Role of autophagy-related proteins and cellular microRNAs in chikungunya and dengue virus infection
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DOI:
10.33612/diss.108290836

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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An autophagy-specific siRNA screen reveals BNIP3 as a regulator of chikungunya virus infection

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CHAPTER 4

Abstract

Chikungunya virus (CHIKV) is an alphavirus transmitted to humans by blood-feeding mosquitoes of the genus *Aedes* sp., which has rapidly spread around Asia, Europe and the Americas in the last decade. CHIKV infection causes a febrile disease associated with acute and chronic arthritogenic symptoms for which vaccines and specific treatments remain unavailable. Autophagy–related (ATG) proteins are a set of host factors that are grouped on the basis of their involvement in autophagy. ATG proteins and autophagy receptors, however, have also been shown to function singularly or in functional complexes in other cellular pathways unrelated to autophagy. In case of CHIKV, ATG proteins have been shown to both inhibit or enhance virus replication. Here, an ATG-specific siRNA screen was performed to evaluate the importance of ATG proteins and autophagy receptors in controlling CHIKV infection. We observed that 10 out of 50 ATG proteins interfere with CHIKV replication. Amongst these, depletion of the mitochondrial protein and autophagy receptor BCL2 Interacting Protein 3 (BNIP3) was found to increase CHIKV infectivity and progeny virus particle production. We also show that BNIP3 controls CHIKV infectivity independently of autophagy and cell death pathways. Detailed analysis of the replicative cycle of CHIKV revealed that BNIP3 interferes with infection during the early stages of RNA replication. The antiviral role of BNIP3 was found conserved across two distinct CHIKV genotypes and the closely related Semliki Forest virus. Altogether, this study describes a novel and previously unknown function of the autophagy receptor BNIP3 in the control of the early stages of the alphavirus replication cycle.
1. Introduction

Chikungunya virus (CHIKV) is a re-emerging alphavirus that is transmitted to humans by Aedes spp. mosquitoes and causes outbreaks of febrile disease in countries around Asia, Africa, Europe and the Americas (1,2). The caused disease is hallmarked by fever, headache, nausea, and severe joint and muscle pain. Usually, it resolves within 1-2 weeks after infection. In a relatively high number of cases (35-52%), however, the disease progresses to a chronic debilitating illness that can persist for months to years (3). Current approaches to treat CHIKV-infected patients involve unspecific therapies to relieve disease symptoms. There is no licensed vaccine available to prevent infection (4).

CHIKV is an enveloped virus with a single-stranded positive-sense RNA genome. The genome encodes for four non-structural proteins (nsP1 to nsP4) and five structural proteins (C, E3, E2, 6K/TF, E1) (5,6). The infectious cycle of the virus begins with the attachment of the E2 viral glycoprotein to cellular receptors, which subsequently triggers the internalization of the viral particle via clathrin-mediated endocytosis (7). After fusion with early endosome limiting membrane, the nucleocapsid is released into the cytoplasm where the genomic RNA is translated into the non-structural proteins (7,8). The non-structural proteins facilitate RNA replication, including the production of a subgenomic RNA that is further used as a template for the translation of the structural proteins (9). The E1-E2-E3 envelope proteins are inserted into the endoplasmic reticulum (ER) membrane and are processed during their transport to the plasma membrane to form mature viral spike proteins (10). In parallel, progeny genomic RNA is packaged by the C protein to form a nucleocapsid (11). Interaction of the newly formed nucleocapsid with the viral E1/E2 spike proteins at the plasma membrane drives the assembly, budding and release of progeny virus particles (11).

Viruses are obligatory parasites and depend on the host cell for reproduction. Therefore, they have evolved to exploit host cell pathways to their benefit. Many host factors have been described to control CHIKV infection, including autophagy-related proteins (ATG). ATG proteins are orchestrating autophagy, a lysosomal degradative transport route that involves the formation of double-membrane vesicles known as autophagosomes, which sequester cytoplasmic cargoes targeted to destruction (12). Specific cargoes such as mitochondria or ER are selectively targeted to autophagosomal degradation through the so-called autophagy receptors (13). Intriguingly, the ATG proteins BECLIN1 (BECN1) and ATG7, and the autophagy receptor calcium binding and coiled-coil domain 2 (NDP52) stimulate CHIKV infection, whereas the autophagy receptor p62 reduces viral infectivity (14,15). These results appear contradictory, but there is increasing evidence showing that many ATG proteins function in cellular process that are unrelated to autophagy. Indeed, we previously revealed that ATG13 and FIP200 play a role in picornavirus replication, independently from their function in autophagy as part of the ULK complex (16).

Here, we used an image- and siRNA-based screen approach to comprehensively assess the role of 50 ATG proteins and autophagy receptors in CHIKV replication. Downregulation of most ATG proteins was found to promote CHIKV replication implying that autophagy in general counteracts CHIKV infection. The most pronounced effect was seen for BCL2 Interacting Protein 3 (BNIP3), a mitochondrial protein known for its...
functioning as an autophagy receptor in mitophagy (17) and in participating in cell death pathways (18,19). We found that downregulation of BNIP3 increases CHIKV infectivity thereby leading to an enhanced production of progeny virus particles. This novel function of BNIP3 in controlling CHIKV infectivity does not rely on an intact autophagy/mitophagy machinery and is not associated with CHIKV-induced cell death. We demonstrate that BNIP3 interferes with infection at a step after membrane hemifusion but prior to abundant RNA replication. The antiviral properties of BNIP3 are conserved between circulating CHIKV strains of distinct genotypes and can be extended to the closely related alphavirus Semliki Forest virus (SFV).

2. Materials and methods

Cell lines. U2OS cells (ATCC® HTB-96™) and ATG7 knockout (ATG7KO) U2OS (20) were maintained in DMEM, high glucose, GlutaMAX™ Supplement (Gibco). Green monkey kidney Vero-WHO (ECACC 88020401) and Vero-E6 (ATCC CR-1586) cells were cultured in high glucose DMEM (Gibco), while BHK-21 (ATCC CCL-110) were cultured in RPMI 1640 medium (Gibco). Stable U2OS cells expressing GFP-WIPI2B, provided by T. Proikas-Cezanne (University of Tuebingen, Germany) (21), were maintained in high glucose DMEM, plus GlutaMAX™ Supplement (Gibco), supplemented with 600 µg/ml of G418 Sulfate (Gibco, 11811031). All media were supplemented with 10% heat inactivated fetal bovine serum (Lonza) and 100 U/ml penicillin/streptomycin (Gibco). All cells were maintained at 37°C in humidified atmosphere containing 5% CO₂.

