scientific report

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

Delphine Judith^{1,7}, Serge Mostowy^{2,8,†}, Mehdi Bourai³, Nicolas Gangneux^{1,7}, Mickaël Lelek^{4,9}, Marianne Lucas-Hourani³, Nadège Cayet⁵, Yves Jacob⁶, Marie-Christine Prévost⁵, Philippe Pierre^{10,11}, Frédéric Tangy³, Christophe Zimmer^{4,9}, Pierre-Olivier Vidalain³, Thérèse Couderc^{1,7+} & Marc Lecuit^{1,7,12,13++}

¹Institut Pasteur, Biology of Infection Unit, ²Institut Pasteur, Bacteria-Cell Interactions Unit, ³Institut Pasteur, Viral Genomic and Vaccination Unit, ⁴Institut Pasteur, Computational and Imaging and Modeling Group, ⁵Institut Pasteur, Ultrastructural Microscopy Platform, ⁶Institut Pasteur, Genetic, Papillomavirus and Human Cancer Unit, ⁷Inserm U1117, Institut Pasteur, Biology of Infection Unit, ⁸Inserm U604, INRA USC2020, Institut Pasteur, Bacteria-Cell Interactions Unit, ⁹CNRS UMR2582, Institut Pasteur, Computational and Imaging and Modeling Group, Paris, France, ¹⁰Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, ¹¹Inserm U631, CNRS UMR6102, Centre d'Immunologie de Marseille-Luminy, Marseille, ¹²Paris Descartes University, Sorbonne Paris Cité, Institut Imagine, and ¹³Necker-Enfants Malades University Hospital, APHP, Division of Infectious Diseases and Tropical Medicine, Paris, France

Chikungunya virus (CHIKV) is a recently re-emerged arbovirus that triggers autophagy. Here, we show that CHIKV interacts with components of the autophagy machinery during its replication cycle, inducing a cytoprotective effect. The autophagy receptor p62 protects cells from death by binding ubiquitinated capsid and targeting it to autophagolysosomes. By contrast, the human autophagy receptor NDP52—but not its mouse orthologue—interacts with the non-structural protein nsP2, thereby promoting viral replication. These results highlight the distinct roles of p62

and NDP52 in viral infection, and identify NDP52 as a cellular factor that accounts for CHIKV species specificity.

Keywords: autophagy; Chikungunya virus; p62; NDP52; species specificity

EMBO reports (2013) 14, 534–544. doi:10.1038/embo.2013.51

INTRODUCTION

Chikungunya virus (CHIKV) is a re-emerged arbovirus belonging to the *Alphavirus* genus that causes febrile arthralgia in humans [1]. Alphaviruses are enveloped viruses with a positive-strand RNA genome, encoding non-structural (nsP1 to nsP4) and structural proteins, the capsid, 3 envelope glycoproteins (E1, E2 and E3) and 6k peptide. Alphaviruses replication in the cytoplasm of vertebrate cells is cytolitic [2]. During infection, nsPs associate with viral RNA to form replicative complexes (RC), and allow replication through a double-stranded (ds)RNA replicative intermediate. RCs are associated with membranous structures called cytopathic vacuoles located in the perinuclear area [3]. RCs of Sindbis (SINV) and Semliki forest (SFV) viruses, two alphaviruses, harbor endo- or lysosomal compartment markers [4,5]. They also use the plasma membrane as a site of replication [6,7]. Alphavirus infection is associated with cell shutoff and apoptosis. nsP2, an essential and multifunctional component of RCs, serves as a trigger for cell shutoff and induction of apoptosis in SINV- and CHIKV-infected cells [8,9]. These functions are related to nsP2 nuclear location and assigned to its carboxy-terminal domain [9–11].

