

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Mammalian Cell Lines and Plasmids

HeLa cells²⁶ (provided by V. Stollar) were cultured in DMEM containing 10% FBS and 1x Penicillin/Streptomycin. HeLa/GFP-LC3 cells⁶ were cultured in media containing 10 µg/mL G418. Primary mouse embryonic fibroblasts (MEFs) were generated as described²¹, maintained in DMEM containing 15% fetal bovine serum, 1x penicillin/streptomycin, 118 µM β-mercaptoethanol, and 1x MEM non-essential amino acids, and passaged no more than 6 times. Plasmids expressing mCherry-Parkin¹³, Myc-Smurf1, Myc-Smurf1ΔHECT, and 6xMyc-Smurf1 C699A²¹, Flag-Smurf1 and Flag-Smurf1 Smurf1 C699A²⁷, and YFP-Smurf1 and YFP Smurf1 C699A²⁸ have been described. Flag-Smurf1ΔHECT (deletion of amino acids 346-731), HA-Smurf1ΔC2 (deletion of amino acids 1-119), YFP-Smurf1ΔC2 (deletion of amino acids 14-117) were created using the Quickchange XL kit (Stratagene) according to the manufacturer's protocol. Plasmids expressing mCherry-Smurf1, mCherry-Smurf1ΔHECT, and mCherry-Smurf1ΔC2 were created by cloning Smurf1 into pmCherry-C1 (Clontech) using standard PCR-based methods.

Viral Strains and Infections

The Sindbis virus (SIN) strain SVIA (ATCC) is derived from a low-passage isolate of the wild-type AR339 SIN strain²⁹. Generation and amplification of the recombinant SIN strains SIN-mCherry.capsid and SIN-mCherry.capsid/GFP-LC3, using the dsTE12Q vector³⁰ have been described⁶. Briefly, viral RNA was *in vitro* transcribed from the

recombinant dsTE12Q-mCherry.capsid plasmid and transfected into BHK-21 cells using well-established methods³¹. SIN stocks and supernatants of virally-infected cells in all experiments were titered by plaque assay titration on BHK-21 cells. The HSV-1 ICP34.5 deletion mutant strain, HSV-1 17termA, has been described³², and viral stocks were titered on Vero cells. All viral infections for screening and follow up experiments were performed at a multiplicity of infection (MOI) of 5 plaque-forming units (PFUs) per cell, with the exception of viral growth in MEFs which was performed at a MOI of 0.1 and correlative light and electron microscopy which was performed at a MOI of 10.

siRNA Screening

Primary Screen: A genome-wide siRNA library (Dharmacon) containing 21,125 SMARTpools (each containing 4 siRNAs targeting an individual gene) was seeded in triplicate in glass-bottom 96-well plates (Greiner) in 30 μ L Optimem (Gibco) at a final concentration of 50 nM using a BioMek liquid handler. Lipofectamine 2000 (Invitrogen) was added in 20 μ L Optimem at a final dilution of 1:200 with a Biotek dispenser, followed by addition of 6.4×10^3 HeLa/GFP-LC3 cells in 100 μ L normal media using a MultiDrop dispenser for reverse transfection. After 48 hrs to allow gene knockdown, the supernatant was removed by low speed centrifugation of plates inverted in reservoirs. Control wells containing non-silencing siRNA (NC-2, Dharmacon) in column 1 were mock infected with 30 μ L Optimem, and control wells in column 12 and all sample wells (columns 2-11) were infected with SIN-mCherry.capsid virus in 30 μ L using a Biotek dispenser. After 1 hr incubation (virus adsorption), 120 μ L of media containing 2% FBS was added to each well. After 11 hrs of infection, 10 μ L of 2% media containing Hoechst 33342 (Invitrogen) (5 μ g/mL final) was added to each well, plates were incubated for 40

min at 37°, followed by supernatant removal by plate inversion and low speed centrifugation, followed immediately by fixation with pre-warmed 2% paraformaldehyde (PFA) (Electron Microscopy Sciences) in 1x D-PBS (Gibco). Cells were fixed overnight at 4°, PFA was then replaced with 1x PBS (Gibco), and plates were sealed and stored at 4° until imaging (discussed below).

Confirmation and Secondary Screens: Assay plates for the colocalization confirmation screen and for the secondary cell survival screen were prepared exactly as for the primary screen, except that each of the 4 individual siRNA oligos from the primary hit pool were arrayed in a custom library purchased from Dharmacon. For the cell survival secondary screen, six white clear bottom assay plates (Costar) were prepared per library plate with non-coding (NC) siRNA oligos in columns 1 and 12 for each plate, and triplicate samples were either mock-infected or infected as in the primary colocalization screen above, but with SIN strain dsTE12Q in 30 μ L Optimem. After 24 hrs of infection, media was removed from plates and 50 μ L CellTiter-Glo reagent (Promega) (diluted 3:1, 1x PBS/1% TritonX-100: CellTiter-Glo), which correlates viable cell numbers with ATP levels, was added to each well using a multidrop dispenser. Plates were shaken for 5 min at room temperature on an orbital shaker, and luminescence values were obtained on an Envision 2103 multilabel reader. Cell viability data were analyzed as described below in the section “*Statistical analysis*”. For the mitophagy secondary screen, cells were reverse-transfected in suspension at a final concentration of 1.3×10^4 HeLa cells, 80 ng mCherry-Parkin plasmid, and 0.08 μ L Lipofectamine 2000 per 100 μ L. One-hundred μ L of this suspension was added to triplicate glass-bottom 96-well assay plates (Greiner) with siRNA and transfection reagent prepared as for the confirmation screen for colocalization

described above. Cells were incubated for 48 hrs to allow gene knockdown, and media was replaced with 150 μ L normal media containing 10 μ M CCCP (Sigma). After 24 hrs, cells were fixed in 2% PFA, and stained as detailed below in “Immunofluorescence”.

High Content Image-Based Screening. Images were captured with a Pathway 855 automated microscope (BD Biosciences) using a 40x air objective in Hoechst (Ex: 360/10, Dichroic: 400DCLP, Em: 435LP), GFP (Ex: 488/10, Dichroic: Fura/FITC, Em: 515LP), and mCherry (Ex: 560/55, Dichroic: 595LP, Em: 645/75) channels, using wide-field epifluorescence. Raw images were segmented for cell regions and subcellular puncta using the Imaging and Advanced Imaging packages in Pipeline Pilot software (Accelrys). For colocalization measurements, cell boundaries were identified using watershed segmentation with Hoechst-positive nuclei as markers, and cytosolic Hoechst signal as background intensity. mCherry intensity statistics per cell area were measured for each cell in all wells. The top 1% of signal intensity in all cells from mock-infected wells on each plate was set as the background threshold for mCherry expression. Individual cells in infected wells were classified as infected if the 25th percentile of the mCherry intensity histogram was higher than the background threshold defined in uninfected wells. GFP and mCherry puncta were segmented using a difference of Gaussian method on natural log-transformed images to permit detection of puncta in cells with varying expression intensities. Centroid X,Y coordinates and area measurements for each punctum from infected cells that contained both green and red puncta were analyzed for colocalization as described below. For the mitophagy secondary screen, images were analyzed similarly to colocalization analysis, except that cytoplasmic background was defined using AlexaFluor 350 Phalloidin (Invitrogen, 1:100 dilution), and additional

parameters were measured including total mitochondrial area per cell and mean intensity within mitochondrial regions.

Statistical Analysis. All statistical analyses were performed with the open-source R software package (www.r-project.org). Colocalization events were defined as touching or overlapping green and red dots in the same cell. The number of colocalization events in each cell was modeled as a Poisson distribution for each well. Generalized linear models³³ were used to determine whether an siRNA treatment significantly increased or decreased the number of colocalization events by comparing the siRNA-treated well to negative control wells. The total possible number of colocalization events (number of green dots multiplied by number of red dots) was included in the generalized linear models to adjust for potential differences in the number of dots in each cell. The z-scores from the generalized linear models were used as summary statistics for each siRNA-treated well for selection of hits. Primary hits for siRNAs that decrease colocalization were defined as those with z-scores for all three replicates below 2 standard deviations (S.D.) from the mean of all replicates, and/or those whose median z-score (for those with values for at least 2 replicates) was below 3 S.D. from the mean of all median z-scores. Primary hits for siRNAs that increase colocalization were defined as those with z-scores for all three replicates above 1.5 S.D. from the mean of all replicates. Primary hits were considered validated in the colocalization confirmation screen if at least 2 siRNAs from the set of 4 individual siRNAs targeting each gene had a p-value < 0.05 vs. negative controls in the generalized linear models.