Viruses: production and titration. Wild type (WT) CHIKV-La Reunion (LR, OPY1 strain) and 5’GFP-CHIKV-LR were produced from infectious cDNA clones generously provided by A. Merits (University of Tartu, Estonia) and from European Virus Archive goes global (EVAg), respectively (22). CHIKV strain S27 was kindly provided by S. Guenther (Bernhard-Nocht-Institute for Tropical Medicine, Germany) and CHIKV strain 99659 was provided by M. Diamond (Washington University, St. Louis, USA). SFV was obtained from M. Kielian (Albert Einstein College School of Medicine, New York, USA). For viruses derived from an infectious clone, plasmid DNA was linearized with NotI (Invitrogen) and used to synthesize RNA by use of an SP6 RNA polymerase (NEB). The in vitro transcribed RNA was subsequently electroporated (Bio-Rad gene pulser Xcell machine; 850V, 25 µF, no resistance) into BHK-21 cells and virus-containing supernatants were collected at 48 h post-electroporation. Subsequently, sub-confluent monolayers of Vero-E6 cells were infected at multiplicity of infection (MOI) 0.01 to generate working stocks. To produce working stocks for the other viruses, Vero-WHO cells were infected at MOI 0.01. At 48 h post-infection (hpi), the cell supernatants were harvested and clarified from cell debris by low-speed centrifugation, snap frozen in liquid nitrogen and stored at -80°C. Infectious virus titers were determined by standard plaque assay on Vero E6 cells. The number of CHIKV genome equivalent copies present in solution was determined by RT-qPCR, by amplification of the E2 gene as described before (23).

siRNA based screen. For the primary siRNA screen, a customized ON-TARGETplus SMARTpool human siRNA library (Dharmacon, Horizon™) targeting 50 ATG genes
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(Table S1) was used to reverse-transfect U2OS cells in a 96-well plate format. 2 pmol of siRNA were used for single gene knock-down, whereas for multiple gene knockdowns 3 pmol, 4.5 pmol, or 6 pmol were used for silencing of 2, 3 or 4 genes, respectively. Reverse transfection was performed using 0.1µl of Lipofectamine RNAiMax transfection reagent (Invitrogen) per well, according to the manufacturer’s protocol. After 20 min, 3,000 U2OS cells were seeded per well in complete culture medium to a final volume of 100 µl (16). At 48 h post-transfection (hpt), cells were infected with 5’GFP-CHIKV-LR at MOI 10. At 17 hpi, cells were fixed with 4% paraformaldehyde (PFA) and nuclei stained with Hoechst33342 (Sigma-Aldrich, B2261).

Automated image acquisition and analysis
Images were acquired using a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific) at the Cell Screening Core of the University Medical Centre Utrecht, The Netherlands. For image acquisition, the Hoechst, FITC filters and 10x lens were used. For image analysis, the Cellomics SpotDetector V3 algorithm was used to determine GFP expression in approximately 1,500 cells per well. Three parameters were measured and calculated, 1) percentage of GFP positive cells, 2) average GFP intensity in GFP-positive cells, and 3) GFP intensity per well. Autofocus and cell number were determined in the Hoechst channel, and the FITC filter was used to detect GFP. An equal fixed exposure time was automatically set for all the samples (16).

Analysis of the screen data
Median Z-score values were used to standardize the measurements of each parameter from 4 independent experiments. Z-score values below −1.96 and above 1.96 were considered as statistically significant, with \( p < 0.05 \). To measure the extent of variation from the control, the ratios versus the siScramble-transfected cells were calculated for each parameter. Averages of the ratios were used to create a heat map using the conditional formatting tool from Microsoft Excel 2016.

siRNA-mediated silencing and infection. Individual or pooled siRNAs targeting BNIP3 (ID: LU-004636-00), ATG7 (ID: J-020112-08-0002) or NIX (ID: L-011815-00-0005), or non-targeting siRNA (siScramble, ID: D-001810-10) were obtained from Dharmacon (Horizon™). Reverse and forward transfection of U2OS and ATG7KO U2OS cells was performed using Lipofectamine RNAiMAX (Invitrogen) with a final siRNA concentration of 20 nM according to manufacturer’s protocol in either Nunc™ Lab-Tek™ II Chambered Coverglass or 24 and 6 well plates. At 48 hpt, cells were infected with CHIKV or SFV at the indicated MOI.

Flow cytometry, mitochondrial mass measurement and cell death assays.
Cells infected with CHIKV were harvested at the indicated time points by detachment with 0.5% trypsin-EDTA (Gibco). Thereafter, the cells were fixed with 4% PFA and suspended in FACS buffer (PBS, 5% FBS, 1% EDTA). To detect infected cells, GFP or viral E2 surface protein expression was assessed by flow cytometry. Anti-E2 stem antibody (rabbit polyclonal) was provided by G. Pijlman (Wageningen University, The Netherlands). Secondary staining was performed using Alexa647-conjugated goat anti-rabbit antibody (Life Technologies).
To evaluate mitochondrial mass, U2OS cells were incubated for 20-25 min with MitoSpy™-Green FM or MitoSpy™-Red CMXRos (Biolegend), diluted in plain cell culture medium prior to collection of the cells for flow cytometry.

Cell death experiments were carried out using a staining protocol based on the eBioscience Annexin-V apoptosis detection kit PE and Fixable Viability Dye eFluor™ 780 (Invitrogen), following manufacturer’s recommendations. Cells treated with menadione at a final concentration of 100 µM were included as a control for the experiment. In all experiments, 10,000 to 30,000 events were acquired per condition in a BD FACSVersor or a BD-LSR-II instrument (BD Biosciences) and analyzed using the Kaluza Analysis software, Version 2.1 (Beckman Coulter).