Autophagy is a cellular catabolic process, which sequesters cytosolic components within double-membrane vesicles and targets them for degradation in lysosomes [12]. While autophagy was initially thought to be non-selective, evidence suggests a selective

¹Institut Pasteur, Biology of Infection Unit,

²Institut Pasteur, Bacteria-Cell Interactions Unit,

³Institut Pasteur, Viral Genomic and Vaccination Unit,

⁴Institut Pasteur, Computational and Imaging and Modeling Group,

⁵Institut Pasteur, Ultrastructural Microscopy Platform,

⁶Institut Pasteur, Genetic, Papillomavirus and Human Cancer Unit,

⁷Inserm U1117, Institut Pasteur, Biology of Infection Unit,

⁸Inserm U604, INRA USC2020, Institut Pasteur, Bacteria-Cell Interactions Unit,

⁹CNRS UMR2582, Institut Pasteur, Computational and Imaging and Modeling Group, 25 rue du Docteur Roux, 75014 Paris, France

¹⁰Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Parc Scientifique de Luminy, 13288 Marseille Cedex 9, France

¹¹Inserm U631, CNRS UMR6102, Centre d'Immunologie de Marseille-Luminy, Parc Scientifique de Luminy, 13288 Marseille Cedex 9, France

¹²Paris Descartes University, Sorbonne Paris Cité, Institut Imagine, 12 rue de l'école de Médecine, 75006 Paris, France

¹³Necker-Enfants Malades University Hospital, APHP, Division of Infectious Diseases and Tropical Medicine, 149 rue de Sèvres, 75015 Paris, France

[†]Present address: Section of Microbiology, MRC Centre for Molecular Microbiology and Infection, Imperial College London, Armstrong Road, London SW7 2AZ, UK

⁺Corresponding author. Tel: +33 1 45 68 87 62; Fax: +33 1 40 61 34 21;

E-mail: therese.couderc@pasteur.fr

⁺⁺Corresponding author. Tel: +33 1 40 61 30 29; Fax: +33 1 40 61 34 21;

E-mail: marc.lecuit@pasteur.fr

Fig 1 | Autophagy components promote CHIKV infection and control virus-induced cell death in HeLa cells. Cells were mock infected or infected and immunoblotted for actin, p62 (A) or LC3 (B). Cells were infected for 15 h and labeled using antibodies to p62 and capsid (C). Cells were transfected with GFP-LC3-B, infected for 15 h and labeled with anti-capsid antibody (D). Mice were infected for 3 days, then whole-cell lysates of muscle were immunoblotted for LC3 or actin (E) and muscle sections were labeled with p62 and capsid antibodies (F). Cells were treated with DMSO (CTRL) or rapamycin and then infected. Viral replication (G) and production (H) were assessed and whole-cell lysates were immunoblotted for capsid or actin (I). Cells were treated with CTRL, Beclin1 (left) or Atg7 (right) siRNA, then infected for 24 h. Cell mortality (that is, fold change relative to mock-infected cells) (J), viral replication (K) and production (L) were assessed and whole-cell lysates of siRNA-treated cells were immunoblotted for Beclin1, Atg7, capsid and actin (M). Images and graphs shown are representative of at least three independent experiments and data presented in graphs correspond to mean + s.d. ($n=3$). Scale bar, 10 μ m. CHIKV, Chikungunya virus; CTRL, control; GFP, green fluorescent protein; DMSO, dimethylsulphoxide; siRNA, short interfering RNA.

Fig 2 | Autophagy receptors p62 and NDP52 localize to distinct pools of capsid and have distinct effects on CHIKV infection in HeLa cells. Cells were infected for 15 h and labeled using antibodies to NDP52 and capsid (A), to p62, NDP52 and capsid (B), to NDP52, LC3-C or capsid (C), to p62, ubiquitin (FK2) and capsid (D), or to NDP52, ubiquitin (FK2), and capsid (E). Cells were treated with CTRL or p62 siRNA, then infected for 24 h. Cell mortality (that is, fold change relative to mock-infected cells) (F), viral replication (left) and qRT-PCR (right) (G), and viral production (H) were assessed and whole-cell lysates were immunoblotted for p62, capsid or actin (I). Cells were treated with CTRL or NDP52 siRNA, then infected for 24 h. Cell mortality (that is, fold change relative to mock-infected cells) (J), viral replication (left) and qRT-PCR (right) (K) and production were assessed (L). Whole-cell lysates of siRNA-treated cells were immunoblotted for NDP52, capsid or actin (M). Images and graphs shown are representative of at least three independent experiments and data presented in graphs correspond to mean $\pm$ s.d. ($n = 3$). Scale bar, 10 μ m. C, capsid; CHIKV, Chikungunya virus; CTRL, control; NDP52, nuclear dot protein 52 kDa; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siRNA, short interfering RNA.

autophagic degradation of cytosolic material, including intracellular pathogens [13]. By simultaneously binding to ubiquitin and LC3/GABARAP proteins, autophagy receptors such as p62 (SQSTM1) and NDP52 (nuclear dot protein 52 kDa) can mediate docking of ubiquitinated targets to autophagosomes [14–16]. While recent studies suggest that p62 and NDP52 might mediate antibacterial autophagy through different pathways [16–19], their respective contributions to selective autophagy remains unclear.

