Supplemental Information

Species-specific impact of the autophagy machinery on Chikungunya virus infection

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Supplemental Figure Legends

Figure S1, related to Figure 1.

(A) Mock-infected HeLa cells were labeled using anti-p62 antibody. (B) HeLa cells were infected for 15h, fixed for STORM and labeled using antibodies to p62 and capsid. Widefield and STORM images of p62 localizing with capsid are shown. Scale bar=200nm in magnified image (Left). (C) HeLa cells were infected for 20h and fixed for transmission electron microscopy. Small vesicles containing and surrounded by nucleocapsids are shown. Scale bar=100nm. (D) HeLa cells were mock-infected (mock) or infected (CHIKV) for 20h and fixed for immuno-electron microscopy. p62 or capsid were labeled using primary antibody and protein A-gold 15nm (black arrows) or 10nm (white arrows) respectively. A small vesicle containing and surrounded by p62 and capsid is shown in infected cells. Scale bar=100nm. (E) HeLa cells were treated with DMSO (CTRL) or rapamycin then infected. Cell mortality (i.e. fold-change relative to mock-infected cells) was measured 9, 15, or 24h after infection for infected and mock-infected cells. (F) HeLa cells were treated with DMSO (CTRL) or wortmannin and infected for 15h and 24h and viral replication was quantified by flow cytometry. (G) HeLa cells were treated with DMSO (CTRL) or wortmannin and infected for 15h or 24h of infection and viral production was measured by titration of infectious virus in supernatants of infected cells. Data shown are representative of at least three independent
experiments. Graphs are shown as mean + SD (n=3) of one representative of at least three independent experiments. Scale bar=10µm.

**Figure S2, related to Figure 2.**

(A) Three-dimensional reconstitution of widefield image z-stacks of HeLa cells infected for 15h was performed using ZEN LE 2009 Software. Cells were labelled with antibodies to NDP52 or capsid. Scale bar=5µm. (B) HeLa cells were infected for 15h, fixed for STORM and labeled with antibodies to NDP52 or capsid. A STORM image of NDP52 in close vicinity of capsid is shown. Scale bar of magnification (left) =200nm. (C) HeLa cells were infected with CHIKV for 15h, and labeled using antibodies to NDP52, TGN46 or CHIKV capsid. (D) HeLa cells were infected with CHIKV for 15h, and labeled using antibodies to p62, NDP52 or CHIKV capsid. Scale bar=10µm. (E) HeLa cells were transfected with GFP-LC3-B and infected for 15h, and labeled using antibodies to NDP52 or capsid. (F) HeLa cells were infected for 15h, and labeled using antibodies to NDP52 or E2. Data shown are representative of at least three independent experiments. Scale bar=10µm.

**Figure S3, related to Figure 2.**

(A) HeLa cells were infected for 15h, and labeled using antibodies to p62 or CHIKV E2. (B) HeLa cells were transfected with GFP-LC3-B then infected for 15h, and labeled with anti-E2 antibody. (C) HeLa cells were infected for 15h, and labeled using antibodies to p62, ubiquitin (FK2) or capsid. Scale bar=10µm. (D) HLFs were infected for 15h and labeled using antibodies to p62, FK2 or capsid. Scale bar=10µm. (E) HLFs were treated with control (CTRL) or p62 siRNA then infected for 24h and cell mortality (i.e. fold-change relative to mock-infected cells) was measured for infected and mock-infected cells. (F) HLFs cells were treated with control (CTRL) or p62 siRNA then infected for 24h and viral production was
assessed by titration of infectious virus in supernatants of infected cells. Whole-cell lysates of siRNA-treated cells were immunoblotted for p62 and actin to show the efficiency of siRNA depletion. (G) HLFs were treated with control (CTRL) or NDP52 siRNA then infected for 24h and cell mortality (i.e. fold-change relative to mock-infected cells) was measured for infected and mock-infected cells. (H) HLFs were treated with control (CTRL) or NDP52 siRNA then infected for 24h and viral production was assessed by titration of infectious virus in supernatants of infected cells. Whole-cell lysates of siRNA-treated cells were immunoblotted for NDP52 or actin to show the efficiency of siRNA depletion. Data shown are representative of at least three independent experiments. Graphs are shown as mean + SD (n=3) of one representative of at least three independent experiments. Scale bar=10µm.