**Western blot.** Cellular proteins were extracted using the RIPA buffer Lysis System (Santa Cruz). The total protein content was determined using the Bradford Ultra reagent (Expedeon). Proteins were denatured by heating at 95°C for 5 min and separated by SDS-PAGE. Afterwards, the proteins were transferred onto PVDF membranes (Immobilon-P, Millipore). Blocking was done at room temperature with either 5% bovine serum albumin (BSA, Roche), 5% milk or Odyssey buffer (1:1) diluted in TBST buffer (0.02M Tris-base, 0.15 M NaCl, 0.1 % Tween 20, pH 7.4) for 1 h prior to antibody incubation. Membranes were incubated with specific primary antibodies in blocking buffer from 16 to 24 h at 4ºC. Anti-LC3 (rabbit monoclonal, Novus biologicals), anti-BNIP3 (mouse polyclonal, ANa40, Santa Cruz), anti-GAPDH (mouse monoclonal, 6C5, Abcam), anti-tubulin (mouse monoclonal, Sigma), or anti-ATG7 (rabbit, 2631, Cell Signaling Technology), anti-RFP (mouse monoclonal, 6G6, Chromotech), anti-vinculin (mouse monoclonal, hVIN1, Sigma-Aldrich) and anti-nsP2 (mouse monoclonal, ABM3F3.2E10, Abgenex) antibodies were used. Rabbit polyclonal antibodies against CHIKV E1, Capsid and the aforementioned E2 stem, were kindly provided by G. Pijlman (Wageningen University, The Netherlands). Membranes were subsequently probed for 2 h at room temperature with the following secondary antibodies conjugated to either HRP or Alexa-680: donkey anti-rabbit HRP (Alpha Diagnostic International), goat anti-mouse HRP (Sigma, A8924), goat anti-rabbit Alexa-680 or goat anti-mouse Alexa-680. Detection of HRP-conjugated antibodies was carried out using Pierce™ ECL Western Blotting Substrate (Thermo Scientific) or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific), and images were taken in an ImageQuant LAS 4000 series. Membranes probed with fluorescent antibodies were imaged using the Odyssey Imaging System (LI-COR Biosciences). Protein signal intensities were quantified using either the ImageJ or the ImageQuant TL 8.1 software.

**mCherry-BNIP3 overexpression.** An expression vector encoding for BNIP3 fused to mCherry (pmCherry C1- BNIP3) was kindly provided by I. Novak (University of Split, Croatia). An empty pcDNA-mCherry vector was used as negative control. U2OS cells were transfected in either 6 or 24 well plates with using 2 µg or 0.5 µg of DNA, and 4 µl or 1 µl of Lipofectamine 3000 (Invitrogen), respectively, following the recommendations of the manufacturer. At 6 hpt, the medium was removed and the cells were transfected with either the siScramble or the siRNA targeting BNIP3 for 16 h as described above (see 4). A shorter time-point was chosen for analysis as extensive
cell death was observed in the control condition (due to overexpression of BNIP3) at latter time-points.

**Quantification of intracellular viral RNA.** Viral RNA was isolated from total cell lysates using the RNeasy Mini Kit (QIAGEN), following manufacturer’s recommendations. Absolute viral RNA copies were quantified by RT-qPCR. For this purpose, total RNA was reverse transcribed using Omniscript RT kit (QIAGEN), and DNA was amplified with HotStarTaq DNA Polymerase (QIAGEN) using sets of primers targeting either the E1 or the nsP1 genes in independent PCR reactions, as described previously (24,25).

**Immunofluorescence.** Transfected and infected cells seeded beforehand on glass coverslips were fixed with 4% PFA and permeabilized with 0.1% Triton X100 in PBS. Next, cells were blocked with 2% BSA and stained for CHIKV infection by using the anti-E2 stem antibody. In all cases, cells were mounted with ProLong Gold Antifade mountant with DAPI (Invitrogen). Images were acquired in a DeltaVision™ microscope (GE Healthcare, Life Sciences) and analyzed using the Fiji-ImageJ software (NIH) (26).

**Microscopic membrane fusion assay.** U2OS cells were reverse-transfected, as described above, and seeded in Nunc™ Lab-Tek™ II Chambered Coverglass (Thermo Scientific) at a density of 9,000 cells per well. Fusion assay was performed at 48 hpt, using purified CHIKV labeled with the lipophilic fluorescent probe 1,1'-Diocadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD', Invitrogen), as previously described (7). Prior to infection, cells were washed three times with serum-free, phenol red-free MEM (Gibco) and kept in phenol red-free MEM with 1% of glucose. DiD-labeled CHIKV was added and cells were incubated for 20 min at 37°C in order to allow virus cell entry and membrane fusion. As a control, diethylpyrocarbonate (DEPC; Sigma-Aldrich) treated DiD-labeled CHIKV was used, as described before (7). At 20 min, unbound virus was removed by washing the cells three times with serum-free, phenol red-free MEM. Cells were kept in serum-free, phenol red-free MEM containing 1% glucose during analysis (Leica Biosystems 6000B microscope). A total of 15 random snapshots (55-70 cells in total) were taken per condition, and images were examined by eye for fusion-positive cells.

**Statistical analysis.** All data were analyzed using the GraphPad Prism software version 5. Data are presented as means ± SEM, unless stated otherwise. Statistical differences were determined using the two-tailed student’s t-test or the dependent t-test. A p value ≥ 0.05 was considered as statistically significant.