Selective autophagy is a well-recognized innate immune response to infection [20–22]. Autophagy might exert anti- or pro-viral roles and its impact on alphaviruses has been investigated [23–26]. Autophagy has been reported to limit the pathogenesis of CHIKV-infected mouse cells [27]. Yet, in human cultured cells, CHIKV triggers an autophagic response that promotes viral replication [28]. The molecular mechanisms underlying these species-specific differences are not known, and the role of autophagy on CHIKV remains unclear. Here we have uncovered distinct but complementary roles for the autophagy receptors p62 and NDP52 in the context of viral infection, and provide molecular evidence for the species specificity of CHIKV.

RESULTS AND DISCUSSION

Autophagy promotes CHIKV infection in human cells

We first investigated whether CHIKV induces autophagy in HeLa cells. A decrease of p62 protein and an increased conversion of LC3-I to LC3-II, both indicative of autophagy induction, were observed in infected cells (Figs 1A,B). Puncta of p62 clustered around the cytosolic capsid (Fig 1C) and capsid colocalized partially with GFP-LC3-B (Fig 1D). Stochastic optical reconstruction microscopy provided a high-resolution image of p62 association with capsid (supplementary Fig S1A online). Ultrastructural analysis revealed double-membrane vesicles, containing and surrounded by nucleocapsids, immunolabelled for capsid and p62 (supplementary Figs S1B,C online). Moreover, CHIKV induced autophagy in a mouse model for CHIKV (Figs 1E,F) [29].

Autophagy induction with rapamycin in infected human cells significantly increased CHIKV replication (15 h: 1.6 ± 0.2 -fold, $P \leq 0.005$; 24 h: 1.6 ± 0.1 -fold, $P \leq 0.01$) and production (15 h: 2.4 ± 0.3 -fold, $P \leq 0.05$; 24 h: 2.9 ± 0.3 -fold, $P \leq 0.05$), as well as the level of capsid (15 h: 1.6 ± 0.06 -fold, $P \leq 0.001$; 24 h: 1.5 ± 0.11 -fold, $P \leq 0.01$) (Figs 1G–I). No significant difference in cell viability between treated and untreated cells was observed (supplementary Fig S1D online). In contrast, treatment with wortmannin, a well-characterized inhibitor of PI-3 kinase activity and autophagy, restricted infection (supplementary Figs S1E,F

online). Depletion of canonical mediators of autophagy, Beclin1 (Atg6) and Atg7, significantly increased virus-induced cell death (Beclin1: 1.7 ± 0.3 -fold, $P \leq 0.05$; Atg7: 1.8 ± 0.1 -fold, $P \leq 0.001$) (Fig 1J) and decreased CHIKV replication (Beclin1: 1.3 ± 0.2 -fold, $P \leq 0.05$; Atg7: 1.8 ± 0.04 -fold, $P \leq 0.001$), production (Beclin1: 1.7 ± 0.2 -fold, $P \leq 0.05$; Atg7: 2.4 ± 0.4 -fold, $P \leq 0.05$) (Figs 1K,L), and level of capsid (Beclin1: 1.5 ± 0.1 -fold, $P \leq 0.001$; Atg7: 3.1 ± 1.1 -fold, $P \leq 0.01$) (Fig 1M). Altogether, these data suggest a cytoprotective effect and an overall proviral role of autophagy in CHIKV-infected human cells.