For analysis of cell survival, raw luminescence values for all wells on each plate were normalized by the median of the negative control on the same plate to adjust for

plate-to-plate variation. To control for non-specific cytotoxic effects of siRNAs in uninfected cells, we used triplicate plates with SIN infection and triplicate plates without SIN infection. The normalized luminescence values between the triplicate infected and triplicate uninfected wells for the same siRNA were compared using a t-test to determine whether each siRNA treatment decreased cell survival after infection. Genes for which at least two individual siRNAs had a p-value < 0.05 were considered as hits in the cell survival screen.

For automated analysis of mitophagy, mitochondrial mass for each cell was approximated by the following formula:

$$\frac{\text{total mitochondrial area per cell}}{\text{cell area}} \times \text{mean intensity within mitochondrial mask. Two-}$$

way ANOVA models were used to test which siRNAs decreased Parkin-mediated mitophagy: Mitochondrial Mass $\sim \beta_0 + \beta_1\text{Parkin} + \beta_2\text{siRNA} + \beta_3\text{Parkin} \times \text{siRNA}$. In this model, b_3 is the coefficient of interaction between Parkin expression (Parkin positive or negative) and a siRNA treatment (siRNA-treated or on-plate negative controls), and b_3 indicates whether an siRNA treatment affects Parkin-mediated mitophagy. For example, positive b_3 indicates that the corresponding siRNA decreases Parkin-mediated mitophagy. Genes for which at least two individual siRNAs had a p-value < 0.05 were considered as hits in the mitophagy screen.

Cell Viability Assays

The cell survival secondary screen in HeLa cells is described above. Cell viability was determined in SIN-infected MEFs by trypan blue exclusion. A minimum of 200 cells was counted per sample, and triplicate samples were counted per condition.

Bioinformatics

To assess the statistical enrichment of the various functional gene sets including molecular function, biological process categories, and pathway-associated protein-protein interaction (PPI) networks for the confirmed colocalization hit genes relative to their representation in the global set of genes examined in the siRNA screen, p-values were computed using the hypergeometric test³⁴ and implemented in the R programming language as described in Hitomi et al.³⁵. The gene sets were compiled from multiple sources: molecular function and biological process categories from Gene Ontology (GO)³⁶ and Panther^{37,38}; protein class from Panther; signaling and metabolic pathways from MSigDB³⁹ and Panther.

For the functional enrichment map, the enrichment results were represented as a network graph, with nodes denoting enriched gene sets or categories. Node size is proportional to the siRNA hits in each gene set, while the node color intensity corresponds to the gene set enrichment score ($-\text{Log}_{10}(\text{p-value})$). Thickness and color intensity of edges denote the extent of mutually overlapping genes between gene sets. Clusters of strongly connected components in the network were identified using Tarjan's algorithm⁴⁰.

To identify enriched pathway-associated networks, first-order PPI network modules were generated from the MSigDB collection of canonical pathways and assessed for over-representation among the set of siRNA hits. Network constructions were implemented in the Perl programming language and incorporated graph theoretical representations by abstracting gene products as nodes and interactions as edges. PPI data were derived from a collection of genome-wide interactome screens and curated literature

entries in HPRD⁴¹. For the autophagy-centric network, we used high-confidence PPI data (HCIPs) from a recent proteomics analysis of the human autophagy system¹¹ and data from HPRD.

To determine whether siRNAs hits in each screen (viral colocalization, cell survival, or mitophagy screen) were enriched for miRNA seed sequences, we determined whether each siRNA used in the virus capsid/autophagosome colocalization confirmation screen contains miRNA seed sequences using the 7mer-8m seed-match type which corresponds to positions 2-8 on mature miRNA (downloaded from the TargetScan database at www.targetscan.org), and then used Fisher's exact tests to analyze for miRNA seed sequence enrichment in the siRNAs that scored positive in each screen versus the siRNAs that did not score positive in each screen. TargetScan, which is a de novo prediction algorithm, was used to identify the predicted targets for each miRNA seed sequence found in each of the siRNAs evaluated in the virus capsid/autophagosome colocalization confirmation screen.

Mitophagy Assays

For details of the secondary screen for mitophagy, please refer to the section "siRNA Screening." For Parkin-mediated mitophagy assays in HeLa cells, siRNA (50 nM final concentration) and 0.4 ml Lipofectamine 2000 were mixed in 200 μ L Optimem in wells of 4-chamber glass slides (Nunc), and 5×10^4 HeLa cells in 400 μ L normal media without antibiotics (co-transfected in suspension with 320 ng mCherry-Parkin and 0.32 ml Lipofectamine 2000) were added to wells and incubated for 48 hrs. For MEF studies, 1×10^4 cells were seeded in 4-chamber glass slides and reverse-transfected with 800 ng of the indicated plasmids mixed with 2 μ L Lipofectamine 2000. After 24 hrs, the media

was replaced with MEF media containing DMSO or CCCP. All mitophagy experiments were performed by treatment with 10 μ M CCCP for 24 hrs.

Western Blot and Co-immunoprecipitation Analyses

For Smurf1:SIN capsid and p62:SIN capsid co-immunoprecipitation studies, HeLa/GFP-LC3 cells, *Smurf1*^{+/+} MEFs, or *Smurf1*^{+/+} MEFs were mock-infected or infected with SIN (strain SVIA, MOI=5) in 15 cm dishes for 11 hrs, then lysed in 600 μ L TNT lysis buffer (50mM Tris-Cl pH 7.4, 150mM NaCl, 1mM EDTA, 1% TritonX-100, and protease and phosphatase inhibitors) per dish. Forty μ L of lysates were reserved for Western blots as loading controls. Samples were incubated for 2.5 hrs with anti-Smurf1 antibody (Novus Biologicals, cat. # H00057154-M01, 1:200 dilution), anti-p62 antibody (Progen, cat. # GP62-C, 1:200 dilution), or IgG control, immunoprecipitated with Dynabeads protein G (Invitrogen) for 30 min, and boiled for 5 min with Laemmli buffer containing 2.5% β -mercaptoethanol. For Smurf1:p62 co-immunoprecipitation, *Smurf1*^{+/+} MEFs were reverse-transfected in 10 cm dishes with 5 μ g of the indicated plasmids using 12.5 μ L of Lipofectamine 2000. Twenty-four hours after transfection, cells were lysed in 300 μ L TNT lysis buffer per dish, and immunoprecipitation was performed with anti-p62 antibody as above. Western blot analyses were performed on lysates and co-immunoprecipitates as described⁶ with the following antibodies: anti-p62 for human p62 (BD Biosciences, cat. # 610832, 1:500 dilution), for mouse p62 (Novus Biologicals, cat. # H00008878-M01, 1:500 dilution) anti-Smurf1 (Novus Biologicals, cat. # H00057154-M01, 1:500 dilution), anti-Sindbis virus capsid (provided by M. McDonald and R. Kuhn, rabbit, 1:5000 dilution), anti-LC3 (Novus Biologicals, cat. # NB100-2220, 1:700

dilution), anti-Atg7 (Sigma, cat. # A2856, 1:500 dilution) and anti-actin (Santa Cruz, cat. # sc-47778, 1:2000 dilution).

Sample Preparation for Histology, Immunostaining, and Electron Microscopy

For immunofluorescence staining, HeLa cells and MEFs were fixed in 2% PFA in PBS, permeabilized in 0.5% TritonX-100/PBS, then blocked with 1% BSA in PBS. HeLa cells were stained with mouse monoclonal anti-Tom20 antibody (Santa Cruz, cat. # sc-17764, 1:500 dilution) and labeled with a donkey anti-mouse-AlexaFluor 488 secondary antibody (Invitrogen). MEFs were stained with a rabbit polyclonal anti-Tom20 antibody (Santa Cruz, cat. # sc-11415, 1:500 dilution) and labeled with donkey anti-rabbit-AlexaFluor 488 or AlexaFluor 594, or goat anti-rabbit AlexaFluor 350 (Invitrogen, 1:500 dilution). Immunohistochemical staining of paraffin-embedded tissue sections from *Smurf1*^{+/+} (WT) or *Smurf1*^{-/-} (KO) mice²¹ was performed using a polyclonal guinea pig anti-p62 antibody (Progen, cat. # GP62-C, 1:1000 dilution) or anti-ubiquitin antibody (Santa Cruz sc-9133, 1:500). Primary antibodies were detected with the ABC Elite kit (Vector Laboratories) according to the manufacturer's instructions. Tissue samples and virally-infected MEFs were prepared for electron microscopy (EM) as described⁴², except for correlative light and electron microscopy (described below).

