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Supplemental Information

Autophagy Protects against Sindbis Virus Infection of the Central Nervous System

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Supplemental Data

Figure S1

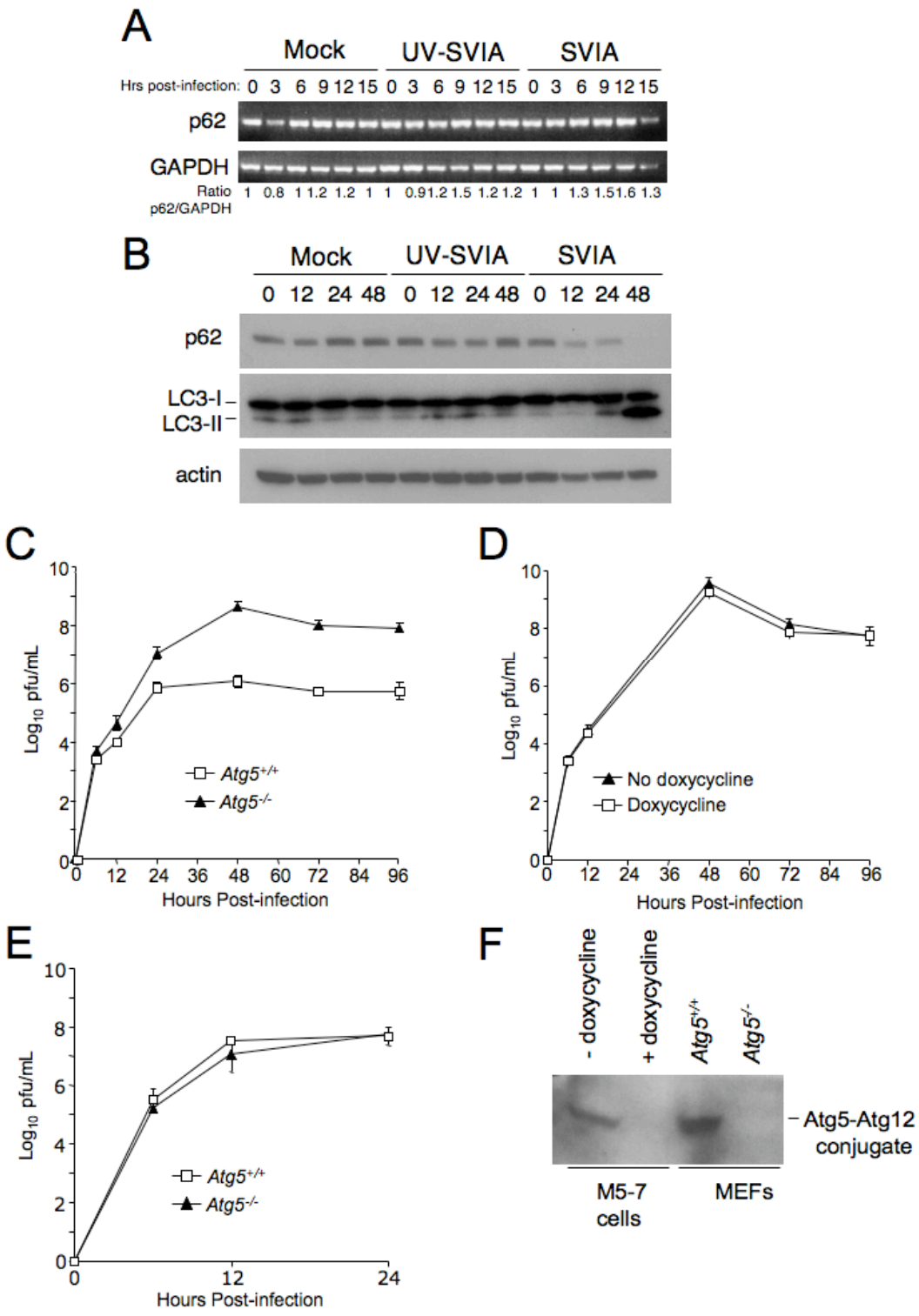


Figure S2