Distinct effects of p62 and NDP52 on CHIKV infection

As NDP52 is thought to function similarly to p62, we investigated its subcellular location in infected HeLa cells. NDP52 was observed in close proximity to a fraction of capsid (Fig 2A; supplementary Figs S2A,B online) in association with the TGN46-positive perinuclear region identified as the *trans*-Golgi network (TGN) (supplementary Fig S2C online). Strikingly, NDP52 formed clusters with capsid in cytoplasmic areas where p62 was absent (Fig 2B; supplementary Fig S2D online). Moreover, NDP52-capsid clusters did not colocalize with GFP-LC3-B (supplementary Fig S2E online), but clustered with LC3-C (Fig 2C). E2 also localized near NDP52 in the perinuclear region (supplementary Fig S2F online) but neither with GFP-LC3-B nor with p62 (supplementary Figs S3A,B online). As p62 and NDP52 are both ubiquitin-binding proteins, we investigated their location relative to capsid and ubiquitin. Co-localization of p62 with capsid was detected in $71\% \pm 2.0\%$ of infected cells, in which p62 puncta were observed, and in $98\% \pm 0.8\%$ of these cells, ubiquitin partially colocalized with capsid co-clustered with p62 (Fig 2D; supplementary Fig S3C online). Similar results were obtained in primary human labial fibroblasts (HLFs) (supplementary Fig S3D online). In contrast to p62 (Fig 2D), NDP52 did not localize with ubiquitinated capsid (Fig 2E).

We next investigated the respective roles of p62 and NDP52 during CHIKV infection. As with depletion of canonical autophagy proteins Beclin1 and Atg7, p62 depletion significantly increased virus-induced cell death (1.7 ± 0.1 -fold, $P \leq 0.01$) (Fig 2F). In contrast to canonical autophagy proteins, p62 depletion significantly increased CHIKV replication (GFP: 1.3 ± 0.05 -fold, $P \leq 0.01$; quantitative reverse transcription polymerase chain reaction (qRT-PCR): 1.9 ± 0.4 -fold, $P \leq 0.01$), production (1.5 ± 0.1 -fold, $P \leq 0.01$) (Figs 2G,H) and capsid level (1.5 ± 0.08 -fold, $P \leq 0.001$) (Fig 2I). Similar results were obtained in HLFs (supplementary Figs S3E,F online). Depletion of NDP52 significantly increased virus-induced cell death (1.7 ± 0.2 -fold, $P \leq 0.01$) (Fig 2J). However, in contrast to p62 depletion, NDP52

Fig 3 | p62 associates with ubiquitinated capsid and controls cell death induced by cytotoxic capsid in HeLa cells. Cells were infected for 24 h and immunoprecipitation experiments were performed using antibodies to FK2, capsid or nonspecific IgG controls. Immunoprecipitated proteins were revealed using antibodies to FK2 or capsid (A,B). Cells were transfected with Ub-HA and capsid-3XFLAG. Immunoprecipitation experiments were performed 24 h p.i. using antibodies to FLAG or nonspecific IgG controls. Immunoprecipitated proteins were revealed with anti-HA antibody (C). Immunoprecipitation experiments were performed using antibodies to p62 or nonspecific IgG controls. Immunoprecipitated proteins were revealed using antibodies to p62, FK2 or capsid (D,E). Cells were infected for 3 h and then transfected with empty plasmid (CTRL) or p62_{WT}-3XFLAG or p62_{deltaUBA}-3XFLAG. Immunoprecipitation experiments were performed 24 h p.i. with anti-FLAG antibody. Immunoprecipitated proteins were revealed using antibodies to FLAG or capsid (F). Cells were treated with CTRL or p62 siRNA, then infected for 24 h. Immunoprecipitation experiments were performed using antibodies to FK2 or nonspecific IgG controls. Immunoprecipitated proteins were revealed using anti-capsid antibody (G). Cells were infected for 15 h and labeled with antibodies to p62, LAMP-1 or capsid (H). Cells were infected for 9, 15 or 24 h and treated with DMSO (CTRL) or bafilomycin 3 h before the end of experiment. Whole-cell lysates were immunoblotted for capsid or actin (I). HeLa cells were transfected with empty plasmid (CTRL), control protein (cherry-3XFLAG) or capsid-3XFLAG at fixed (750 ng) or at the indicated concentrations. Cell mortality (that is, % of dead cells) was measured 24 h post transfection (J,K). Cells were treated with CTRL or p62 siRNA and transfected with NT or capsid-3XFLAG. Cell mortality (that is, fold change relative to mock-infected cells) was measured 48 h post transfection (L). Cells were infected with CHIKV for 3 h and transfected with empty plasmid (CTRL), p62_{WT}-3XFLAG, p62_{deltaUBA}-3XFLAG or p62_{deltaLIR}-3XFLAG. Cell mortality (that is, fold change relative to mock-infected cells) was measured 24 h post transfection (M). Images and graphs shown are representative of at least three independent experiments and data presented in graphs correspond to mean + s.d. ($n = 3$). Scale bar, 10 μ m. CHIKV, Chikungunya virus; CTRL, control; IP, immunoprecipitation; NT, empty plasmid; p.i., post infection; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siRNA, short interfering RNA; Ub, ubiquitin; WB, western blot.