Sample Preparation for Correlative Light and Electron Microscopy

2.5×10^5 HeLa/GFP-LC3 cells were plated in a 35 mm dish containing a gridded glass coverslip (MatTek) and infected 24 hrs later with SIN/mCherry.capsid at a MOI of 10 for 8.5 hrs. Cells were fixed in a buffer containing 4% PFA, 0.05% glutaraldehyde, and 0.1 M sodium cacodylate for 10 min and imaged with a light microscope immediately thereafter as described in the next methods section. After imaging, the buffer was

replaced with EM fixative buffer containing 2.5% glutaraldehyde/0.1 M sodium cacodylate and samples were incubated at 4° overnight. Coverslips were removed using coverslip removal solution (MatTek), post-fixed with 1% osmium tetroxide containing 0.8% potassium ferricyanide, stained with 1% uranyl acetate, dehydrated in a graded ethanol series, and infiltrated with EMbed-812 resin. Coverslips were embedded by placing them (cell side down) onto BEEM capsules filled with resin. Coverslips were removed from the polymerized resin by immersion in liquid nitrogen. A dissecting scope was used to find the grid sections of interest and the area around this region was scored with a razor blade followed by removal of surrounding resin. Thin-sections were post-stained with uranyl acetate and lead citrate. EM images were captured as described in next section.

Microscopy Studies in MEFs and Correlative Light and Electron Microscopy

For fluorescence microscopy in screening experiments, please refer to section “siRNA screening.” Images for quantification of mitochondrial phenotypes in MEFs were captured with a Zeiss Axioplan2 microscope using a Zeiss PLAN-APOCHROMAT 63x objective and image manipulations were performed with ImageJ (NIH). For analysis of gross mitochondrial phenotypes, cells were scored by a blinded observer as either normal (i.e. reticular mitochondrial network) or as abnormal with accumulation of highly fragmented mitochondria. Mitochondrial surface area fraction was calculated using a custom macro in ImageJ as follows: Cells were segmented and cell surface area was determined by manually thresholding on non-specific background staining to create a cellular mask. Mitochondria within the cellular mask were then automatically thresholded and total mitochondrial surface area was calculated from the mitochondrial mask. The

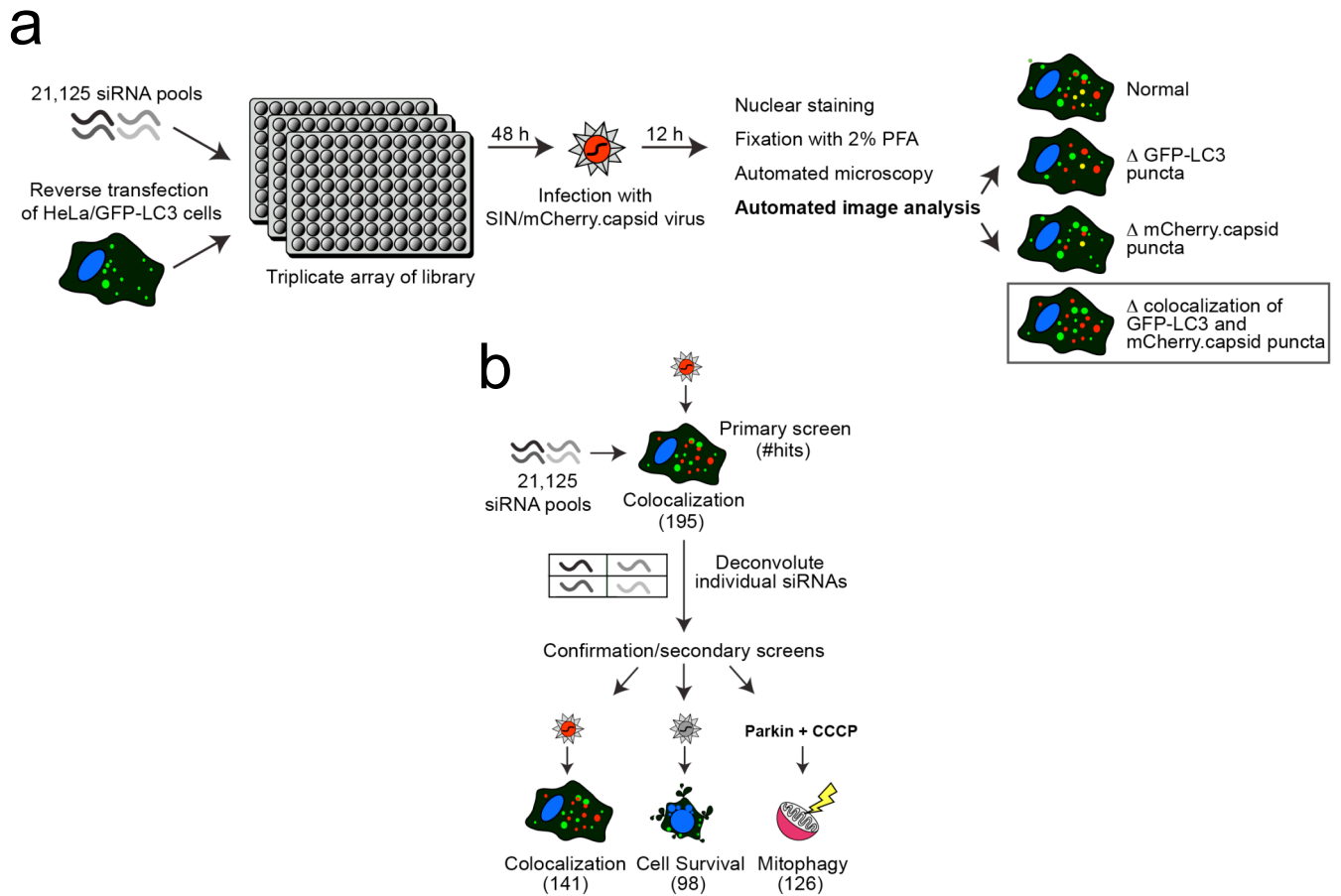
mitochondrial area fraction is the mitochondrial surface area divided by the cell surface area. At least 50 cells with CCCP-induced changes in mitochondrial morphology were analyzed per condition.

Image capture for correlative light and electron microscopy was performed on a DeltaVision RT microscope using an Olympus UPLFLN 40x 0.75 NA objective. Images stacks of approximately 10 z-sections at 2 μm intervals were captured and deconvolved with AutoDeblur (Bitplane). 3D reconstructions and image analyses were performed with Imaris (Bitplane). Confocal microscope images were acquired with a Zeiss 63x 1.4 NA PLAN-APOCHROMAT objective lens using a Zeiss LSM510 NLO with excitation at 488 nm for GFP and AlexaFluor 488 and 543 nm for mCherry and AlexaFluor 594. DAPI and AlexaFluor 350 images were acquired by two-photon excitation at 790 nm using a Chameleon XR (Coherent) NIR laser. EM images were captured using an FEI Tecnai G2 Spirit Biotwin or a JEOL 1200 EX, both with a SIS Morada 11 mpixel side mount CCD camera.

References

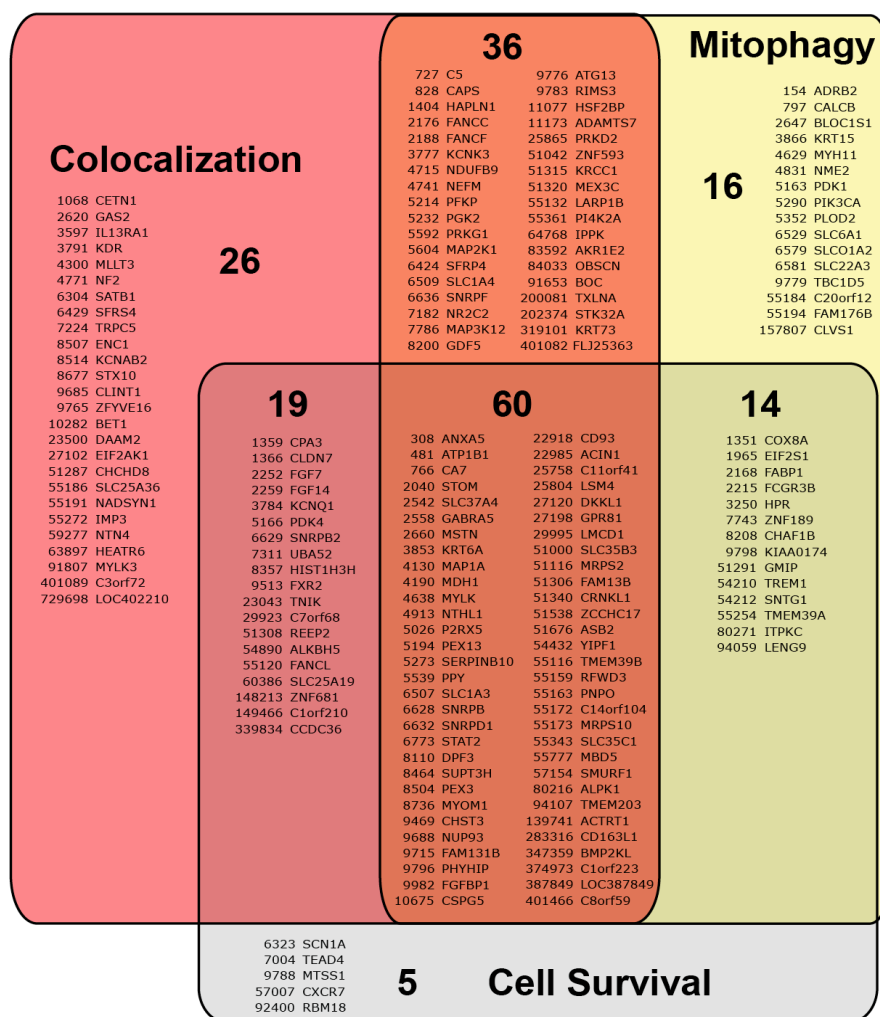
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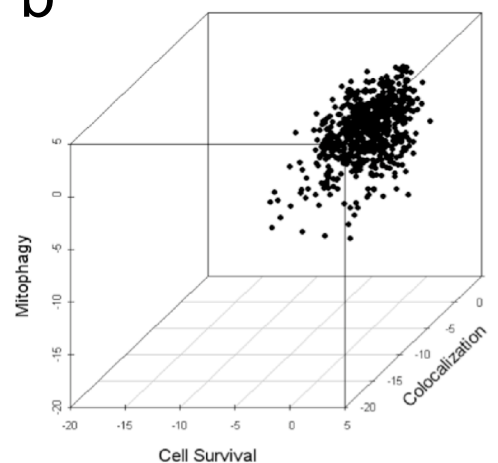