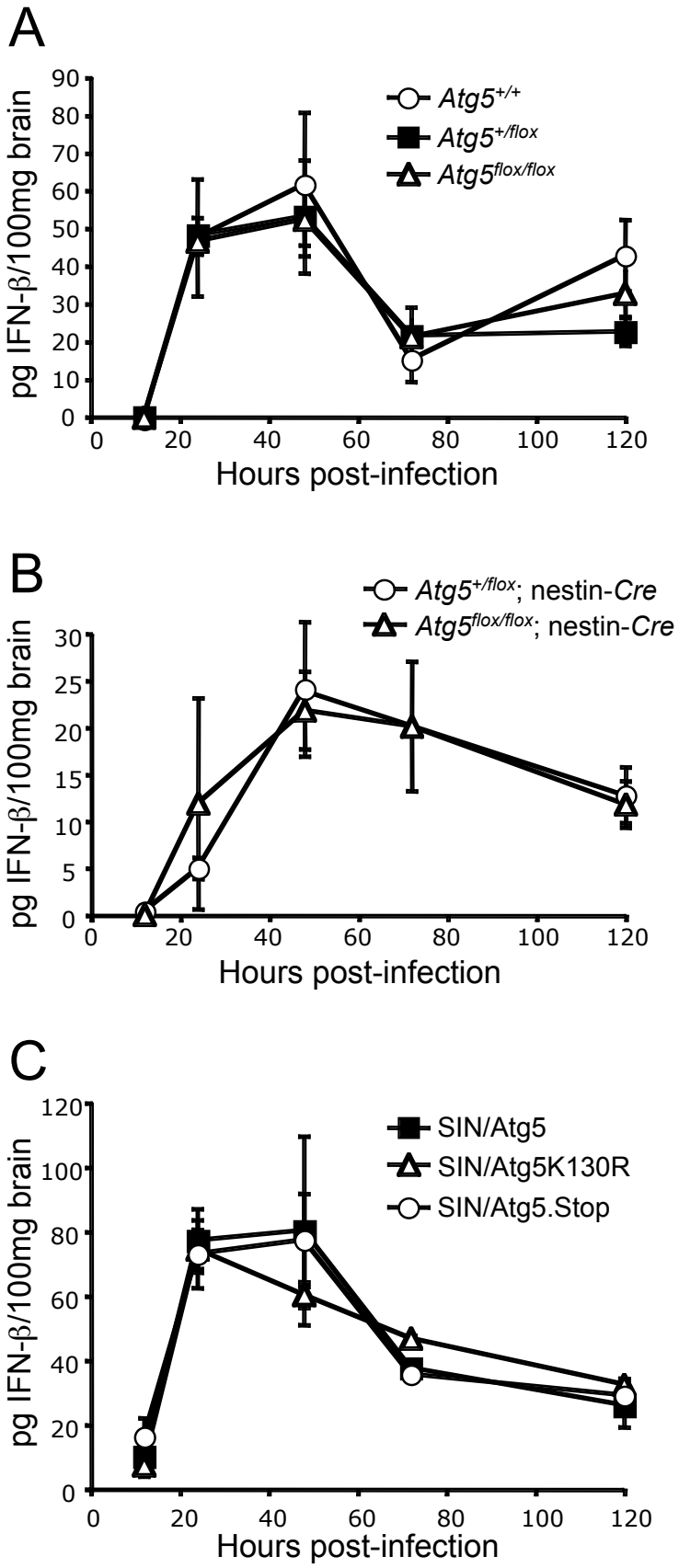

A
pg IFN-β/100mg brain
Hours post-infection
Atg5+/+
Atg5+/flox
Atg5flox/flox
B
pg IFN-β/100mg brain
Hours post-infection
Atg5+/flox; nestin-Cre
Atg5flox/flox; nestin-Cre
C
pg IFN-β/100mg brain
Hours post-infection
SIN/Atg5
SIN/Atg5K130R
SIN/Atg5.Stop