3. **Results**

**An ATG proteome specific siRNA screen identifies BNIP3 as a modulator of CHIKV infection**

In order to assess the role of the ATG proteome in CHIKV infection and replication, we performed an unbiased image- and siRNA-based screen in CHIKV-infected U2OS cells.
U2OS cells are derived from a human bone osteosarcoma and have been successfully used for studying multiple aspects of the CHIKV replication cycle (27–29). For the screen, we used a customized siRNA library consisting of 50 siRNAs targeting different individual ATG genes and autophagy receptors (Table S1). Some of these siRNA probes were additionally assorted, such that their combinations silenced functionally redundant proteins (Table S1). The siRNAs were reverse-transfected in U2OS cells, a cellular system that has been validated and reported by us previously (19). To confirm our previous results, we measured the knockdown efficiency of 4 randomly selected siRNAs, i.e., ATG4B, ATG4C, GAB1, GAB2, by analysing the mRNA levels of their targets by RT-qPCR (Fig. S1A). As expected, siRNA transfection led to a clear reduction (>73.4%) in the levels of the targeted transcripts, when compared to siScramble-transfected cells. For the screen, we employed a GFP-reporter CHIKV (22) to allow read-out on the basis of GFP expression. Images were acquired using an automated microscope and the GFP signal was analysed to determine 1) the number of infected cells (GFP-positive cells), 2) the level of replication in infected cells (GFP intensity in infected cells) and 3) the GFP intensity per cell averaged by wells (GFP intensity). The ideal infection conditions to perform the screen were set at MOI 10 and at 17 hpi to both ensure a high signal to noise ratio, and be able to monitor inhibition as well as enhancement of virus infection. Using this set-up, the percentage of GFP-infected cells in the four independent screen repeats was 26.5±2.9 in cells transfected with the siScramble, demonstrating that we could indeed measure inhibition as well as enhancement of infection under knockdown conditions. Furthermore, data analysis of the screen results revealed that there is no cytotoxicity as the cell numbers in the transfected wells were comparable to those observed in the control cells (Fig. S1B). To analyse the effect of each siRNA, the data of each replica was normalized against the control (i.e., cells transfected with siScramble) and the Z-score values were computed for each parameter to determine the statistical significance (Fig. 1A and 1B). The siRNA targeting ATP6V1A, a subunit of the lysosomal ATPase, was used as a positive control for the screen. ATP6V1A is responsible for endosome acidification and silencing of this protein is expected to prevent CHIKV infection (30). Indeed, ATP6V1A depletion was found to significantly decrease CHIKV replication (Fig. 1A-1B). The analysis of the screen results revealed that most ATG proteins have a (mildly) restricting role in CHIKV replication (Fig 1A). A significant effect was detected for 10 ATG proteins, that is, their Z-score value was below -1.96 or above 1.96 (Fig. 1A and Fig. 1B). Depletion of UVRAG significantly inhibited CHIKV infection (Fig. 1A-B) whereas depletion of 9 different proteins significantly enhanced infection. These were ULK1, ULK4, ATG4C, ATG7, LC3B, GABARAPL2, ATG9B, NBR1 and BNIP3. BNIP3 had the most pronounced effect with a 1.9 and 2.4-fold increase in the percentage of infected cells and GFP intensity per well, respectively, compared to the siScramble control (Fig. 1A-B).
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Figure 1. siRNA-based screen to determine the relevance of ATG proteins and autophagy receptors in CHIKV-infected U2OS cells. (A) Heat-map showing the ratio of GFP-positive cells (%), the average GFP intensity per well, and the average GFP intensity in infected cells, for each knockdown as compared to cells transfected with a siRNA scramble (bottom). The significant hits (p < 0.05 in at least one of the parameters) are indicated with a star. Data shown represents the mean of four independent experiments. (B) Representative distribution of the Z-score values as compared to the siScramble based on the percentage of GFP-positive cells. Z-scores between -1.96 and +1.96 (dotted lines) represent significant hits with p <0.05. BNIP3 is indicated in blue. (C) Bar plot showing the fold change (FC) in infection relative to non-transfected (NT) cells of the respective MOI. U2OS cells were reverse-transfected with a siRNA pool targeting BNIP3 or a siRNA control (siScramble) for 48 h. Subsequently, cells were infected with the 5'GFP-CHIKV-LR strain at the indicated MOIs. Cells were collected 10 hpi, fixed and infection (i.e., GFP-positive cells) was determined by flow cytometry. (D) Bar plot showing the FC in genome equivalent copies per ml (GECs/ml) at 10 hpi relative to NT cells. Quantification was done by RT-qPCR. (E) Bar plot showing the FC in infectious virus particles per ml (PFU/ml) at 10 hpi as compared to NT cells. Analysis was done by plaque assay. (F) Bar plot showing the FC in specific infectivity (GECs/ml divided by the PFU/ml) compared to NT cells. Data represents mean ± SEM of at least three independent experiments. Student’s test: *** p<0.001, ** p<0.01, * p<0.05, no symbol implies non-statistically significant.
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Given the potent antiviral effect of BNIP3, we decided to elucidate the role of this protein in CHIKV infection. To this end, we determined the mRNA levels of BNIP3 by RT-qPCR in siScramble and siBNIP3-transfected cells. This quantification shows that the siRNA targeting BNIP3 reduced its mRNA levels by 95% (Fig. S2A). Thereafter, we attempted to confirm the knockdown of protein expression by western blot using a commercial antibody, but we were unable to detect endogenous BNIP3 expression, an issue which is commonly encountered for this protein (31,32). As an alternative approach, we used a mCherry-tagged BNIP3 expression plasmid and evaluated mCherry levels as a measure of BNIP3 expression by flow cytometry and western blot. To this end, cells were transfected with the plasmid encoding for mCherry-tagged BNIP3. At 6 hpt, cells were transfected with either the siScramble or the siRNA against BNIP3 and incubated for another 16 h prior to analysis. As an additional control, cells were transfected with an expression plasmid solely encoding mCherry. We observed that transfection with the siRNA targeting BNIP3 led to a strong reduction in both the number of cells expressing the mCherry-fused BNIP3 protein (Fig. S2B) and the mCherry-BNIP3 expression levels (Fig. S2C), demonstrating that BNIP3 is efficiently downregulated by the siRNA used in this study.

In the screen, the role of ATG proteins on CHIKV infection was assayed at 17 hpi, which represents two rounds of replication. To investigate whether BNIP3 controls CHIKV infectivity in the first round of replication, we next assessed the number of infected cells at an earlier time-point, i.e., at 10 hpi using MOI 1 and MOI 10. Similar to 17 h of infection, knockdown of BNIP3 expression increased the number of infected cells by 2.0±0.4-fold and 1.9±0.5-fold following infection at MOI 1 and 10, respectively (Fig. 1C) as compared to the siScramble-transfected cells. This indicates that BNIP3 controls CHIKV infection within the first round of replication. Moreover, these results reveal that our transfection procedure has no effect on the overall infectivity of the virus, since no differences in infection were observed between the non-transfected and the cells transfected with siScramble (Fig. 1C).

Next, we examined whether the higher number of infected cells is associated with a higher production of viral particles. Hereto, we analysed the number of secreted genome equivalent copies (GEC) per ml of supernatant of BNIP3-depleted U2OS cells at 10 hpi following CHIKV infection at MOI 1 and 10 and compared to siScramble-transfected cells. Importantly, Fig. 1D shows that GEC/ml secretion is 2.6±0.2 and 2.6±0.5-fold increased under conditions of BNIP3 silencing at MOI 1 and 10, respectively. In parallel, to investigate whether BNIP3 interferes with the infectious properties of secreted virus particles we also evaluated the presence of infectious virus particles by plaque assay. Under conditions of BNIP3 knockdown, a 2.2±0.3-fold increase in the secretion of infectious virus particles was seen at MOI 1 and 3.8±1.0-fold increase following infection at MOI 10 when compared to siScramble-transfected cells (Fig. 1E). Subsequent calculation of the GEC to PFU ratio revealed no differences (Fig. 1F), indicating that depletion of BNIP3 increases the overall production of progeny virus particles without interfering with the infectious properties of released particles. The increase in the secretion of virus progeny was still present at 16 hpi, as a 3.4±1.0- and 2.8±1.4-fold increase was observed in BNIP3-depleted cells when compared to siScramble-transfected cells (Fig. S2D). Taken together, these data show that BNIP3 is a novel host factor that controls CHIKV infection in U2OS cells.
BNIP3 controls CHIKV infection through an autophagy-independent mechanism