Supplementary Figure 1. Schematic illustration of primary, confirmation, and secondary screens. **a**, Schematic overview of primary screen. The screen was designed to identify siRNAs that resulted in the image pattern shown in box on right (“ Δ colocalization of GFP-LC3 and mCherry.capsid puncta”). See Supplementary Methods for details of experimental design and statistical analysis. **b**, Schematic overview of confirmation and secondary screens. Four individual siRNAs per gene were used to target the 195 genes identified in the primary screen that decreased SIN capsid/GFP-LC3 colocalization. These deconvoluted pools of siRNAs were used to repeat the colocalization screen and in this confirmation screen, 141 of 195 genes had 2 or more individual siRNAs that scored positive. The same pools of siRNAs were used in a screen to detect decreased cell survival in SIN-infected HeLa cells relative to uninfected HeLa cells, and in this secondary cell survival screen, 98 of the 195 genes had 2 or more individual siRNAs that scored positive. The siRNA pool was also used to detect decreases in mCherry.Parkin-mediated mitophagy in HeLa cells treated with CCCP, and in this secondary mitophagy screen, 126 of the 195 genes had 2 or more individual siRNAs that scored positive.

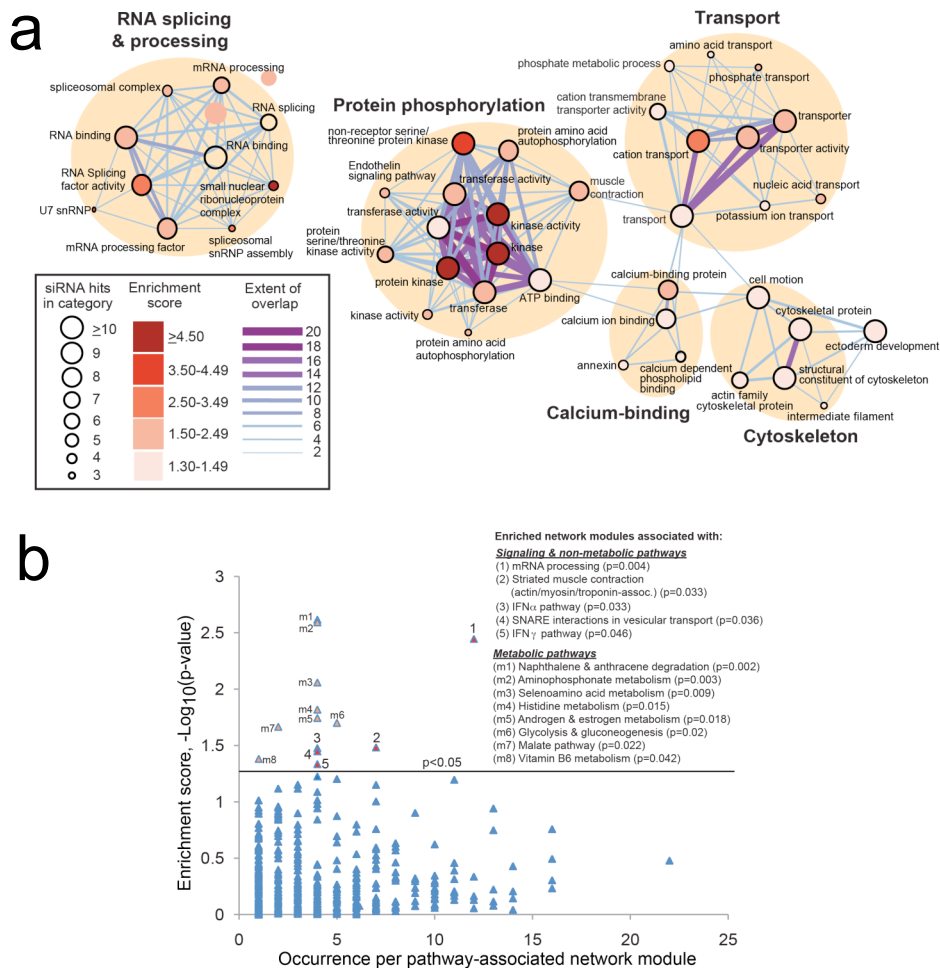
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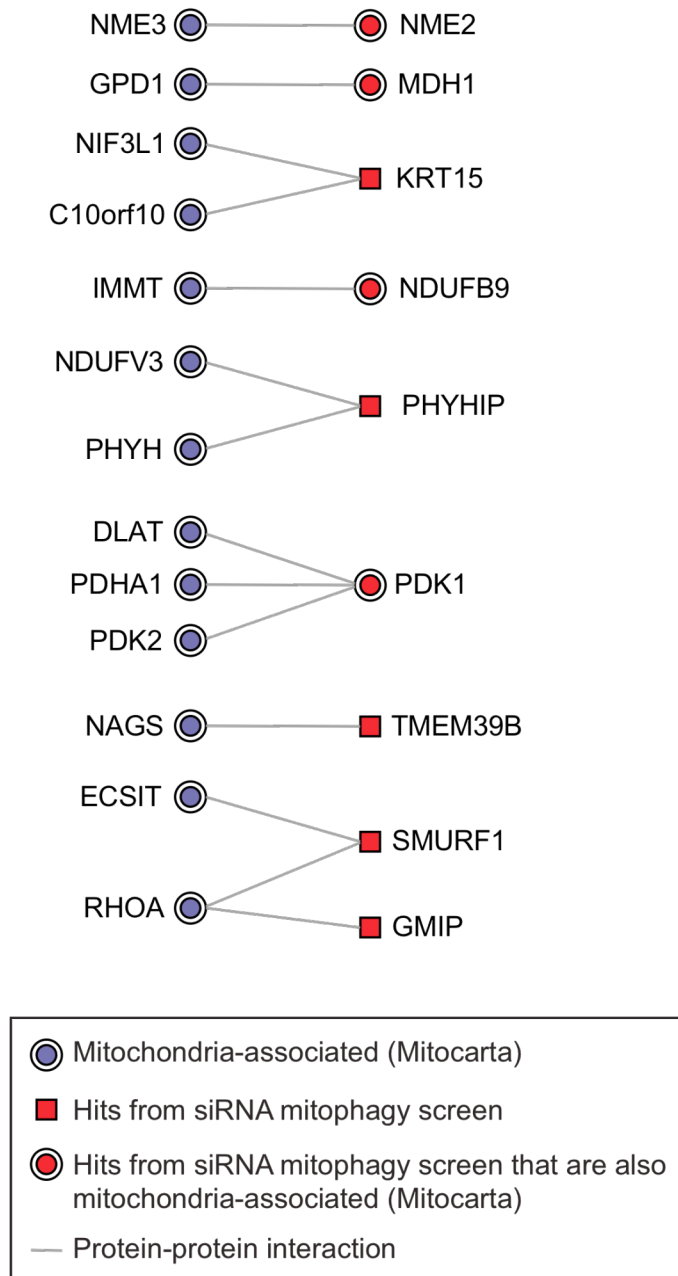
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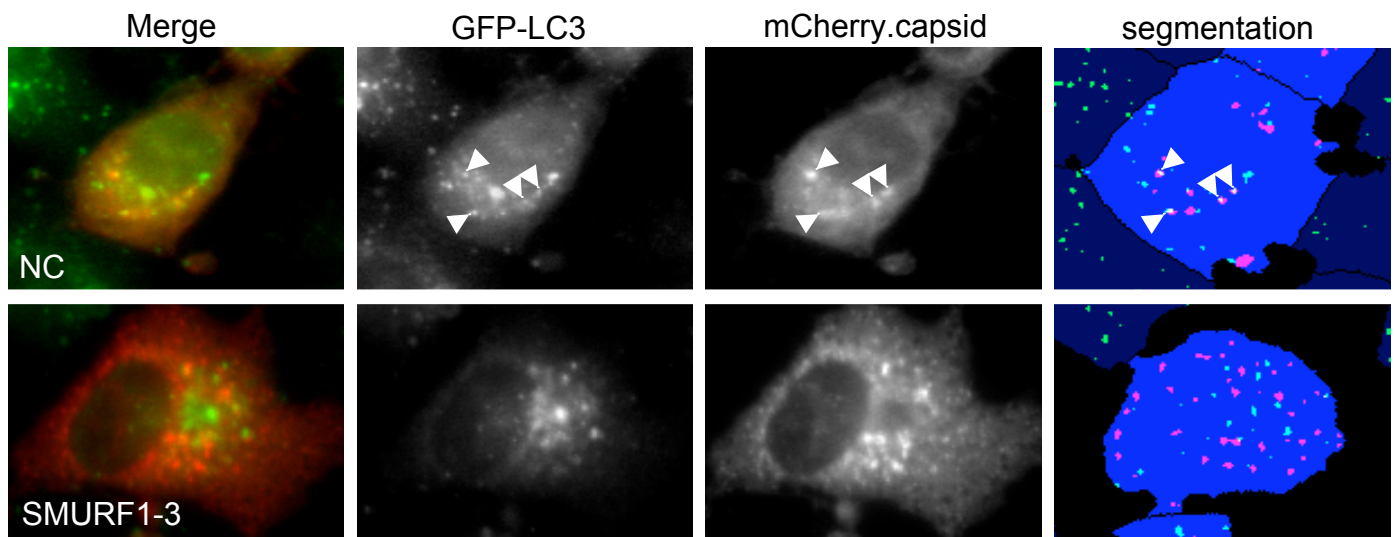
Supplementary Figure 2. Overlap between colocalization screen hits and secondary screen hits for cell survival and mitophagy. **a**, List of gene symbols and locus ID's for confirmed hits in colocalization confirmation screen, cell survival screen, and mitophagy screen, arranged according to their overlap (or lack of overlap) in each screen. **b**, Scatter plot of log-transformed p-values in the confirmation screen for viral capsid/autophagosomal colocalization (Supplementary Table 2), the secondary screen for survival of virus-infected cells (Supplementary Table 6) and the secondary screen for Parkin-mediated mitophagy (Supplementary Table 7). Spearman correlations were as follows: 0.20 for cell survival vs. colocalization ($P=3.8E-08$); 0.09 for colocalization vs. mitophagy ($P=0.019$); and 0.10 for cell survival vs. mitophagy ($P=0.0049$).