depletion significantly decreased CHIKV replication (GFP: 1.4 ± 0.2 -fold, $P \leq 0.05$; qRT-PCR: 2.3 ± 0.1 -fold, $P \leq 0.001$), production (1.9 ± 0.1 -fold, $P \leq 0.01$) (Figs 2K,L) and capsid level (2.1 ± 0.6 -fold, $P \leq 0.01$) (Fig 2M). Similar results were obtained in HLFs (supplementary Figs S3G,H online). Together, these results show that p62 and NDP52 protect cells from virus-induced death, but that they differentially affect CHIKV infection in human cells.

p62 targets capsid to autophagolysosomes

p62 localized with ubiquitinated capsid in infected cells (Fig 2D). In addition, capsid and ubiquitin were reciprocally co-immunoprecipitated from lysates of infected cells (Figs 3A,B) and from capsid and ubiquitin co-transfected cells (Fig 3C). p62 co-immunoprecipitated with capsid (Fig 3D), which was recognized by an anti-ubiquitin antibody (Fig 3E). p62 immunoprecipitation of capsid depends on its ubiquitination, as p62_{deltaUBA} mutant (which lacks the p62 ubiquitin binding region) did not immunoprecipitate capsid, in contrast to p62_{WT} (Fig 3F). Moreover, the proportion of ubiquitinated capsid increased upon depletion of p62 but not upon depletion of SMURF1, an ubiquitin ligase implicated in alphaviral infection (Fig 3G; supplementary Fig S4 online) [30]. p62 and capsid were associated in LAMP-1-positive compartments (Fig 3H) and capsid level increased in bafilomycin-treated infected cells, confirming that it is degraded by autophagy (Fig 3I).

To elucidate the mechanism of virus-induced cell death upon p62 depletion, we investigated the cytotoxicity of capsid. Capsid was cytotoxic (Fig 3J), in a dose-dependent manner (Fig 3K) and its cytotoxicity is increased in p62-depleted cells (Fig 3L). Moreover, infected cells overexpressing p62_{WT} displayed lower virus-induced cell death (1.6 ± 0.1 -fold, $P \leq 0.01$), while overexpression of a p62 variant, either p62_{deltaUBA} or p62_{deltaLIR} (p62 lacking the LC3 binding region), displayed higher virus-induced cell mortality (p62_{deltaUBA}: 1.6 ± 0.1 -fold, $P \leq 0.01$; p62_{deltaLIR}: 1.5 ± 0.1 -fold, $P \leq 0.01$) (Fig 3M).

Taken together, these results suggest that p62 targets ubiquitinated capsid for autophagic degradation and that the cytoprotective effect of p62 is linked to its ability to bind ubiquitinated capsid. It has been reported that p62 targets SIN

capsid in an ubiquitin-independent and SMURF1-dependent manner [24,30]. These observations highlight differences between CHIKV and SINV, and are consistent with the ability of p62 to target both ubiquitinated and non-ubiquitinated substrates to autophagic degradation [31].

Human NDP52 binds nsP2

We next investigated the role of NDP52 in infected human cells. We have identified NDP52 as an interactor of nsP2 by yeast two-hybrid screen [9]. We show here that NDP52 co-immunoprecipitates with nsP2 in infected cells (supplementary Fig S5A online). By using the yeast two-hybrid system, we found that the coiled-coil domain of NDP52 and the C-terminal domain of nsP2 are both required for NDP52–nsP2 interaction (Fig 4A; supplementary Fig S5B online). Moreover, NDP52 colocalized partially with nsP2 at the perinuclear region (Fig 4B), in the vicinity of the areas enriched in capsid (supplementary Fig S5C online). Co-localization of NDP52 with nsP2 was detected in $68.9\% \pm 1.1\%$ of infected cells (supplementary Fig S5D online). Similar results were obtained in HLFs (supplementary Fig S5E online).