Figure S3A

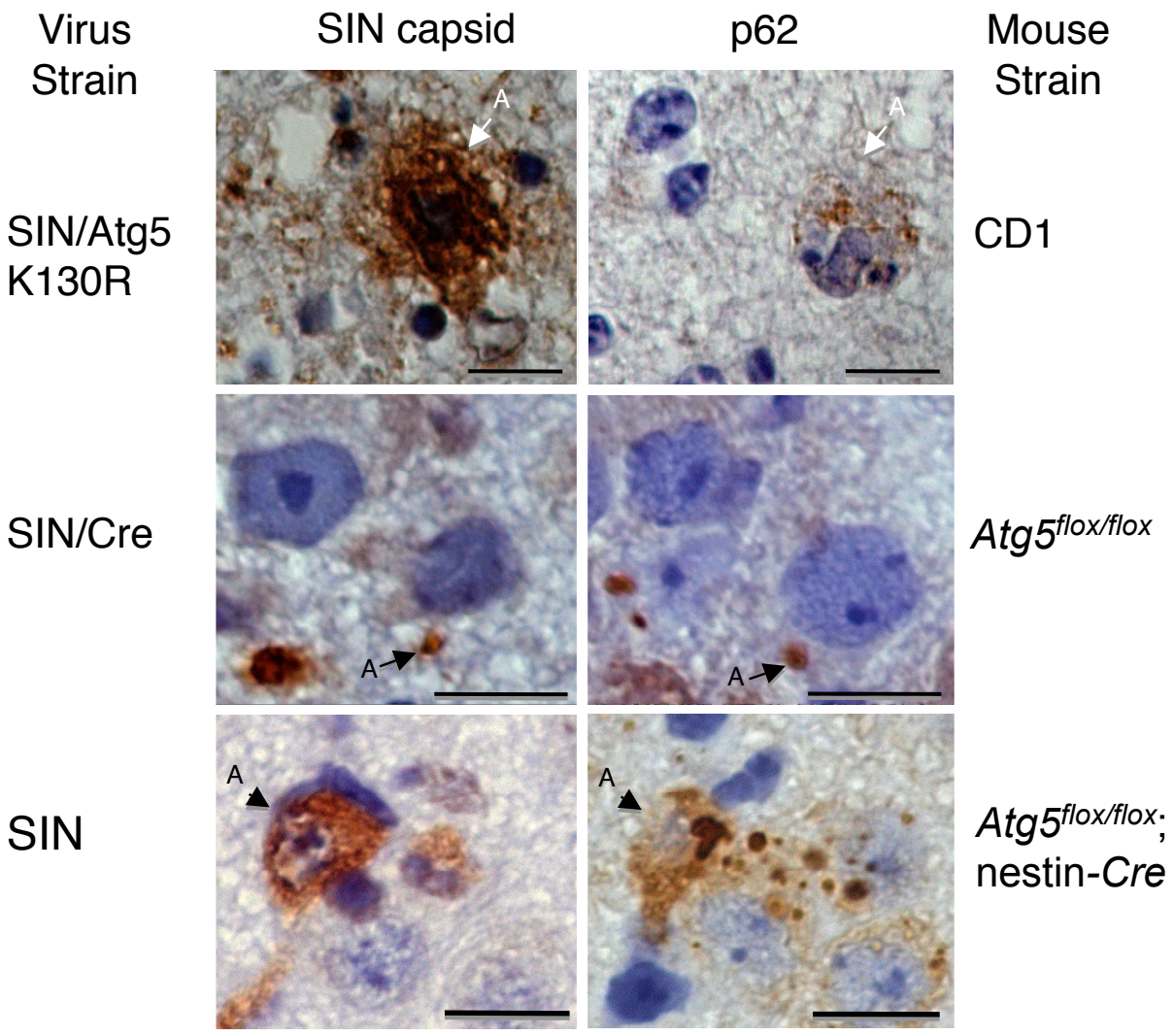