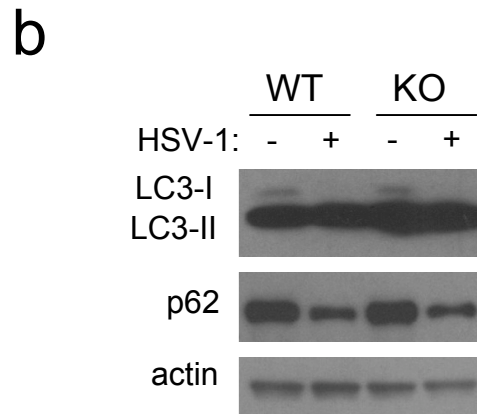
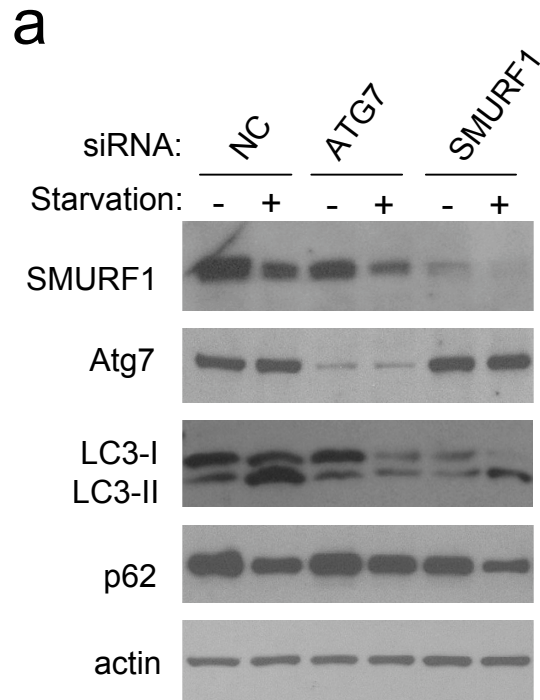
Supplementary Figure 3. Functional enrichment map of siRNA hits from the viral capsid/autophagosome colocalization screen. **a**, Enrichment analyses of gene sets including molecular function and biological process categories from Panther and Gene Ontology (GO) and protein class and pathway assignments from Panther, are represented graphically as a network map. Nodes denote enriched gene sets, with the size of each node corresponding to the total number of siRNA hits in each gene set or category. Node color intensity is scaled according to the enrichment score ($-\log_{10}(p\text{-value})$). All nodes shown represent gene sets that are significantly enriched (hypergeometric $P < 0.05$) in the set of siRNA hits from the colocalization screen. Thickness and color intensity of edges denote the number of overlapping genes between gene sets. For clarity, only gene sets with at least 2 overlapping genes are shown. Clusters of functionally related and mutually connected gene sets are grouped in shaded circles. Supplementary Table 5 is a list of siRNA hits organized into the various categories and clusters shown here. **b**, Enrichment analyses of pathway-associated network modules. First-order protein-protein interaction (PPI) networks of pathway components from MSigDB collection of pathways were examined for enrichment in the set of siRNA hits from the viral capsid/autophagosome colocalization screen.



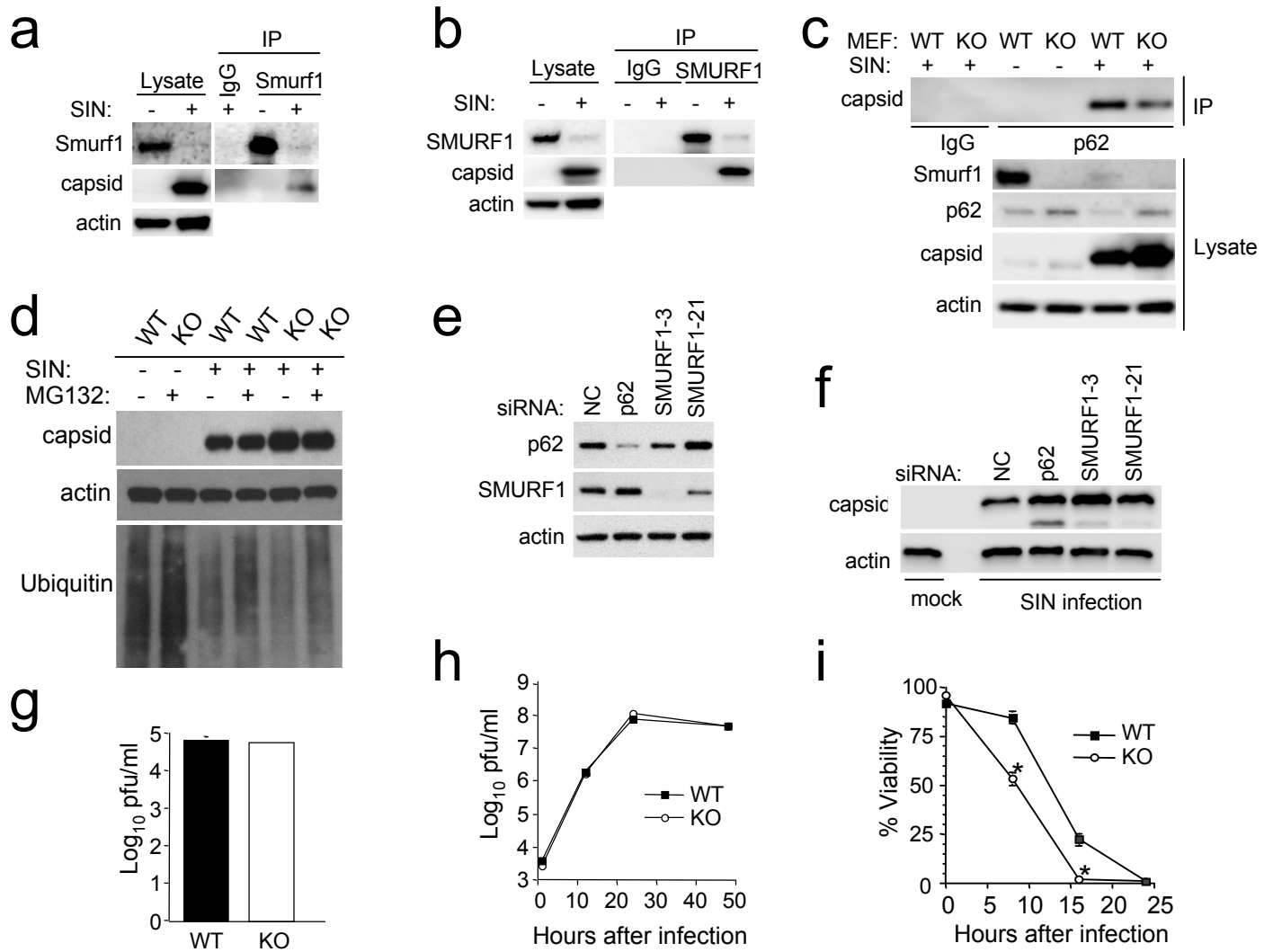
Supplementary Figure 4. Interaction map of mitochondria-associated components and mitophagy screen hits. Mitochondria-associated components were identified from MitoCarta. The interaction map shows first-order protein-protein interactions (PPI) between these mitochondria-associated components and mitophagy screen hits, using PPI data from HPRD.



