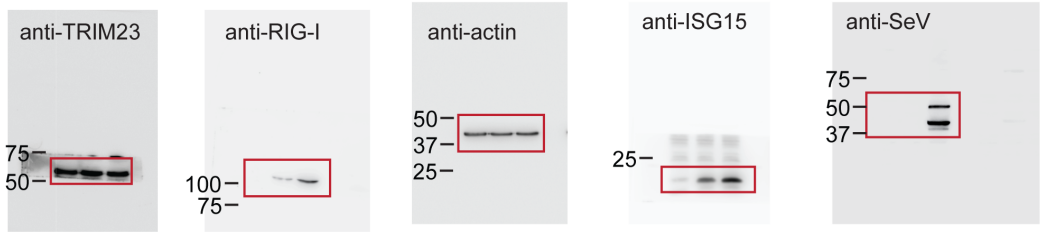
Figure 6g:



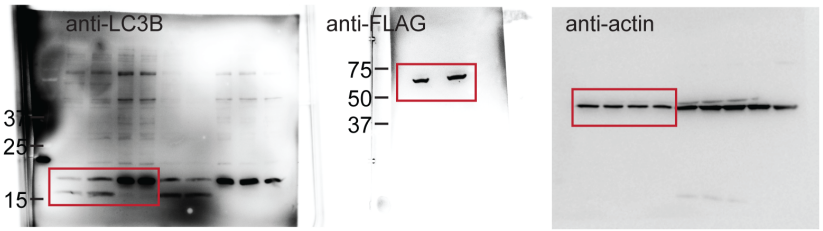
Supplementary Figure 1b:



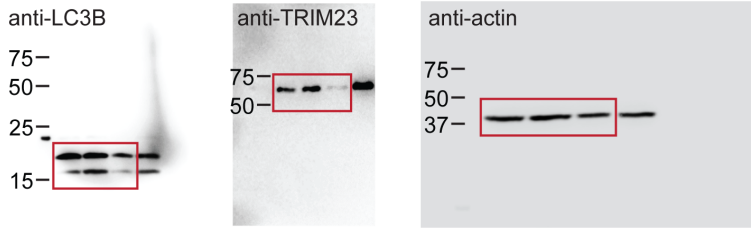
Supplementary Figure 2d:



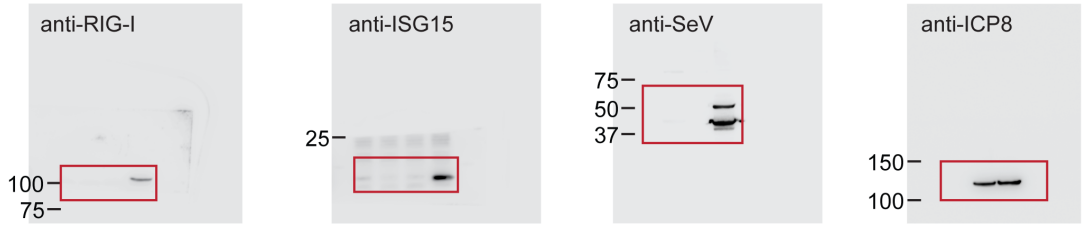
Supplementary Figure 3c:



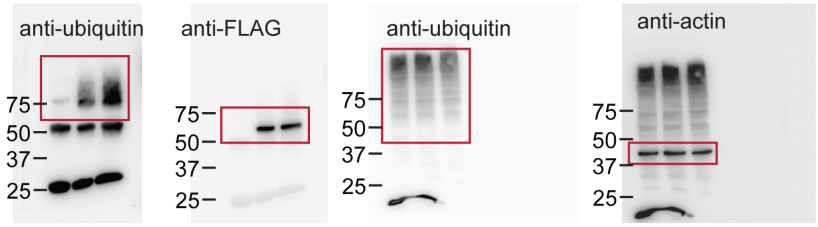
Supplementary Figure 3i:



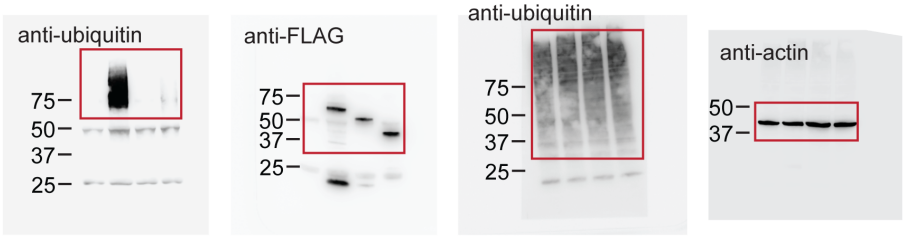
Supplementary Figure 4g:



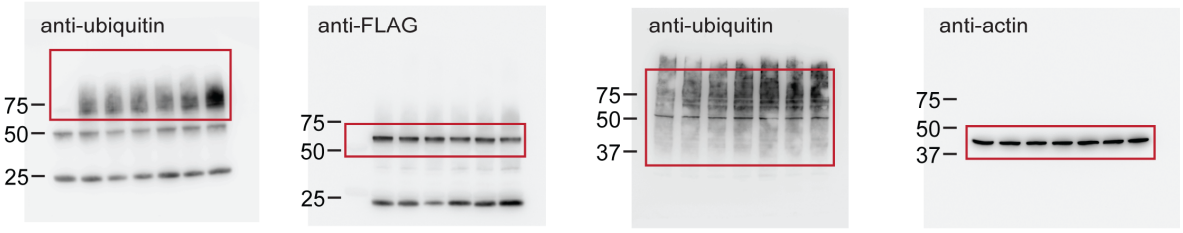
Supplementary Figure 5a:



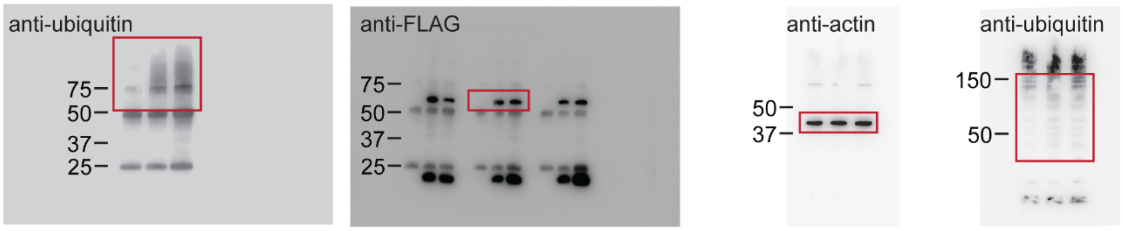
Supplementary Figure 5b:



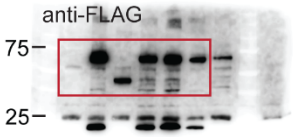
Supplementary Figure 5d:



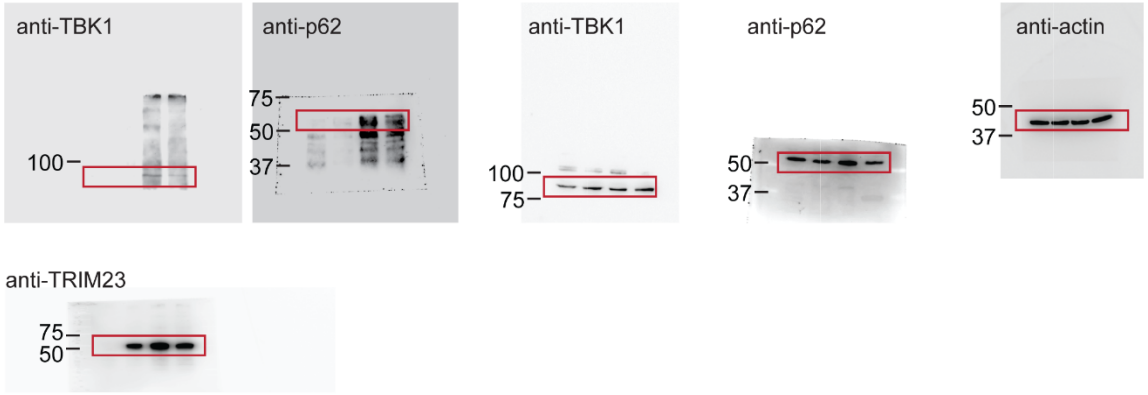
Supplementary Figure 6a:



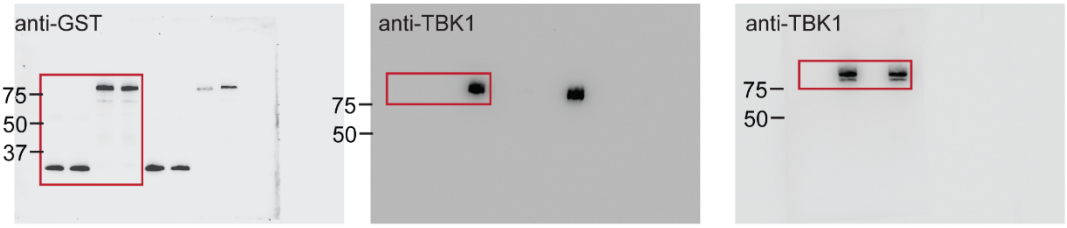
Supplementary Figure 6b:

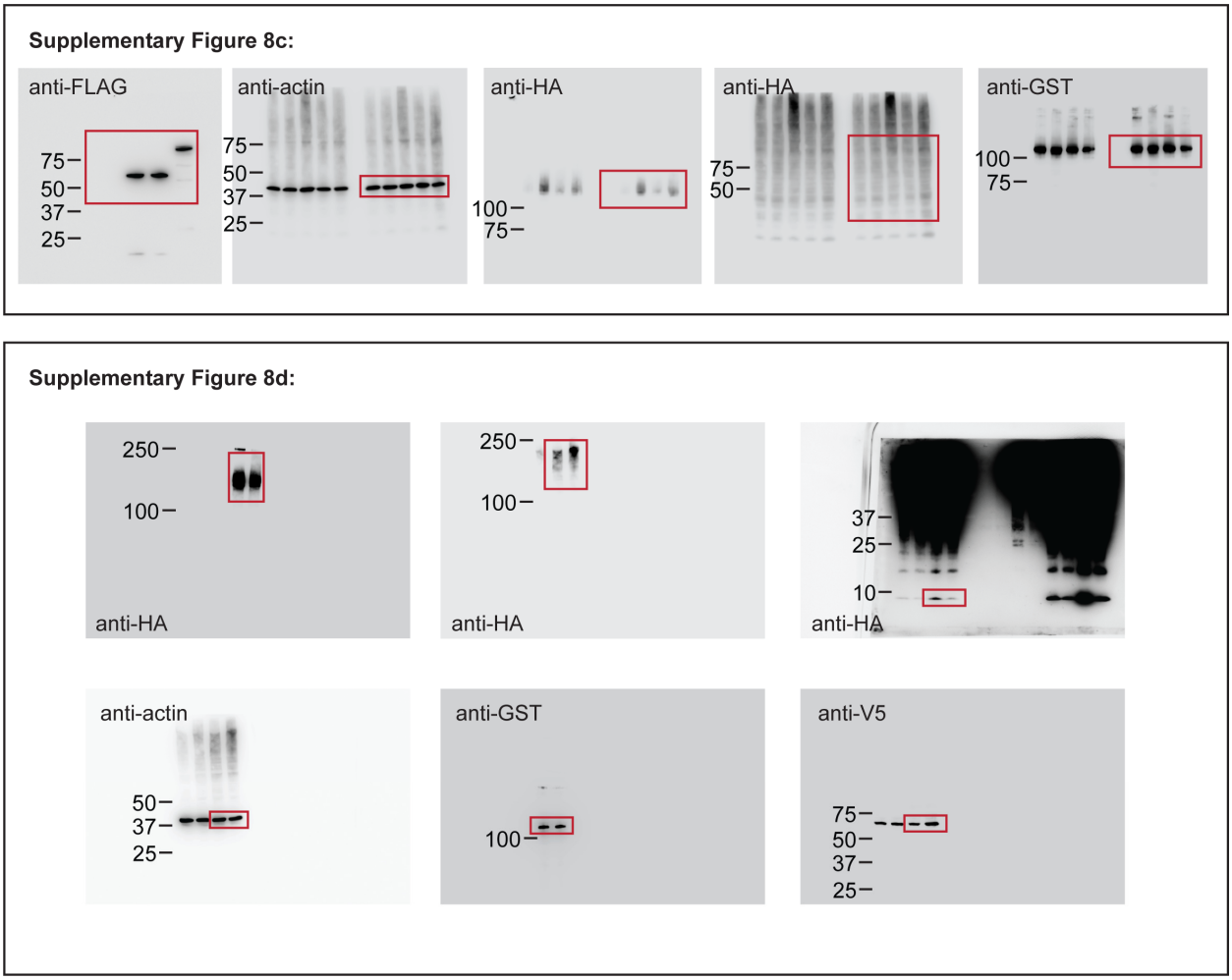


Supplementary Figure 7d:



Supplementary Figure 7e:





Supplementary Figure 9. Complete western blot images of the indicated figures of the manuscript. Red boxes indicate the cropped areas shown in the indicated figures. Numbers indicate molecular weight markers (in kDa).