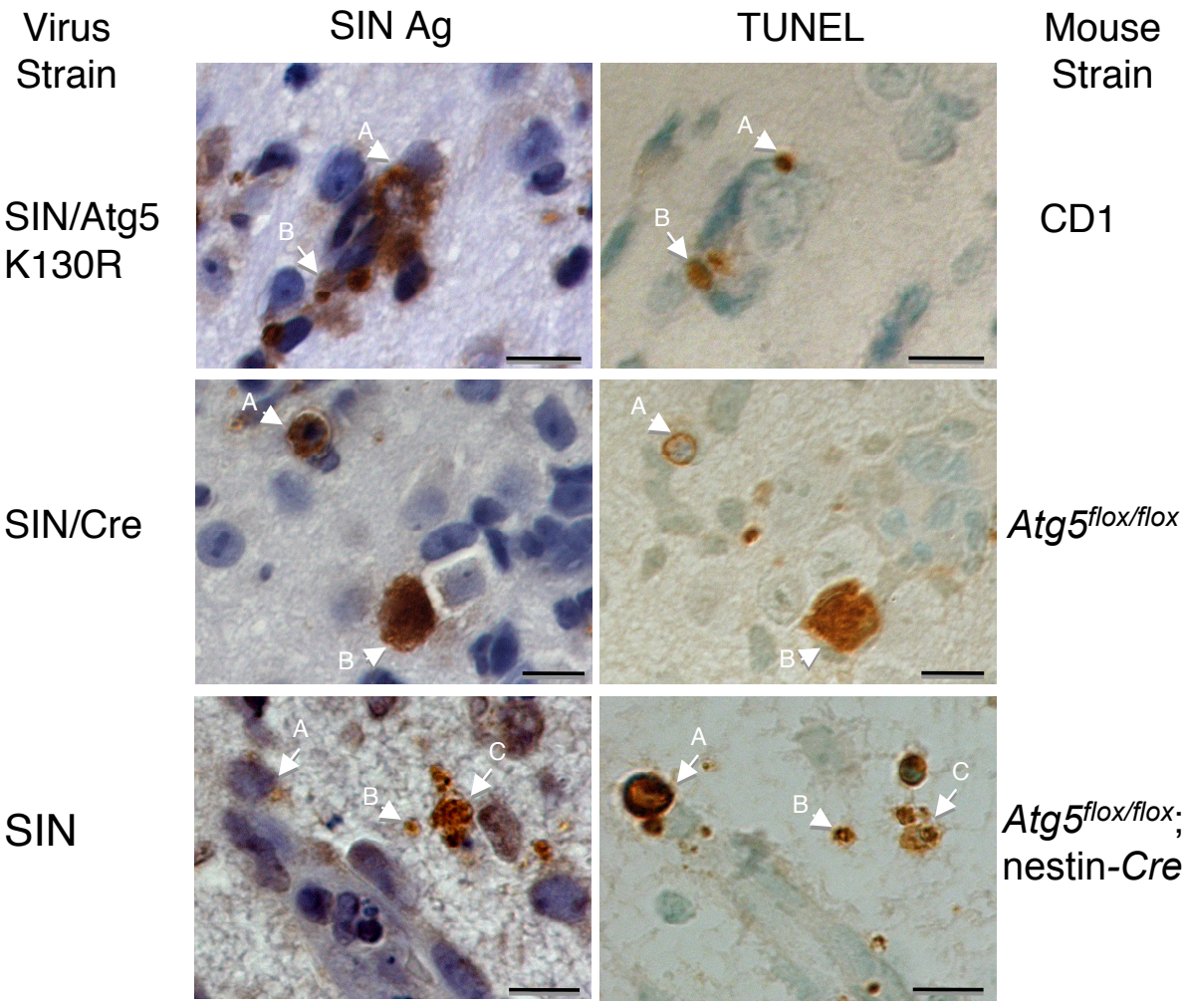