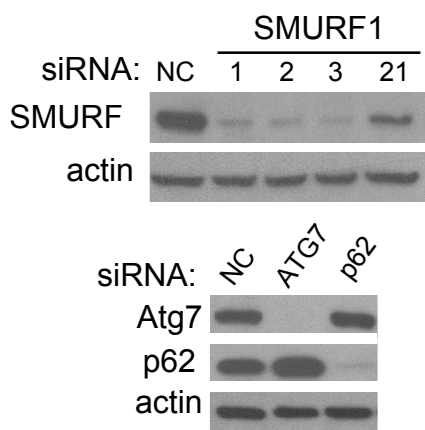
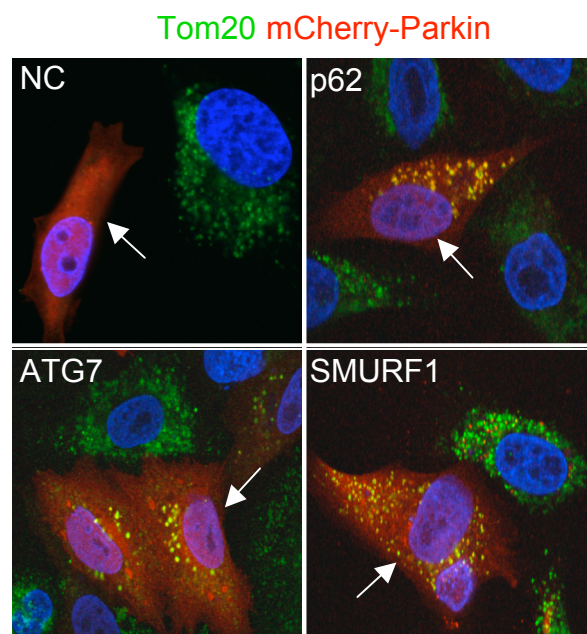
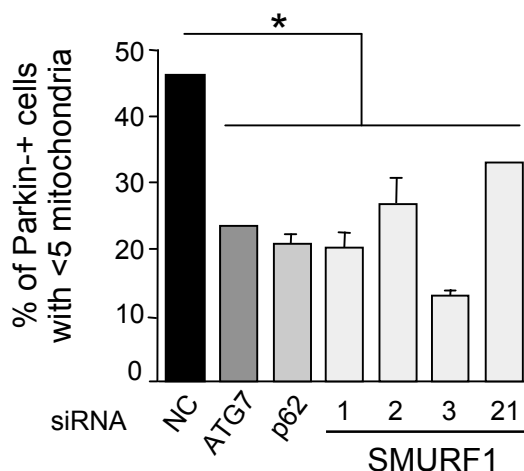
Supplementary Figure 5. Representative example of raw image data and segmentation from colocalization confirmation screen. Representative fluorescent micrographs of non-silencing control (NC) siRNA- or SMURF1-3 siRNA-treated cells from viral capsid/autophagosome colocalization confirmation screen. Shown are raw images representing merged green (depicting GFP-LC3) and red (depicting mCherry.capsid) channels and individual green (GFP-LC3) and red (mCherry.capsid) channels as well the same images after segmentation for cell regions and subcellular puncta as described in the Supplementary Methods. The segmented images were used for automated colocalization analysis. Arrowheads denote representative colocalized red and green puncta that would be scored as a colocalization event in the screen analysis.



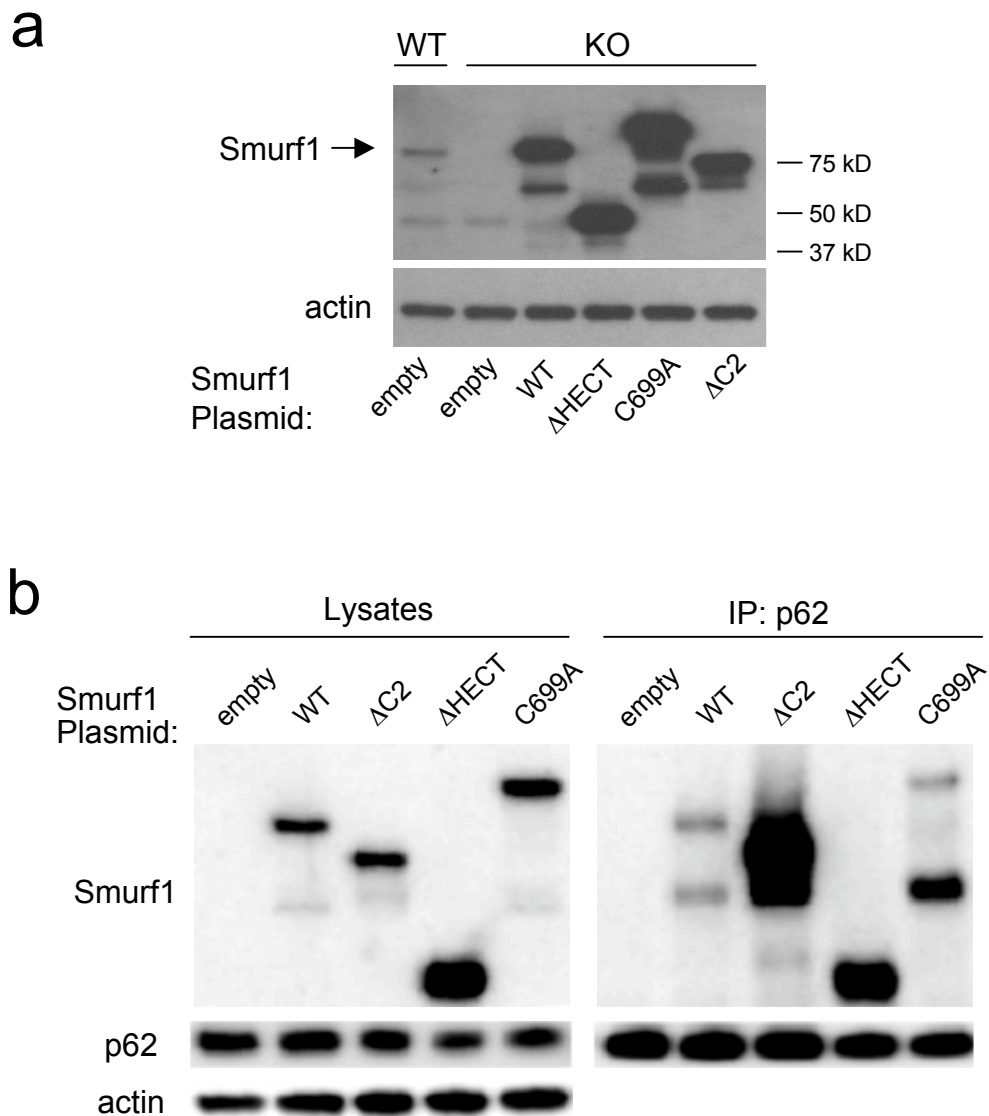
Supplementary Figure 6. Smurf1 is not required for induction of autophagy during starvation or HSV-1 infection. **a**, Western blot analysis of LC3-II/LC3-I and p62 levels in HeLa cells treated with the indicated siRNA for 48 hrs, and then subjected to treatment in either normal media (starvation -) or EBSS (starvation +) for 4 hrs. Western blot analyses of SMURF1 and Atg7 demonstrate efficiency of siRNA knockdown. **b**, Western blot analysis of LC3-II/LC3-I and p62 levels in WT or *Smurf1*^{-/-} MEFs mock-infected or infected with HSV-1 17termA (MOI = 5) for 16 hrs.