Figure S4, related to Figure 3.

(A) HeLa cells were treated with control (CTRL) or SMURF1 siRNA, then infected or not for 24h. Immunoprecipitation experiments were performed using antibodies to FK2 or nonspecific IgG controls and lysates from mock- or virus-infected cells. Cell lysates prior to the immunoprecipitation step and immunoprecipitated proteins were revealed using antibodies to SMURF1 or CHIKV capsid or actin. (B) Lysates from mock- or virus-infected HeLa cells prior to the immunoprecipitation step were immunoblotted using antibodies to FK2 or capsid. Data shown are representative of at least three independent experiments.

Figure S5, related to Figure 4.

(A) Immunoprecipitation experiments were performed using antibodies to NDP52 or nonspecific IgG controls (a rabbit anti-FLAG) (data not shown) and lysates from infected or mock-infected HeLa cells. Immunoprecipitated proteins were revealed using antibodies to NDP52 and nsP2. (B) Yeast cells expressing Gal4 DNA binding domain (BD) fused alone
(empty vector) or to CHIKV nsP2 or SINV nsP2 or SFV nsP2 were cotransformed with a plasmid encoding the Gal4 transactivation domain (AD) fused to NDP52 or the indicated mutants. (C) HeLa cells were infected for 15h, and labeled using antibodies to NDP52, CHIKV capsid or nsP2. (D) HeLa cells were infected for 15h, and labeled using antibodies to NDP52 or nsP2. (E) HLFs were infected for 15h, and labeled using antibodies to NDP52 or nsP2. (F) HeLa cells were infected for 15h, then labeled using antibodies to NDP52, capsid and dsRNA. (G) HeLa cells were infected for 15h and labeled using antibodies to NDP52, capsid or puromycin. (H) HeLa cells were treated with control (CTRL) or NDP52 siRNA, transfected with empty plasmid (CTRL) or CHIKV nsP2-3XFLAG for 48h and treated with puromycin. Puromycin incorporation into newly synthesized protein were revealed using antibodies to puromycin. (I) HeLa cells were transfected with 500 ng of empty plasmid (CTRL) or CHIKV nsP2-3XFLAG or 500ng of CHIKV nsP2-3XFLAG and various concentrations of NDP52 (as shown along the X-axis). Twenty-four hours post transfection cell mortality (i.e. % of dead cells) was measured. Data shown are representative of at least three independent experiments. Scale bar=10µm.

**Figure S6, related to Figure 5.**

(A) Comparison of the *Homo sapiens* NDP52 amino acid sequence with *Mus musculus* NDP52 amino acid sequence. Alignment was performed using UNIPROT (http://www.uniprot.org/). (B) HeLa cells were infected or not for 24h, and immunoprecipitation experiments were performed using antibodies to NDP52 or nonspecific IgG controls and lysates from mock- or virus-infected cells. Immunoprecipitated proteins were revealed using antibodies to LC3-C. (C) MEFs cells were infected for 3h and transfected with empty plasmid (CTRL), hNDP52, hLC3-C or hNDP52 and hLC3-C. Fifteen hours post-infection, cells were labeled using antibodies to hNDP52, hLC3-C or nsP2. Scale bar=10µm.
(D) MEF cells were transfected with empty plasmid (CTRL) or CHIKV nsP2-3XFLAG for 24h and treated with puromycin. Whole-cell lysates of treated cells were immunoblotted for nsP2 or actin. Puromycin incorporation into newly synthesized protein was revealed using antibodies to puromycin. (E) Yeast cells expressing Gal4 DNA binding domain (BD) fused alone (empty vector) or to CHIKV nsP2 or SINV nsP2 or SFV nsP2 were cotransformed with a plasmid encoding the Gal4 transactivation domain (AD) fused to NDP52 or the indicated mutants.