Virus Strain
SIN capsid
p62
Mouse Strain
SIN/Atg5 K130R
CD1
SIN/Cre
Atg5flox/flox
SIN
Atg5flox/flox; nestin-Cre
A

Figure S3B


Virus Strain
SIN Ag
TUNEL
Mouse Strain
SIN/Atg5 K130R
CD1
SIN/Cre
Atg5flox/flox
SIN
Atg5flox/flox; nestin-Cre
A
B
C

Supplemental Figure Legends

Movie S1, relates to Figure 1. Live-cell imaging of *Atg5*$^{+/+}$ MEFs infected with SIN-mCherry.capsid/GFP-LC3 spanning from 16 hours post-infection to 17 hours post-infection. mCherry.capsid structures appear first (arrowheads), followed by GFP-LC3 engulfment. Engulfment of two separate capsid structures is shown.

Figure S1. Analysis of Autophagy-Related Parameters and Viral Replication in SIN-Infected Cells, relates to Figure 1.

(A) p62 mRNA levels during SIN infection. Semi-quantitative RT-PCR to detect p62 transcripts in wild-type primary MEFs at serial time points after mock, SVIA or UV-inactivated SVIA (UV-SVIA) infection. The intensity of each band was determined by densitometry and p62/GAPDH ratios (in arbitrary units) are indicated below each lane. Similar results were observed in three independent experiments.

(B) Autophagy induction by live SIN infection in mouse Neuro-2A cells. Measurement of p62 levels and LC3-II conversion by Western blot analysis in mouse Neuro-2A cells at serial time points after mock, SVIA, or UV-inactivated SVIA (UV-SVIA). Actin was used as a protein loading control. Similar results were observed in three independent experiments.

(C-E) *In vitro* replication of SIN in *Atg5*-deficient cells. Growth curves of SVIA in *Atg5*$^{+/+}$or *Atg5*$^{-/-}$ MEFs (C); *Atg5*-expressing (no doxycycline) or non-*Atg5*-expressing M5-7 cells treated with 1μg/mL doxycline (D); or *Atg5*$^{+/+}$or *Atg5*$^{-/-}$ ES cells (E). Data shown represent the geometric mean ± SEM for three samples per time point. For C-E, similar results were observed in three independent experiments.

(F) Western blot analysis of Atg5-Atg12 conjugate levels (detected with an anti-Atg5 antibody) in *Atg5*$^{-/-}$ and *Atg5*$^{+/+}$ MEFs used in (C) and M5-7 cells used in (D) in the presence or absence of 1μg/mL doxycycline.

Figure S2. Atg5 Disruption Does Not Affect Type I IFN Levels in SIN-infected Mouse Brains, relates to Figure 4.

(A-C) Type I IFN levels as measured by ELISA detection of IFN-β in CD1 mouse brains infected with the indicated recombinant Sindbis virus strains (A), *Atg5*$^{flox/flox}$ or littermate controls (*Atg5*$^{+/flox}$ and *Atg5*$^{+/+}$) infected with Sindbis virus expressing Cre recombinase (B) or one week-old littermates from *Atg5*$^{+/flox}$; nestin-*Cre* transgenic mice crossed with *Atg5*$^{flox/flox}$ mice and infected with the dsTE12Q strain of Sindbis virus (C). For A-C, data shown represent mean ± SEM for groups of 3-4 mice per time point. Similar results were obtained in three independent experiments. No IFN-α could be detected by ELISA in the brains of any infected mice (data not shown).

Figure S3. Histopathological Analyses of SIN-infected Mouse Brains, relates to Figure 5.

(A) p62 accumulates in neurons that express SIN capsid. Representative high-power micrographs showing adjacent sections of mouse brain stained for SIN capsid and cellular p62. Left labels indicate virus strain used for infection and right labels indicate mouse strain. Each row depicts precisely the same region of the brain in adjacent sections confirmed by coordinates in lower power micrographs. Representative cells that

display both SIN capsid immunoreactivity and cellular p62 immunoreactivity are labeled with corresponding letters in each image. Scale bars, 10 μm

(B) SIN-Infected neurons are TUNEL-positive. Representative high-power micrographs showing adjacent sections of mouse brain stained for SIN antigen and TUNEL. Left labels indicate virus strain used for infection and right labels indicate mouse strain. Each row depicts precisely the same region of the brain in adjacent sections confirmed by coordinates in lower power micrographs. Representative cells that display both SIN antigen immunoreactivity and TUNEL-positivity are labeled with corresponding letters in each image. Scale bars, 10 μm