Before investigating the role of BNIP3 in CHIKV infection, we first assessed whether an autophagic response is elicited in CHIKV-infected U2OS cells as our screen indicated that autophagy may restrict CHIKV infection (Fig. 1). Hereto, the levels of lipidated LC3-II/LC3-I (autophagy flux index) by western blot analysis in the absence and presence of the lysosomal inhibitor bafilomycin A$_1$ were evaluated. In these experiments, we used starvation-induced autophagy as a positive control. In the absence of bafilomycin A$_1$, no differences in in the autophagic flux indexes were observed during CHIKV infection from 5 to 10 hpi when compared to mock-treated cells (Fig. S3A-B), which suggests that autophagy is not induced upon CHIKV infection in U2OS cells. A decrease in the autophagic flux index, however, was observed at 16 hpi, which suggests that the autophagic flux is inhibited late in infection (Fig. S3A-B). As expected, nutrient deprivation led to an increase in the autophagic flux index in absence and presence of bafilomycin A$_1$ (Fig. S3A-B). To confirm that autophagy is not induced upon CHIKV infection, we also evaluated the effect of CHIKV on WIPI2 puncta formation in U2OS cells stably expressing GFP-WIPI2. WIPI2 puncta are formed once an autophagic response is triggered (34). After 5 h, in the absence of infection, the percentage of cells that showed GFP-WIPI2 puncta (cells with >1 GFP-WIPI2 puncta) was 3.4±1.4 and 13.4±4.4 in the absence and the presence of bafilomycin A$_1$, respectively (Fig. S3C-D). At 10 h, 4.6±3.1 and 9.3±4.2% GFP-WIPI2 cells were found in the absence and the presence of bafilomycin A$_1$. Furthermore, at 16 h, the percentages of cells with GFP-WIPI2 puncta were 1.0±1 and 6.0±0.5 in the absence and the presence of bafilomycin A$_1$, respectively. Importantly, no significant differences were seen in the number of cells with GFP-WIPI2 puncta in the presence of CHIKV any evaluated time-point (Fig. S3C-D). In contrast, and as expected, nutrient deprivation triggered autophagy, in the 10 h time-point 81.5±6.0 and 89.4±8.3% of the cells showed WIPI2-GFP puncta in the absence and presence of bafilomycin A$_1$, respectively. Similar results were obtained at the other two time-points (Fig. S3C-D). These results confirmed the LC3 western blot results and show that CHIKV infection does not provoke a major autophagic response in U2OS cells.

Thus far, BNIP3 appears to be exclusively involved in mitophagy and not in autophagy (17). Yet, to verify this notion, we next evaluated the effect of BNIP3 depletion on basal and starvation-induced autophagy in U2OS cells in the absence and presence of bafilomycin A$_1$. Western blot analysis revealed that there were no significant differences between siScramble-transfected cells treated with bafilomycin A$_1$ and siBNIP3-depleted cells treated with bafilomycin A$_1$ (Fig. 2A). This suggests that the autophagy flux is comparable in both conditions (Fig. 2A). When autophagy was induced by nutrient deprivation, however, BNIP3 depletion resulted in elevated autophagic flux in comparison with the siScramble-transfected cells (Fig. 2B). These observations indicate that although basal autophagy is not altered by BNIP3, nutrient depletion-induced autophagic flux is influenced by BNIP3 expression. Subsequently, we assessed whether BNIP3 depletion somehow affects the autophagic flux during CHIKV infection. Fig. 2C shows that there are no differences in autophagic flux indexes between siScramble and BNIP3-depleted cells infected for 10 h with CHIKV at MOI 10, which
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suggests that the antiviral function of BNIP3 during CHIKV infection does not involve an alteration of the autophagic flux. To further confirm that BNIP3 controls CHIKV infectivity independently of autophagy, we next investigated the effect of ATG7 silencing, a key regulator of autophagy (35), on CHIKV infection in combination or not with BNIP3 knockdown. siRNA-mediated depletion of ATG7 (Fig. 2D) enhanced CHIKV infection (Fig. 2E), which confirms our screen results (Fig. 1). Importantly, combined depletion of ATG7 and BNIP3 further increased CHIKV infectivity when compared to ATG7-depleted cells (Fig. 2E). As an alternative approach, we also used CRISPR/Cas9-generated ATG7 knockout U2OS cells (ATG7KO) (20), and silenced BNIP3. In line with the above results, the number of CHIKV infected ATG7KO cells transfected with siRNA against BNIP3 (29.2±3.5%) was increased compared to siScramble-transfected ATG7KO cells (19.2±3.7%) (Fig. 2F). Taken together, these results show that BNIP3 depletion has a positive effect on CHIKV infectivity, also when autophagy is inhibited, which indicates that BNIP3 does not rely on an intact ATG machinery to control CHIKV infection.

**BNIP3 depletion does not affect the overall mitochondrial mass and activity**

Given that BNIP3 has been described to participate in mitochondrial clearance and in other regulatory mechanisms related to mitochondrial dynamics (17,36,37), we next investigated whether BNIP3 depletion influences the overall mitochondrial mass during CHIKV infection. To this end, two distinct mitochondrial probes, i.e., MitoSpy Green and MitoSpy Red CMXRos, were used to assess changes in total mitochondrial mass and mass of polarized mitochondria, respectively. Starvation-induced increase in mitochondrial size as a consequence of enhanced mitochondrial fission (38) was used as a positive control for the assay (Fig. S4A). No changes in the cellular mitochondrial content were seen upon CHIKV infection (Fig. 3A-B). Furthermore, similar results were obtained in cells depleted of BNIP3, suggesting that this protein is not controlling mitochondrial homeostasis over the course of CHIKV infection.