**Figure S7, related to Conclusion.**

The cytotoxicity of capsid leads to cell death. Autophagy receptor p62 targets toxic ubiquitinated capsid to autophagolysosomal degradation through its interaction with LC3-B and in association with Beclin1 and Atg7, promoting cell survival. nsP2 is required for RNA replication and is responsible for cell shutoff leading to cell death. By binding nsP2, NDP52 is implicated in RCs in the TGN-derived membranes in association with LC3-C, Beclin1 and Atg7, and restricts cell death by retaining nsP2 in the cytoplasm, which limits cell shutoff.

**Figure S8 related to Materials and Methods.**

HeLa cells were infected with CHIKV for different time points and viral production was assessed by titration of infectious virus in supernatants of infected cells.
**Supplemental experimental procedures**

**Cell culture and virus strains**

HeLa cells were obtained from ATCC (CCL-2) and cultured in Eagles’ Minimal essential medium (EMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Invitrogen). Vero cells were obtained from ATCC (CCL-81) and cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 5% fetal calf serum (FCS) and 1% penicillin-streptomycin (Invitrogen). Primary human fibroblasts (HLFs) were obtained from labial biopsies and were cultured in quantum 333 complete fibroblast medium (PAA, Ref#U15-813). Atg5 \(^{+/+}\) and Atg5 \(^{-/-}\) immortalized mouse embryonic fibroblasts (MEFs), a gift from Dr N. Mizushima, were described previously [1]. MEFs were obtained from C57BL/6 (B6) (gift from M. Albert, [2, 3]) and cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin. CHIKV and CHIKV-eGFP are infectious viruses generated using full-length infectious cDNA clones provided by S. Higgs [4]. The CHIKV-eGFP infectious cDNA clone was constructed from CHIKV cDNA clone. The CHIKV-eGFP expresses green fluorescent protein (eGFP), which is encoded by a gene inserted at the 3’ end of the CHIKV cDNA clone. Both viruses were amplified on mosquito C6/36 cells as described [5], and supernatants were harvested and frozen at -80°C before plaque assay titration and further use. Experiments performed to quantify viral replication by qRT-PCR or by GFP intensity per cell, viral production by titration of infectious virus and level of intra-cellular capsid by western-blot are done with CHIKV-eGFP. In these experiments, cell mortality was also defined in mock-infected or infected cell cultures. All other experiments were performed with CHIKV.

**Animal studies**
Nine-to-twelve-day old B6 mice were obtained from Charles River laboratories (France). Mice were bred according to the Institut Pasteur guidelines for animal husbandry and were kept in Level-3 isolators. Mice were inoculated by intra-dermal route with $10^6$ PFU of CHIKV as described [3] and intracardiacally perfused 3 days after infection. Muscle was either cooled in OCT compound (Tissue-Tek) for cryosectionning or treated with RIPA buffer (Sigma) for protein extracts and Western-blotting, as described below. Cryosections were labeled for p62 and CHIKV capsid, as described below.

**Drug treatments**

HeLa cells were treated for 2h with 50nM of rapamycin (Calbiochem) or DMSO then infected in the same medium. Three hours post infection, inoculum was removed and fresh medium with rapamycin or DMSO was added and maintained throughout the experiment. HeLa cells were treated with 160nM of bafilomycin 3h before the end of experiment. HeLa cells were treated with 10 µg/ml of puromycin (Clontech) for 15 min before fixation then puromycin incorporation was revealed using a puromycin-specific antibody [6].

**siRNA treatment**

HeLa cells were transfected with the following siRNAs using oligofectamine (Invitrogen) according to manufacturer’s instructions (Ambion) for human p62 (Ref# s16962), mouse p62 (Ref# s71143), human NDP52 (Ref# s19994), mouse NDP52 (Ref# s94686) and non-silencing negative control (Ref# AM4635). siRNAs for siRNA sequences for Atg6, and Atg7 were taken from [7]. Seventy-two hours post-transfection, cells were infected with the 3’ CHIKV-eGFP, as described above.