Supplemental Experimental Procedures

Mammalian Cell Lines

Primary MEFs and immortalized MEFs were cultured in 15% FBS media (high glucose DMEM, 15% fetal bovine serum, 1x penicillin/streptomycin, 118μM β-mercaptoethanol, and 1x MEM non-essential amino acids). Primary MEFs were passaged no more than five times. *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ ES cells were cultured in 15% FBS media (high glucose DMEM, 15% ES-grade fetal bovine serum, 1x penicillin/streptomycin, 118μM β-mercaptoethanol, 1x MEM non-essential amino acids, and 1,000u/mL leukemia inhibitory factor). M5-7 cells were cultured in 10% FBS media (high glucose DMEM, 10% fetal bovine serum, 0.1x gentamicin, 1x MEM essential amino acids, and 1x MEM non-essential amino acids). Atg5 expression was suppressed by the addition of 1μg/mL doxycycline. HeLa/GFP-LC3 cells were generated by subcloning the GFP-LC3 fragment from pEGFP-LC3 (provided by T. Yoshimori) into the NheI and EcoRI sites of the

pIRESneo3 vector (Clontech) to generate the plasmid, pGFP-LC3-neo, and using G418 (100 μg/ml) selection to isolate HeLa clones stably transfected with pGFP-LC3-neo. HeLa/GFP-LC3 cells were cultured in DMEM with 10% FBS containing 1x penicillin/streptomycin and 10 μg/ml G418.

Semi-quantitative RT-PCR

RNA was extracted from wild-type MEFs at serial time points following mock, SVIA, or UV-inactivated SVIA infection using TRIZOL according to manufacturers instructions, and first-strand cDNA synthesis was performed using random hexamer primers. PCR was performed (30 cycles) to amplify p62 using the primers ATGGTGCACC CCAATGTGATC and CTCTTGTCTTCTGTGCCTGTG and to detect GAPDH as an internal control using the primers ACCACCATGGAGAAGGCTGG and CTCAGTG TAGCCCAGGATGC.

Recombinant Chimeric SIN Strategies and Primers.

To construct the SIN recombinant chimeric viruses, SIN/Atg5 and SIN/Atg5K130R, the murine *Atg5* and *Atg5K130R* genes (Mizushima et al., 2001) were amplified by PCR using mAPG5/pClneo and mAPG5K130R/pClneo (provided by N. Mizushima) as templates. The PCR products were subcloned into pZeroBlunt (Invitrogen) and ligated into the *Bst*EII site of the SIN recombinant vector dsTE12Q (Liang et al., 1998) to yield the SIN/Atg5 and SIN/Atg5K130R plasmids. To construct SIN/Atg5.Stop, the same strategy was employed; however, two-step PCR was used to amplify the *Atg5* gene without the ATG start codon and a possible false ATG codon 177 base pairs from the

start codon. The SIN/Cre virus was constructed by amplifying the bacterial *Cre* recombinase gene using pCreHyg (provided by D. Leib) as a template. The SIN/Cre.Stop virus was constructed by amplifying the *Cre* gene without the ATG start codon using the same strategy. The SIN-mCherry.capsid virus was generated by overlap-extension PCR using pRSETB-mCherry (provided by R. Tsien) to amplify mCherry, and dsTE12Q/Cre to amplify SIN capsid regions. The SIN-mCherry.capsid/GFP-LC3 virus was generated by amplifying GFP-LC3 from pEGFP-LC3 (provided by T. Yoshimori) subcloned into pZeroBlunt and then into the *BstE*II site of SIN-mCherry.capsid.