Previous studies have showed that NIX/BNIP3L shares 55% sequence-similarity with BNIP3 (39) and functions redundantly with this protein in mitophagy (17). To strengthen the observation that BNIP3 controls CHIKV infection independently from its role in mitophagy, we also investigated whether NIX controls CHIKV infection. Downregulation of NIX did not have an effect on CHIKV replication (Fig. 3C), which is in line with the screen results (Fig. 1). In addition, simultaneous knockdown of NIX and BNIP3 did not further enhance CHIKV replication in comparison to cells exclusively depleted of BNIP3 (Fig. 3C). Altogether, these results underline that BNIP3 controls CHIKV independently of its function in autophagy.
BNIP3 does not control CHIKV infection through its pro-apoptotic function

Next to its role in mitophagy, BNIP3 has also been associated with apoptosis. Specifically, it has been described that BNIP3 can reduce the mitochondrial membrane potential and increase the generation of reactive oxygen species thereby inducing BAX/BAK-dependent apoptosis and possibly other types of cell death (40). CHIKV infection is known to induce apoptosis (41). Thus, we next explored whether BNIP3 silencing influences virus-induced cell death during infection. We hypothesized that if BNIP3 is involved in cell death signalling during CHIKV infection, BNIP3 depletion may diminish or delay cell death upon infection. We evaluated cell death by flow cytometry...
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using a protocol based on Annexin V and fixable viability dye (FVD) staining (Fig. 4A). Three distinct cell populations can be discriminated using this protocol. The first is the Annexin V-positive cells, which constitute cells that expose phosphatidylserine residues in the outer leaflet of the plasma membrane as a consequence of stress or early activation of apoptosis (41). The second cell population comprises cells positive for both Annexin V and FVD, which are considered apoptotic cells (41). The third population, i.e., FVD-positive cells, are considered necrotic cells or cells undergoing other cellular death programmes (41). As control for our experimental readout, U2OS cells were treated with 100 µM menadione, a compound known to induce apoptosis and other types of cell death (42,43). In presence of menadione, 35% of the total cell population showed signs of cell death (percentage of FVD-positive cells) (Fig. S4B). In agreement with the literature (44,45), we observed an MOI- and time-dependent increase in CHIKV-induced cell death as shown by the increased percentage of FVD-positive cells over time (Fig. 4B). Infection of siScramble-transfected cells with CHIKV at an MOI of 1 led to 12.0±6.5% FVD-positive cells at 10 hpi, which increased to 23.9±3.2% at 16 hpi. Following infection at an MOI of 10, 16.1±8.4% and 38.6±7.5% of FVD-positive cells were observed at 10 and 16 hpi, respectively. Furthermore, apoptotic cells were predominantly detected at early time points post-infection at an MOI of 1 and 10 (Fig. 4B). Lastly, Annexin V-positive cells were almost exclusively detected at the early stages of infection (Fig. 4B, 10 hpi). Importantly, BNIP3-depleted CHIKV-infected cells had similar percentages of Annexin V-, FVD- and double positive cells as in the control cells at 10 and 16 hpi, and at both high and low MOIs (Fig. 4B). Consistently, no differences were observed in the proportions of Annexin V-, FVD- and double positive cells between cells depleted for BNIP3 and the siScramble-transfected cells at the time of cell collection (Fig. S4C). Collectively, these results suggest that BNIP3 regulation of CHIKV replication is not associated to mechanisms related to cell death, therefore, it is likely that BNIP3 modulates the virus early in infection, before these pathways are activated.

**CHIKV replication is modulated by BNIP3 early in infection**

Since no function of BNIP3 in regulating virus infection has been described so far, we next decided to precisely determine at which stage of the viral cycle BNIP3 modulates CHIKV infection. Therefore, we tested the influence of BNIP3 knockdown on CHIKV cell entry and membrane hemifusion by live cell imaging using DiD-labeled CHIKV, as described in Materials and Methods. Hemifusion refers to a short lived state in which the outer membrane leaflets have fused but the inner membrane leaflets are still intact, thus referring to a state prior to fusion pore formation and RNA release (46). In the assay, non-fusogenic DEPC-inactivated CHIKV was used as a control. DEPC treatment inactivates the virus, yet, it preserves the conformational integrity of its surface proteins required for cell surface attachment and endocytosis (47). As expected, the results show that the percentage of fusion-positive cells reduced from 36±9% to 1±1% in presence of DEPC-inactivated CHIKV (Fig. 5A). BNIP3, however, does not interfere with virus cell entry and membrane hemifusion as no clear differences in the number of fusion-positive cells were observed between siScramble- and siBNIP3-treated cells (Fig. 5A). This suggests that BNIP3 regulates CHIKV infection at a step after virus cell entry and membrane hemifusion.
Upon RNA delivery into the cell cytoplasm, the CHIKV genome is translated to produce non-structural proteins (nsP1-4) after which the RNA is replicated and a subgenomic RNA is translated to produce the structural C, E2, E1 and E3 proteins. To establish whether BNIP3 interferes with translation of the CHIKV proteins, we next assessed the expression levels of the viral proteins by western blot (Fig. 5B-C) at 10 hpi. BNIP3 silencing was found to increase the total expression of structural proteins E2, E1, and C as well as the expression levels of the non-structural protein nsP2 (Fig. 5B-C). Collectively, these data clearly indicate that BNIP3 depletion results in higher expression levels of viral proteins in CHIKV-infected cells.

Figure 3. BNIP3 silencing does not alter the mitochondrial mass during CHIKV infection. U2OS cells were reverse-transfected as described in the legend to Figure 1C. (A, B) Bar plots showing the FC in mitochondrial mass compared to mock-infected cells as assessed by flow cytometry. Cells were infected with CHIKV-LR at the indicated MOIs for 10 h. (C) Bar plots showing the FC in infection compared to siScramble transfected cells as assessed by flow cytometry. U2OS cells reverse-transfected for 48 h with either siRNAs against NIX, BNIP3, or both, and infected with 5’GFP-CHIKV-LR at MOI 10 for 10 h. Data represents mean ± SEM of at least three independent experiments. NT denotes for non-transfected and siScram for siScramble. Student’s test: *** p < 0.001, no symbol implies non-statically significant.
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To delineate the step of CHIKV replication cycle controlled by BINP3, we next evaluated the effect of BNIP3 depletion on intracellular viral RNA levels. The number of viral RNA copies was determined by RT-qPCR using primer sets targeting nsP1 and E1. The nsP1 primer set only amplifies the genomic RNA whereas the E1 primer set amplifies both the genomic and the subgenomic RNA (24,25). Due to the higher detection sensitivity of RT-qPCR compared to western blot, we analysed RNA levels at 4 and 8 hpi to also assess early events in infection. As expected, the total RNA copy number per
ml significantly increased over time (Fig. S5). At 4 hpi, on average $3.2 \times 10^6$ RNA copies of E1 transcripts and $6.2 \times 10^5$ RNA copies of nsP1 transcripts were detected in cells infected at MOI 1 (Fig. S5). By 8 hpi, the RNA copy numbers increased to on average of $7.0 \times 10^5$ and $1.7 \times 10^8$ for E1 and nsP1 transcripts, respectively, following infection at MOI 1. At MOI 10, the RNA copy numbers were even further enhanced (Fig. S5).