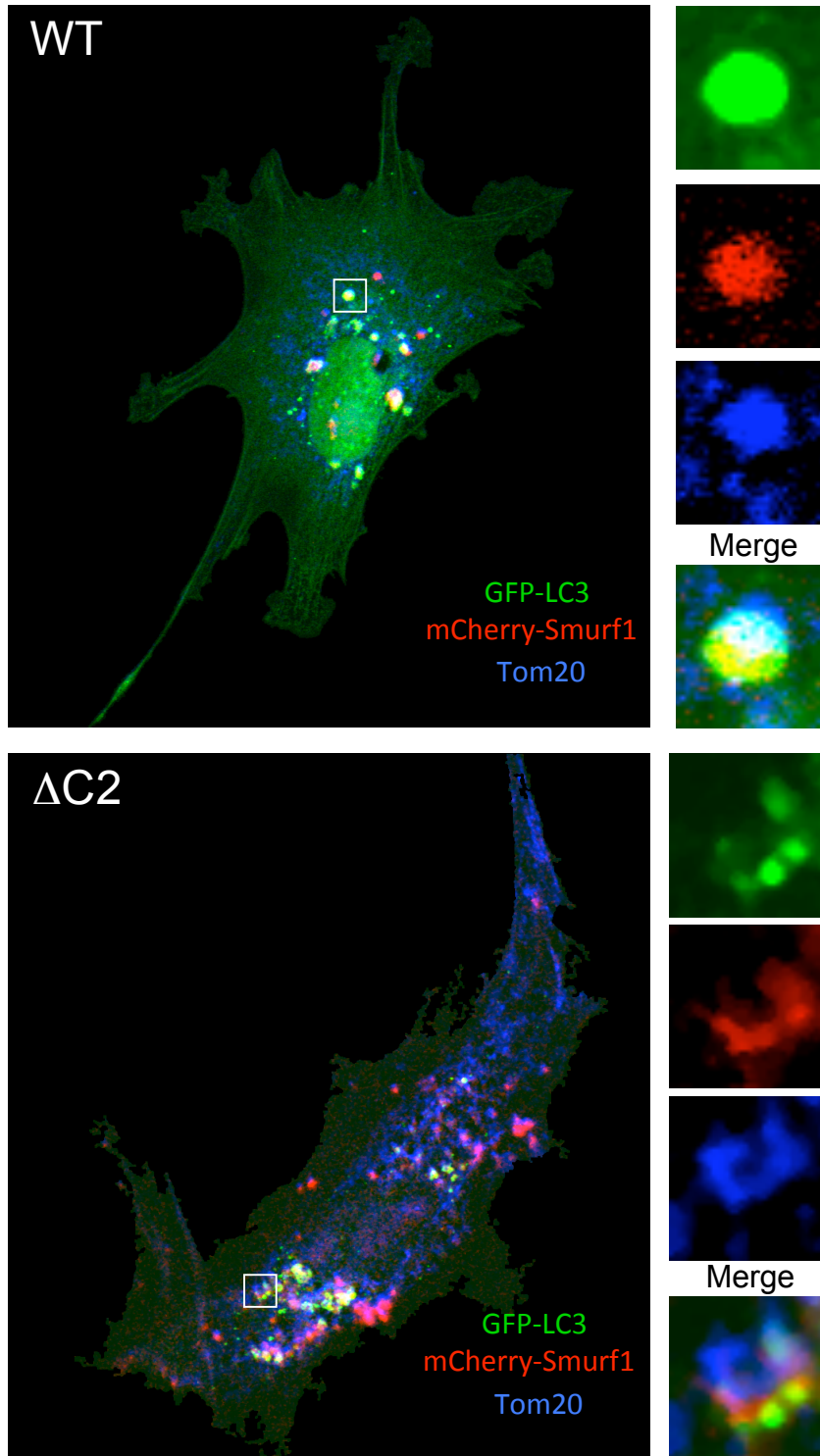
Supplementary Figure 7. Analysis of Smurf1 in Sindbis virus virophagy. **a**, Co-immunoprecipitation (Co-IP) of SIN capsid with Smurf1 in MEFs 10 hrs after SIN infection. For **a-g** and **i**, infections were with SIN strain SVIA, MOI=5 pfu/cell. **b**, Co-IP of SIN capsid with Smurf1 in HeLa/GFP-LC3 cells 11 hrs after SIN infection. **c**, Co-IP of SIN capsid with p62 in *Smurf1*^{-/-} (KO) and *Smurf1*^{+/+} (WT) MEFs 10 hrs after SIN infection. **d**, Western blot analysis of steady-state SIN capsid levels in MEFs of indicated genotype 10 hrs after SIN infection and 2 hrs after treatment with 10 μ m MG132 or vehicle control. **e**, Western blot analysis of p62 and SMURF1 expression in HeLa cells 48 hrs after treatment with the indicated siRNAs **f**, Western blot analysis of steady-state SIN capsid levels in HeLa/GFP-LC3 cells treated with the indicated siRNAs for 48 hrs and infected with SIN for 24 hrs. **g**, Viral titers in *Smurf1*^{-/-} (KO) and *Smurf1*^{+/+} (WT) MEFs 10 hrs after SIN infection. These conditions are identical to those used in **d**. Results shown represent geometric mean \pm s.e.m. for triplicate samples. **h**, Viral growth curve in *Smurf1*^{-/-} (KO) and *Smurf1*^{+/+} (WT) MEFs infected with SIN (SVIA, MOI = 0.1). **i**, Cell viability of MEFs of indicated genotype at serial time points after SIN infection. Results represent mean \pm s.e.m. for triplicate samples per condition, analyzing approximately 200 cells per sample. For **a-i**, similar results were observed in 3-5 independent experiments.

a**b****c**

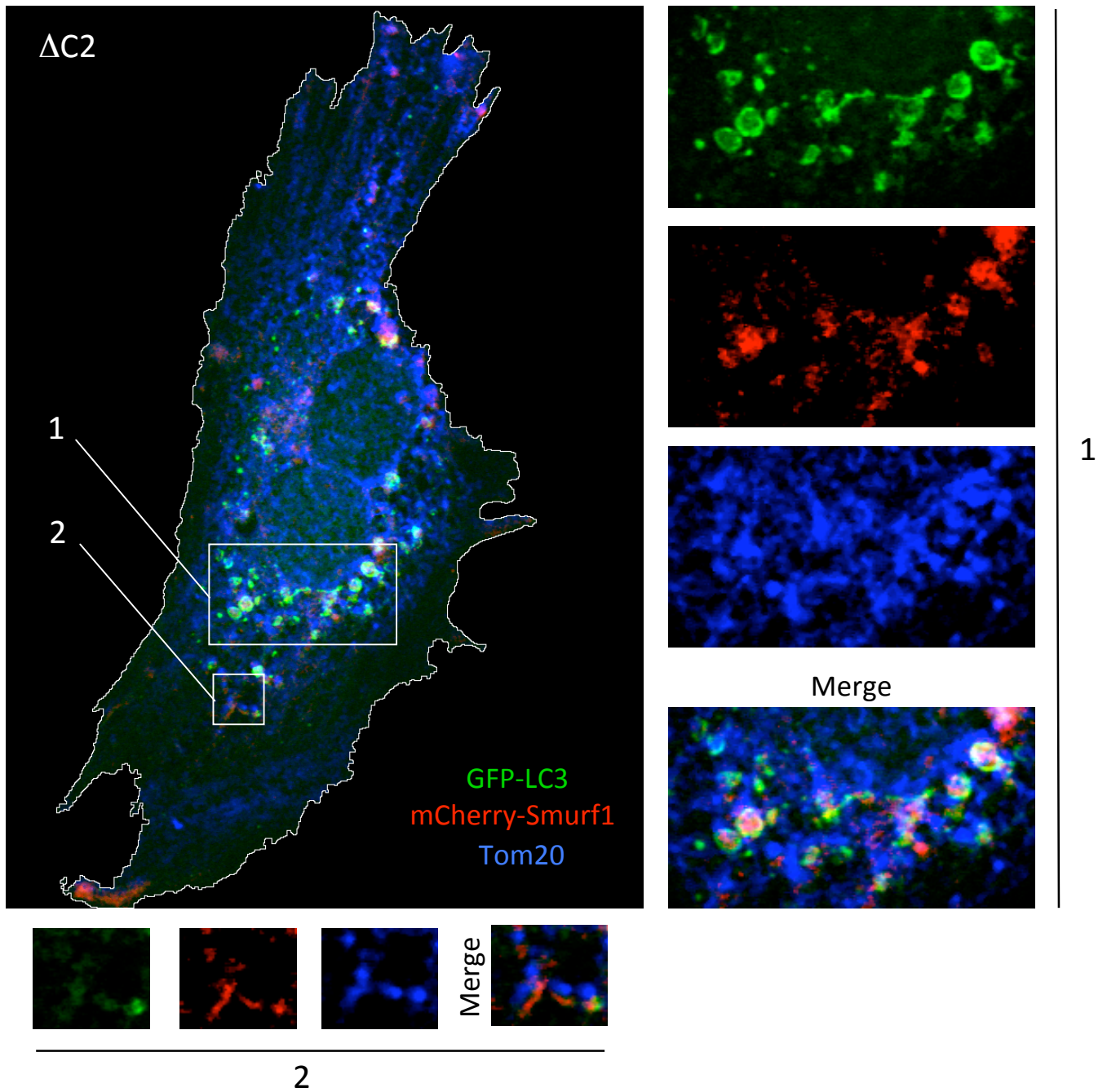
Supplementary Figure 8. Knockdown of SMURF1 results in defective mitophagy in HeLa cells. **a**, Western blot analysis of SMURF1 expression (top) and Atg7 and p62 expression (bottom) in HeLa cells 48 hrs after treatment with the indicated siRNA. **b**, Representative confocal micrographs of HeLa cells transiently transfected with mCherry-Parkin and the indicated siRNA for 48 hrs, and then treated with 10 μ M CCCP for an additional 24 hrs. Mitochondria were visualized by immunofluorescence with an anti-Tom20 antibody. Arrows denote representative Parkin-expressing cells. Scale bar, 15 μ m. **c**, Quantitation of mitophagy as shown in (b). mCherry-Parkin-expressing cells were scored positive for mitophagy if they contained 5 or fewer mitochondria. Data shown are mean \pm s.e.m. for triplicate wells per condition for at least 300 cells per well. *, $P < 0.001$ vs. NC siRNA, t-test. Similar results were observed in more than 5 independent experiments.