The SIN/Atg5.Stop virus insert was amplified using two-step PCR with the following primer combinations GGGGTCACCACAGATGACAAAGATGTGCT and AACATCTTCTTGTCTAACCTTCTGAAAGTG, as well as CACTTTCAGAAGGT TAGACAAGAAGATGTT and AAGGTCACCTCAATCTGTTGGCTGGGGGA. The SIN/Cre virus was constructed with the primers GGGGTCACCATG TCCAATTTACTGACCG and GGGGTCACCCTAATCGCCATCTTCCAGCA using pCreHyg as a template. The SIN-mCherry.capsid virus was generated using the primers CAGAGTACTA GAAGAGCGGCTTAAAACG (containing an endogenous *Sap*I site), and CGCCCTTGCTCACCATGGTGGTGGTGTTGTAGTATTAG (the first 16 nucleotides corresponding to antisense 5' start of mCherry, and last 22 nucleotides corresponding to the antisense SIN genome immediately 5' to capsid start), and ATGAATAGAGGATTCTTTAACATGC (corresponding to the first 25 bp of capsid) and CGTTCTTGACGTCGAACAATCTGTCG (containing an endogenous *Aat*II site within the capsid gene), were used to amplify the region 5' upstream of capsid and the first 361 nucleotides of the capsid gene, respectively, using the dsTE12Q plasmid as a

template. The primers CTACAACACCACCACCATGGTGAGCAAGGGCGAGGAG (the first 16 nucleotides corresponding to the 5' region upstream of capsid, and last 21 nucleotides corresponding to the 5' start of mCherry) and GTTAAAGAATCCTCTATTCATCTTGTACAGCTCGTCCATGCC (the first 21 nucleotides corresponding to the antisense 5' start of capsid, and last 21 nucleotides corresponding the antisense 3' end of mCherry lacking the stop codon) were used to amplify mCherry from pRSETB-mCherry. The three PCR products were linked in two steps using the flanking primers to generate a ~1.6kb fragment that was subcloned into pZeroBlunt, and then into the endogenous *Sap*I and *Aat*II sites of dsTE12Q. The SIN-mCherry.capsid/GFP-LC3 virus was generated using the primers TACAACGGTCAC CATGGTGAGCAAGGGCGAGGAGC and CGATCAGGTGACCTCACAAGCATGG CTCTCTTCC.

siRNA

The following sequences were used for siRNA knockdown of p62: p62-1, GAAAUGGGUCCACCAGGAA; p62-2, GAUCUGCGAUGGCUGCAAU; p62-3, GCAUUGAAGUUGAUAUCGA; and for Atg7: GGGUUAUUACUACAAUGGUG.

Coimmunopreciptation and Radioimmunoprecipiation

For coimmunoprecipitation studies, HeLa/GFP-LC3 cells were infected with SVIA at an MOI of 5 in 60mm dishes for 11 hours and lysed in 600 μl TNT lysis buffer (50mM Tris-Cl pH 7.4, 150mM NaCl, 1mM EDTA, 1% TritonX-100, and protease inhibitors). Forty μl of lysates were reserved for Western blots as loading controls. Samples were pre-

cleared using 40 μl protein G-agarose beads (Santa Cruz) and 6 μl normal guinea pig IgG (Santa Cruz) for 30 minutes to 1 hour, immunoprecipitated overnight with 6 μl anti-p62 antibody (Progen), or IgG control, and boiled for 5 minutes with Laemmli buffer containing 2.5% β-mercaptoethanol. For radioimmunoprecipitation, cells were treated with negative control or p62-2 siRNA for 48 hrs, infected at an MOI of 5 with SVIA, or pelleted and lysed as described below to assess siRNA knockdown. At 3 hours post-infection, cells were depleted of Met/Cys by washing three times with 2 ml Met/Cys-free media (Gibco) containing 2% dialyzed FBS (Gibco), and then incubated with the same media for 2 hours. Cells were then labeled for 1 hour with 21 μCi Trans-35S LABEL, Metabolic Labeling Reagent (MP Biomedicals), and washed 5 times with 1 ml 2% media supplemented with Met and Cys at 2 mM each. Cells were scraped and pelleted at 2.4k RCF, and stored at -80° C until lysis. Cells were first lysed in 100 μl TNT lysis buffer, and insoluble material pelleted at 16k RCF. The insoluble pellet was then solublized in 50 μl TSD buffer (50mM Tris-Cl pH 7.4, 1% SDS, 5mM DTT) by boiling for 10 minutes. Soluble and insoluble lysates were added to a final volume of 600 μl TNT buffer, and immunoprecipitated as described above using rabbit IgG (Santa Cruz) to pre-clear samples, and 6 μl anti-Sindbis virus antigen antibody for immunoprecipitation. Immunoprecipitates were boiled for 5 minutes in Laemmli buffer containing 1.25% β-mercaptoethanol, separated by SDS-PAGE, dried, and exposed by autoradiography at -80° C.