Interestingly, at 4 hpi, the number of viral RNA copies of the E1 and nsP1 transcripts as compared to the cells transfected with the siScramble was significantly higher in BNIP3-depleted cells exposed to MOI 1 and 10. At 8 hpi, the fold change in viral intracellular RNA levels in BNIP3-depleted cells was less prominent although significant in the case of the E1 transcripts (Fig. 5D). Furthermore, no clear differences were observed between the fold enhancement of nsP1 and E1 RNA levels at all conditions tested, suggesting that BNIP3 does not specifically interfere with the replication of either the genomic or the subgenomic RNA. The high RNA copy numbers in BNIP3-depleted CHIKV-infected cells early in infection suggests that BNIP3 interferes with successful delivery of the viral RNA to the site of replication or interferes with the initial formation of the replication complex.

**BNIP3 modulates alphavirus replication**

To address whether BNIP3 also controls the infectivity of other CHIKV genotypes, we next examined the effect of BNIP3 depletion on the infectivity of the originally isolated African (S27) strain in 1953 and the recently isolated Caribbean CHIKV (99659) strain that belongs to the Asian genotype. The CHIKV-LR used in this study belongs to the East/Central/South African genotype. We assessed both the number of infected cells by flow cytometry (Fig. 6A) and infectious viral particle produced following infection at an MOI of 10. At 10 hpi, 19.7±4.2% and 9.7±4.0% of cells were infected with CHIKV-S27 and CHIKV-99659, respectively. For the ancient CHIKV-S27 strain, we observed a non-significant positive trend with a 1.4±0.2- and 2.0±0.4-fold increase in the percentage of infected cells and infectious virus particle production, respectively (Fig. 6B). For the circulating CHIKV-99659 strain, BNIP3 silencing resulted in a significant increase in both the percentage of infected cells (2.5±0.2-fold) as well as the secretion of infectious virus progeny (2.3±0.4-fold) (Fig. 6B). The above data indicates that the function of BNIP3 in controlling CHIKV replication is strain-dependent, and conserved across CHIKV-99659 and CHIKV-LR, two currently circulating CHIKV strains of distinct genotypes. Furthermore, given that SFV is part of the same antigenic complex as CHIKV, we also tested the effect of BNIP3 towards this virus. At 10 hpi, 23.8±9.6% of the cells were infected with SFV following infection at MOI 1. Similar as with CHIKV-S27, BNIP3 silencing increased, albeit not significantly, the percentage of infected cells by 1.4±0.2-fold (Fig. 6B). This translated into a significant 1.9±0.4-fold enhancement in virus progeny release as compared to the siScramble-transfected cells (Fig. 6B). This shows that the antiviral role of BNIP3 in CHIKV infection can be translated to other alphaviruses from the same antigenic complex.
An autophagy-specific siRNA screen reveals BNIP3 as a regulator of chikungunya virus infection

Figure 5. BNIP3 controls CHIKV replication early in infection. U2OS cells were reverse-transfected as in Figure 1C. (A) Cells were incubated with DiD-labeled CHIKV and the number of fusion-positive cells was measured. (B) Representative blots and (C) bar plot showing the FC in nsp2, E2, E1 and C protein expression levels in BNIP3-depleted cells compared to siScramble-transfected cells. GAPDH and vinculin were used as loading controls. (D) Bar plot showing the FC in intracellular E1 and nsp1 RNA levels in BINP3-knockdown cells compared to siScramble-transfected cells. Cells infected at the indicated MOI and time-points. NT denotes for non-transfected, siScram for siScramble. Data represents the mean ± SEM of at least three independent experiments. Student’s test: *** p < 0.001, ** p < 0.01, * p < 0.05, no symbol implies non-statically significant.

Discussion

This study unveils BNIP3 as a host factor controlling alphavirus replication. The previously unknown role of BNIP3 in controlling CHIKV replication was found to be independent from the function of this protein in mitophagy and cellular death. BNIP3 depletion was found to increase the number of infected cells and the production of infectious progeny virus particles. Analysis of the steps involved in CHIKV replication cycle suggests that BNIP3 interferes with CHIKV early in infection, yet after virus cell entry and hemifusion with the endosomal limiting membrane. The antiviral effect of BNIP3 was found conserved among distinct two distinct circulating CHIKV genotypes and the closely related SFV.
Figure 6. BNIP3 antiviral role is conserved across CHIKV genotypes and SFV. U2OS cells were reverse-transfected as described in Figure 1C. Cells were infected at an MOI of 10 (CHIKV-S27, CHIKV-99659) or at an MOI 1 (SFV-MK) for 10 h. (A) Representative flow cytometry dot plot and (B) bar plots showing the FC in the percentage of infected cells (measuring the E2-positive cells by flow cytometry) and infectious titers (determining the PFU/ml by plaque assay) in BNIP3-depleted cells compared to siScramble-transfected cells at 10 hpi. Data represents the mean ± SEM of at least three independent experiments. Student’s test: ** $p < 0.01$, * $p < 0.05$, no symbol implies non-statically significant.

The observation that the intracellular CHIKV RNA levels are increased in BNIP3-depleted cells at 4 hpi, indicates that BNIP3 acts early in infection. Furthermore, these results suggest that the potency of BNIP3 to interfere with the intracellular RNA levels decreases over time as the fold enhancement is lower at 8 hpi than at 4 hpi under conditions of BNIP3 depletion, whereas the total RNA copy number increased during this time window. Intriguingly, no major effect was detected on virus cell entry and hemifusion. We therefore hypothesize that BNIP3 specifically interferes with CHIKV RNA delivery in the cell cytosol or with replication early in infection. Upon translation of the 5’CHIKV-ORF, the viral genomic RNA and the viral replicase complex are relocated to membrane invaginations at the plasma membrane known as spherules (48). It is tempting to speculate that BNIP3 participates at this stage of the viral infectious cycle, either by interfering with the transport of the viral replicase complex or the formation of spherules. An alternative explanation is that BNIP3 interferes with the formation of a fusion pore, proper dissociation of the nucleocapsid or with the formation of early replication complexes thereby decreasing the chance to initiate a productive infection. The molecular mechanisms involved in these steps of CHIKV cycle are, however, ill-understood and future studies are required to determine the precise mode of viral interference employed by BNIP3 in CHIKV-infected cells.
BNIP3 was found to control CHIKV independently of cell death and autophagy pathways implying that BNIP3 is also involved in other as yet unknown cellular processes. These unknown processes might be unveiled by studying the functional domains of BNIP3. To date, several functional domains have been identified in the amino acid sequence of BNIP3: 1) a PEST domain that targets BNIP3 for degradation, 2) a BH3 domain and 3) a transmembrane domain (TM) (49). The BH3 and TM domains are involved in the induction of apoptosis and mitophagy (50). BNIP3 has also an LC3-interacting motif within the PEST domain, which classifies this protein as an autophagy receptor (51). According to our data, we speculate that the role of the conventional function of the TM domain and the LIR motif of BNIP3 are not required for alphavirus infection, however, future research should confirm this hypothesis. Investigating the effect of different BNIP3 mutants in CHIKV replication could help to elucidate BNIP3 function in more detail. Another strategy to delineate this is to investigate the interactome of BNIP3 in CHIKV-infected cells. In this regard, BNIP3 was previously described to bind viral proteins of 2 different families of RNA viruses in a study using a yeast two-hybrid approach (52). Therefore, it may be relevant to investigate whether one or more CHIKV proteins interact with BNIP3.