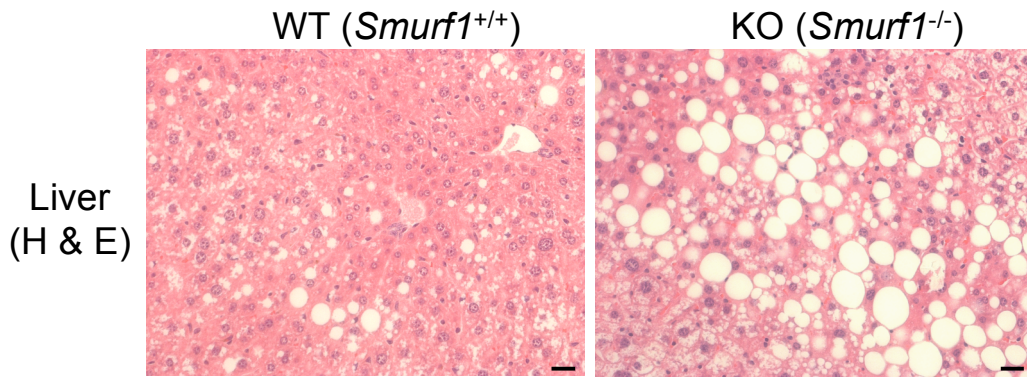
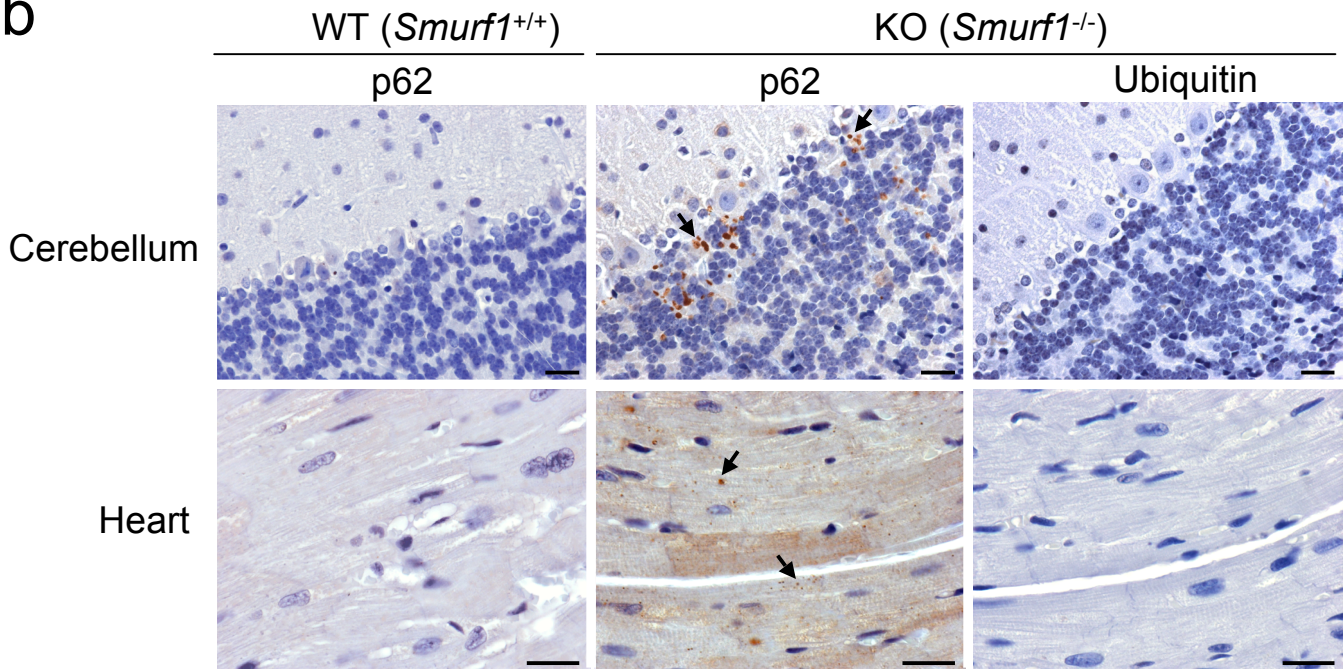
Supplementary Figure 9. The C2 domain and HECT domain of Smurf1 are not required for its interaction with p62. **a**, Western blot analysis of Smurf1 protein expression in *Smurf1*^{+/+} (WT) and *Smurf1*^{-/-} (KO) MEFs 48 hrs after transfection with indicated Smurf1 expression plasmid. Arrow indicates WT Smurf1. **b**, Co-immunoprecipitation of Smurf1 with p62 in *Smurf1*^{-/-} (KO) MEFs transfected with indicated Smurf1 expression plasmid.



Supplementary Figure 10: Detail of subcellular localization of WT Smurf1 or Smurf1 Δ C2 mutant, LC3 and mitochondria after 4 hrs of CCCP treatment. Left panels, Enlarged confocal micrographs of *Smurf1*^{-/-} cells shown in Fig. 4e transiently transfected with GFP-LC3 and mCherry-Smurf1 wild-type (WT) or mCherry-Smurf1 Δ C2 (Δ C2), treated for 4 hrs with CCCP, and stained with anti-Tom20 to label mitochondria. Right panels, enlargement and individual fluorescence channels for insets outlined by white boxes in images on left.



Supplementary Figure 11: Subcellular localization of Smurf1 $\Delta C2$, LC3, and mitochondria after 24 hrs of CCCP treatment. Representative confocal micrograph of a *Smurf1*^{-/-} MEF transiently transfected with GFP-LC3 and mCherry-Smurf1 $\Delta C2$, treated for 24 hrs with CCCP, and stained with anti-Tom20 to label mitochondria. Individual fluorescence channels and merged image for insets (white boxes) are shown to the right and below the image.

a**b**

Supplementary Figure 12. Accumulation of lipid droplets in *Smurf1*^{-/-} hepatocytes and accumulation of p62/SQSTM1 aggregates in *Smurf1*^{-/-} mouse cerebellar neurons and cardiomyocytes. a, H & E staining to detect histopathology in sections from livers of WT (*Smurf1*^{+/+}) and KO (*Smurf1*^{-/-}) mice. Scale bars, 20 mm. **b**, Representative photomicrographs of immunostaining to detect p62/ SQSTM1 expression in sections from cerebellum and hearts of (*Smurf1*^{+/+}) WT and (*Smurf1*^{-/-}) KO mice and ubiquitin expression in adjacent cerebellum and heart sections of (*Smurf1*^{-/-}) KO mice. Arrows denote representative p62/SQSTM1 aggregates in KO mice. No ubiquitin aggregates were detected in adjacent sections. Scale bars, 20 mm. For **a-b**, similar results were observed in the brains, hearts, and livers of 4 mice per genotype.