**Plasmid transfection**
Plasmids GFP-LC3-B (BUG 3046) [7], 3XFLAG, nsP2-3XFLAG, nsP2\textsubscript{R606A}-3XFLAG, and capsid-3XFLAG were transiently transfected in HeLa cells with JET PRIME (Polyplus) according to the manufacturer’s instruction. HeLa cells were infected for 3h then transiently transfected with p62\textsubscript{WT}-3XFLAG, p62\textsubscript{deltaUBA}-3XFLAG and p62\textsubscript{deltaLIR} -3XFLAG or hLC3-C and hNDP52-3XFLAG as described above.

**qRT-PCR**

Total RNA was extracted from cell cultures using RNeasy Mini kit (QIAGEN, Cat No. 74104), according to the manufacturer's protocol. RNA was eluted then stored at -80°C until use. A real-time qRT-PCR was performed using Power SYBR Green RNA to CT one step (Applied Biosystem, Ref#4389986) according to manufacturer's protocol using a 7500 Fast System instrument (Applied Biosystem). Final concentration of each specific primer was 0.175µM (CHIKV F: ACGCAATTGAGCGAGGCAC, CHIKV R: CGGAGGACATTGGGCCCAC). CHIKV genome expression was determined using the 2^ΔΔCt comparative method [8]. The housekeeping gene used was GAPDH (GAPDH F: GGTATCGTGGAAGGACTCATGAC, GAPDH R: ATGCCAGTGAGCTTCCGTTCAG).

**Immunofluorescence studies**

Primary antibodies used include rabbit polyclonal antibodies to p62 (Clinisciences, Ref#PM045), NDP52 (Abcam, Ref#ab68588), LC3 (Novus Biologicals, Ref#NB-100-2331), Atg7 (Sigma, Ref#A2856), LC3-C (Interchim, Ref#R-140-100), FLAG (Sigma; Ref#7425) mouse monoclonal antibodies to mono- and poly-ubiquitinated conjugates (FK2, Enzo life Sciences, Ref#PW8810), p62 lck ligand (BD Biosciences, Ref#610832), LC3 (MBL, Ref#M152-3), dsRNA (English and Scientific Consulting, Ref# 10010200), LAMP-1 (BD Pharmingen, Ref#555798), Beclin1 (BD Biosciences, Ref# 612112), Tubulin-α (Sigma, Ref#T6074), HP1-α (Euromedex, Ref#2HP-2G9-AS), FLAG M2 (Sigma; Ref#F1804) and β-
actin (Sigma, Ref#A1978), and sheep polyclonal antibodies to TGN46 (AbD Serotec, Ref#AHP500GT) and LC3-C (Novus biologicals, Ref#NB110-74785). All secondary antibodies used were Alexa Fluor conjugates (Invitrogen). CHIKV capsid labeling was performed using a mouse monoclonal antibody directed against CHIKV capsids, coupled or not to Alexa-488, and CHIKV-E2 labeling using a mouse monoclonal antibody directed against E2 glycoprotein, coupled to cyanin-3. CHIKV nsP2 labeling was performed using a mouse monoclonal anti-CHIKV nsP2 antibody. CHIKV nsP3 labeling was performed using a rabbit polyclonal anti-CHIKV nsP3 antibody kindly provided by A. Merits [9]. Cells were fixed with 4% paraformaldehyde in PBS for 20 min, then permeabilized for 30 min in 0.2% Triton 100X (Sigma) and blocked in 5% of normal goat serum (Vector Laboratories). Cells were stained with the appropriate primary and secondary antibodies and counterstained with Hoechst (Vector Lab). Cells were observed with an AxioObserver microscope (Zeiss). Pictures and Z-stacks were obtained using the AxioVision 4.5 software.

**Coimmunoprecipitation assays**

Mock- and virus-infected cells were lysed in IGEPAL buffer (20mM Tris (PH 8.0), 1% IGEPAL CA-630 (Sigma), 150mM NaCl, 10% glycerol, and protease/phosphatase inhibitors. Capsid, ubiquitinated proteins and nsP2 were immunoprecipitated from total protein extracts by addition of 1µg of appropriate antibody and 20 µl of a 50% suspension of protein-A-sepharose beads or protein-G-sepharose beads (Amersham Biosciences Biotech) on lysates from mock- or virus-infected cells. Mouse or rabbit anti-FLAG were used as nonspecific IgG controls. Samples were analyzed by SDS-PAGE 10% and immunoblotted with indicated antibody.