Sample Preparation for Histology and Immunostaining

For immunofluorescence staining, GFP-LC3 MEFs were permeabilized in 0.2% TritonX-100/PBS, blocked with 1% goat serum, and stained with rabbit polyclonal anti-SIN antibody (1:400 dilution), and labeled with a goat anti-rabbit-TRITC-labeled secondary antibody (Santa Cruz; 1:100 dilution). Slides were mounted with Gel/Mount™ (Biomeda corp). For immunostaining of frozen brain sections from SVIA-infected GFP-LC3 neonates, slides were heated at 50°C for 10 min before rehydration in 100%, 95% and 70% ethanol, washed 3 x 5 min in PBS, and blocked and immunostained as described above.

Western Blot

For Western blot analyses, cells were lysed for 30 min on ice in TNT lysis buffer and cleared samples were boiled in 1:1 Laemmli buffer, separated on SDS-PAGE denaturing gels, and transferred to PVDF membranes (Biorad). Membranes were blocked with 5% nonfat milk in PBS/0.1% Tween-20 (PBST). Signals were visualized with a Supersignal®West Pico Chemiluminescent Substrate kit (Pierce). For SIN capsid and SIN antigen Western blots in co-immunoprecipitation studies, samples were analyzed using a One-Step Complete IP-Western Kit For Rabbit Primary Antibody (GenScript Corporation), according to the manufacturer's instructions. Specifically, 10 μl of SIN capsid or SIN antigen antibody was incubated with WB1 solution, and samples were transferred to PVDF membranes after SDS-PAGE analysis.

Microscopy and Quantitation

GFP-LC3 MEFs were imaged and punctae were quantified with a Zeiss Axioplan2 microscope using a Zeiss PLAN-APOCHROMAT 63x objective by an observer blinded to experimental condition. For live cell imaging and colocalization studies, *Atg5*$^{+/+}$ or *Atg5*$^{-/-}$ were imaged in an environmentally-controlled chamber with an Olympus 40x objective on a personal DeltaVision microscope with softWoRx software (Applied Precision). Images were captured at 10-minute intervals with 8 z-sections per time point with 2 μm spacing. Images were deconvolved using softWoRx software. Movie generation and image manipulations were performed with ImageJ (NIH). For colocalization studies, HeLa/GFP-LC3 cells and were imaged similarly to live cells but with an oil-immersion 40x objective and with 10 z-sections obtained per sample. Colocalization was quantitated using Imaris software (Bitplane). siRNA identities were blinded to experimenter until after statistical analysis. For quantitation of pathology in infected brain sections, the number of SIN antigen-positive cells per unit area of midline brain sagittal sections was quantitated using Zeiss Axioplan2 Imaging software using a 20x objective. To quantify the number of TUNEL positive-cells per virus-infected focus, the areas of virus-infected foci were measured for each brain sagittal section, and the number of TUNEL-positive cells in each virus-infected focus was counted using a 20x objective. EM studies were performed as described previously (Liang et al., 1999).

Supplemental References

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Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., and Levine, B. (1998). Protection against fatal Sindbis virus encephalitis by Beclin, a novel Bcl-2-interacting protein. J Virol *72*, 8586.

Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y., and Yoshimori, T. (2001). Dissection of autophagosome formation using *Apg5*-deficient mouse embryonic stem cells. J Cell Biol *152*, 657-668.