BNIP3 was discovered in our study as a factor controlling CHIKV replication on the basis of an siRNA screen targeting 50 distinct ATG proteins and autophagy receptors. We further validated and investigated BNIP3 as it had the most pronounced effect on CHIKV infection. Interestingly, most ATG protein depletions were shown to have a positive effect on viral infection, suggesting that autophagy might have an antiviral function during a CHIKV infection. Subsequent experiments revealed that autophagy is not strongly induced upon U2OS cells exposure to CHIKV, but the knockout of the essential autophagy factor ATG7 clearly enhanced CHIKV infection. Collectively, these observations suggest that basal autophagy may target some of the viral or host factor required for CHIKV infectivity. Interestingly, depletion of UVRAG drastically reduced CHIKV infection and therefore promotes CHIKV infection. Future studies should reveal the true importance and function of these proteins in CHIKV replication.

In summary, our study identifies a BNIP3 as a new host factor regulating CHIKV infectivity, but also that of other alphaviruses. In particular, BNIP3 controls alphavirus infection at an early step in their replication cycle. This regulation is not linked to the known function of BNIP3 in autophagy and cell death. Future studies on how BINP3 controls alphavirus infection will be important because they will shed light on the early steps in infection, which still remain poorly characterized.

Acknowledgments

The authors thank Izabela Rodenhuis-Zybert for all her comments and suggestions regarding this study. F.R. is supported by ZonMW TOP (91217002), ALW Open Programme (ALWOP.310), Marie Skłodowska-Curie Cofund (713660) and Marie Skłodowska Curie ETN (765912) grants. J.S. is also supported by a Skłodowska-Curie Cofund (713660). L.E. is supported by Erasmus Mundus EURICA mobility programme and Graduate School of Medical Sciences PhD scholarship (University Medical Center Groningen).
CHAPTER 4

References

An autophagy-specific siRNA screen reveals BNIP3 as a regulator of chikungunya virus infection


49. Vasagiri N, Kutala VK. Structure, function, and epigenetic regulation of BNIP3: a


An autophagy-specific siRNA screen reveals BNIP3 as a regulator of chikungunya virus infection

**Supplementary data**

**Table S1. Genes targeted by the customized siRNA library**

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Figure S1. siRNA transfections downregulate targeted genes and do not induce cell toxicity. (A) Bar plot showing the mRNA levels quantified by RT-qPCR of two random genes ADP-ribosylation factor 1 (ARF1) and Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1) at 48 hpt with specific siRNAs. Data represents two technical replicates for ARF1 or one replicate for GBF1 included in one experiment. (B) The siRNA-based screen was performed as described in the materials and methods. Plot shows the average number of cells per each gene knockdown in the screen. Data represents the mean ± SEM of four independent experiments.
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Figure S2. Validation of BNIP3 as a factor controlling CHIKV replication. U2OS cells were reverse-transfected with either siScramble or siRNA targeting BNIP3. (A) Bar plot showing the FC in BNIP3 mRNA levels compared to the siScramble control. Quantification was done by RT-qPCR at 48 hpt. (B) Flow cytometry dot plot showing the percentage of mCherry-positive cells. (C) Bar plot showing the FC in mCherry expression by western blot in U2OS cells transiently expressing mCherry-BNIP3 or just mCherry, and reverse-transfected with the indicated siRNAs for 16 h. (D) Bar plot showing the FC in infectious virus particles per ml (PFU/ml) compared to NT control of cells transfected and then infected with 5’GFP-CHIKV-LR at MOI 1 and 10 for 16 h. Evaluation was done by plaque assay. Data shown A, B, C and E represents the mean ± SEM of at least three independent experiments. Student’s test: *** p < 0.001, ** p < 0.01, * p < 0.05, no symbol implies non-statically significant.
An autophagy-specific siRNA screen reveals BNIP3 as a regulator of chikungunya virus infection

**Figure S3. CHIKV fails to induce autophagy in U2OS cells.** (A) Representative LC3 western blots and (B) determination of the autophagic flux index (LC3-II/LC3-I ratio) in protein lysates from U2OS cells infected with CHIKV-LR at MOI 10 and at the indicated time-points (C) Representative pictures and (D) quantification of GFP-WIPI2 puncta in U2OS cells stably expressing GFP-WIPI2 that were infected with CHIKV-LR at MOI 10. Cells were analysed by immunofluorescence at the indicated time-points. Baf, Bafilomycin A1. Data shown represent mean ± SEM of at least three independent experiments. Student’s test: *** p < 0.001, ** p < 0.01, no symbol implies non-statically significant.

**Figure S4. Controls for the assays determining mitochondrial mass and cell death by flow cytometry.** (A) Representative flow cytometry histogram of U2OS cells treated with HBSS (starvation) for 10 h and stained using MitoSpy Green. (B) Representative flow cytometry dot plot of U2OS cells treated with menadione at a concentration of 100 µM for 10h and stained using Annexin V and FVD. (C) Bar plot of the frequencies of Annexin V-, FVD- and double-positive cells measured by flow cytometry. Cells reverse transfected with the indicated siRNAs for 48 h and mock-treated for 16 h. Data shown represent mean ± SEM of at least three independent experiments. Student’s test: no symbol implies non-statically significant.
**Figure S5. CHIKV RNA levels increase in time.** U2OS cells were reverse-transfected with siScramble for 48 h and infected with 5’GFP-CHIKV-LR. Bar plot showing the FC in intracellular E1 and nsP1 RNA levels in cells transfected at the indicated MOIs and time-points. Data shown represent mean ± SEM of three independent experiments. Student’s test (data from the 8 hpi time-point were compared to their corresponding sample at 4 hpi): *** p < 0.001, ** p < 0.01, * p < 0.05, no symbol implies non-statically significant.
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