**Western blot analyses and cytoplasmic/nuclear extraction**
Cell extracts were prepared with RIPA buffer (Sigma) and boiled for 5 min in loading buffer. Equal amounts of lysates protein were loaded and separated on either a 10% or a 14% SDS-polyacrylamide gel electrophoresis gel and transferred onto a PVDF membrane before analysis. The protein concentration was assayed from the resulting supernatants by BCA’ method (Pierce). Samples were immunoblotted with the appropriated primary antibodies. Mock- and virus-infected cells were lysed for nuclear and cytoplasmic fractions using NE-PER nuclear cytoplasmic extraction reagents (Pierce) according to the manufacturer’s instructions. Protein levels were quantified by measuring the intensity of the bands by densitometry. Tubulin was used as a positive control of cytoplasm extract and HP1 as a positive control of nuclear extract.

**Amino Acid Sequence alignment**

The *Homo sapien* NDP52 (Q13137) amino acid sequence was aligned with *Mus musculus* NDP52 (A2A6M5) amino acid sequence using UNIPROT (http://www.uniprot.org/).

**Mutated constructs of NDP52**

Cellular ORFs encoding for NDP52, NDP52 (1-127), NDP52 (128-393), NDP52 (394-446) were amplified by standard PCR (Phusion, Finnzymes), and cloned by *in vitro* recombination in pDONR207 using Gateway® technology (Invitrogen). PCR primers displayed 20-30 specific nucleotides matching ORF extremities so that their Tm is close to 60 °C. To achieve recombinational cloning of PCR products, 5’ ends of forward primers were fused to attB1.1 recombination sequence 5’-GGGGACAACTTTGTACAAAAAAGTTGGCATG-3’, while reverse primers were fused to attB2.1 recombination sequence 5’-GGGGACAACTTTGTACAAAGAAAAGTTGGTTA-3’. Recombination of PCR products into pDONR207 was performed following manufacturer's
recommendations (BP cloning reaction, Invitrogen). All constructs were transformed and amplified in *E. coli* DH5α strain. Cloning of nsP2 and capsid from CHIKV (wild-type strain 05115 from La Réunion) was performed similarly, and as previously described [10]. (ViralORFeome clone ID: 715 and 717).

**Deletion constructs of p62**

DNA fragments encoding for full-length p62 and p62ΔUBA (aa 1-388) were amplified by standard PCR (ExTaq, Takara) using as a template a pool of 12,000 human full-length ORFs [11], and then cloned by *in vitro* recombination into pDONR207 following manufacturer’s recommendations (Gateway system; Invitrogen). The QuickChange Lightning kit (Agilent-Stratagene) was used to delete the LIR domain from p62 (aa 321-342). A PCR was run on the pDONR207 plasmid containing p62 using 5’-phosphorylated reverse and forward primers which hybridized respectively upstream and downstream of the LIR-encoding region (ΔLIR-Rev: CCCCTCGGACTCCAAGGCGATCTT; ΔLIR-For: TCAAAAGAAGTGGACCCGTCTACAGGTG). Then, template DNA was digested with Dpn1 enzyme, and the PCR product circularized with T4 DNA Ligase (New-England Biolabs) to generate a pDONR207 plasmid containing p62ΔLIR.

**Yeast two-hybrid screen**

Yeast culture mediums were prepared as previously described [12]. Full-length nsP2 was transferred by *in vitro* recombination (LR cloning reaction, Gateway® technology, Invitrogen) from pDONR207 into yeast two-hybrid vector pDEST32 (Invitrogen) in order to be expressed in fusion downstream of Gal4 binding domain (Gal4-BD). Bait constructs were transformed into AH109 yeast strain (Clontech) using a standard Lithium/Acetate procedure. Spontaneous transactivation of the HIS3 reporter gene was not observed in yeast cells
expressing GAL4-BD-nsP2. Consequently, the screen was performed on a synthetic medium lacking histidine (-His medium) and not supplemented with 3-amino-1,2,4-triazole. A mating strategy was used for screening a normalized library of 12,000 human ORFs cloned [11]. Prior to the screening, this library (gift from Dr. Vincent Lotteau) was transferred by in vitro recombination (LR cloning reaction) into yeast two-hybrid vector pPC86 vector (Invitrogen) in order to express ORFs in fusion downstream of Gal4 activation domain (Gal4-AD), and established into the Y187 yeast strain (Clontech). With the mating, 5 million yeast diploid cells were obtained and grown for 6 days on -His selective medium. [His+] colonies were finally selected, and purified over 3 weeks by culture on selective medium to eliminate false-positives. AD-cDNAs were amplified by PCR from zymolase-treated yeast colonies using primers that hybridize within the pPC86 regions flanking cDNA inserts. PCR products were sequenced and cellular interactors were identified by multiparallel BLAST analysis. From this screen, we recovered 35 [His+] yeast colonies encoding for NDP52. To test for potential interactions between nsP2 and NDP52 fragments, corresponding ORFs were transferred from pDONR207 into pDEST32 and pPC86 destination vectors, respectively. Bait and prey plasmids were co-transfected in AH109 cell line using a standard Lithium/Acetate procedure, and plated on -His selective medium. Transformation efficiency was assessed on a medium supplemented for His but depleted for Trp and Leu.

**Stochastic optical reconstruction microscopy**

HeLa cells were infected with CHIKV and processed for stochastic optical reconstruction microscopy (STORM) [13]. For STORM, infected cells were labeled with CHIKV capsid-ATTO532 or p62. p62 primary antibodies were detected using rabbit secondary antibody coupled to Alexa-647. STORM imaging was performed as previously described [7]. Dual color STORM was achieved using a microscope setup and imaging
protocol similar to that used in previous report [14]. We first acquired 30,000 images of ATTO 532 fluorescence using continuous excitation by a solid state laser with emission wavelength at 532 nm. After most ATTO 532 fluorophores photobleached, we acquired 30,000 images of Cy5 fluorescence using continuous excitation by a diode laser at 635 nm. An appropriate set of filters and dichroics was used for each color. All images were recorded by a EMCCD camera with an exposure time of 100 ms.

For reconstruction of STORM images, we used a Matlab-based particle detection and localization software that we adapted for PALM/STORM (PALMTT) [15]. This software computed the position of individual molecules by fitting 2D Gaussian functions to the raw images. Another home-made software (PALMvis) was used to correct for mechanical drift in each channel using multicolor fluorescent beads as fiducial markers, to register the ATTO 532 and Cy5 images, and to visualize high-resolution images by superposing dots or Gaussian spots of standard deviation $\sigma=30$nm.

**Electron microscopy**

HeLa cells were infected with CHIKV and then processed electron microscopy. For transmission electron microscopy, HeLa cells were fixed with 2.5% glutaraldehyde, postfixed with 1% OsO$_4$, dehydrated with a graded ethanol series and embedded in epoxy resin. Ultra thin section were stained with uranyl acetate and examined by transmission electron microscopy using a JEOL JEM-1010 electron microscope.

For immuno-electron microscopy, Hela cells were infected with CHIKV for 15h and fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4), and embedded in 12% gelatin. Blocks were infiltrated with 2.3 M sucrose for cryoprotection, mounted on specimen holders and frozen in liquid nitrogen. Cryosections were cut with a Leica EM UC6/FC6 Microtome (Leica Microsystems, Vienna, Austria). A double labeling was performed
sequentially on thawed cryosections using for each antibody a different size of protein A gold. Cryosections were labeled first with a rabbit polyclonal antibody against p62, then against anti-capsid and protein-A gold (15 nm and 10 nm) obtained from Utrecht University (Utrecht, The Netherlands). Unspecific binding of the second protein A to the first antibody was blocked by incubation with 1% glutaraldehyde as previously described [16]. The grids were observed on a Jeol JEM 1010 (Japan) transmission electron microscope at 80 kV and images were taken using a KeenView camera (Soft Imaging System, Lakewood, CO, USA) using iTEM5.0 software (Soft Imaging System GmbH).

**Supplemental References**