Previous studies have shown that the cytoplasmic fraction of nsP2 is an essential component of RCs [32]. We assessed whether NDP52 and nsP2 localized with other components of RCs, such as nsP3 and dsRNA. nsP2 localized with nsP3 in a TGN46-positive perinuclear region, as does NDP52 (Fig 4C). NDP52 clustered with dsRNA in a perinuclear area enriched in capsid (supplementary Fig S5F online), where protein translation was detected (supplementary Fig S5G online). TGN-associated RCs (labeled by nsP2), in the vicinity of *de novo* protein synthesis (labeled by puromycin) where NDP52 concentrates, were detected in $68.9\% \pm 2.9\%$ of cells. Strikingly, depletion of NDP52 resulted in the disappearance of nsP2 and puromycin co-labeling, and TGN-associated RCs in the vicinity of protein synthesis were only detected in $30.0\% \pm 1.9\%$ of cells ($P \leq 0.001$) (Fig 4D). These data indicate that NDP52 localizes in the vicinity of TGN-associated RCs, which contain nsPs and dsRNA, near sites of protein translation, and suggest that NDP52 promotes CHIKV replication by its interaction with nsP2. RNA viruses target their proteins to specific cellular compartments to anchor their RCs and

Fig 4 | Human NDP52 binds nsP2, localizes near CHIKV RCs, and restricts cell shutoff promoting cell survival in HeLa cells. Yeast cells expressing Gal4 DNA BD fused alone (empty vector) or to nsP2 were cotransformed with a plasmid encoding the Gal4 AD fused to NDP52 or the indicated NDP52 deletion mutants (top). Yeast cells expressing BD fused to nsP2 or the indicated nsP2 deletion mutants were cotransformed with a plasmid encoding the Gal4 AD fused alone (empty vector) or to NDP52 (bottom) (A). Cells were infected for 15 h, then labeled using antibodies to NDP52 and nsP2 (B), or to TGN46, nsP2 and nsP3 (C). Cells were treated with control (CTRL) (left) or NDP52 (right) siRNA, infected for 15 h, then labeled using antibodies to NDP52, nsP2 and puromycin. Quantitative analysis was performed by counting the percentage of infected cells with TGN-associated RCs ($n = 30$ cells per experiment) (D). Cells were transfected with empty plasmid (CTRL) or nsP2_{wt}-3XFLAG or nsP2_{R606A}-3XFLAG. Cell mortality (that is, % of dead cells) was measured 24 h post transfection (E). Cells were treated with control (CTRL) or NDP52 siRNA, then mock infected (M) or infected (C) for 24 h. Cytoplasmic and nuclear fractions were isolated and immunoblotted for nsP2 and NDP52. The amounts of the cytoplasmic and nuclear fractions of nsP2 were quantified by densitometry and expressed as the ratio of nuclear to cytoplasmic fraction of nsP2 (F). Cells were treated with control (CTRL) or NDP52 siRNA, infected for 15 and 24 h and treated with puromycin. Whole-cell lysates of siRNA-treated cells were immunoblotted for NDP52, capsid or actin. Puromycin incorporation into newly synthesized protein was revealed using antibodies to puromycin (G). Cells were treated with CTRL or NDP52 siRNA, then transfected with NT or nsP2-3XFLAG. Cell mortality (that is, fold change relative to mock-infected cells) was measured 48 h post transfection (H). Images and graphs shown are representative of at least three independent experiments and data presented in graphs correspond to mean $\pm$ s.d. ($n = 3$). Scale bar, 10 μ m. AD, transactivation domain; BD, binding domain; CHIKV, Chikungunya virus; CTRL, control; NDP52, nuclear dot protein 52 kDa; nsP, nonstructural protein; NT, empty plasmid; RC, replicative complexes; siRNA, short interfering RNA; TGN, *trans*-Golgi network.

establish assembly sites. RCs are targeted to these subcellular membranes by nsPs [33]. Although the precise mechanism by which NDP52 promotes CHIKV RNA replication remains to be fully understood, our results suggest that nsP2, via NDP52 that is itself linked to LC3-C, allows the anchorage of RCs to the TGN membrane.

The nuclear fraction of nsP2 mediates cell shutoff and death [8]. When expressed in HeLa cells, nsP2 was cytotoxic (3.7 ± 0.2 -fold, $P \leq 0.001$) (Fig 4E). This cytotoxicity depended on its role in cell shutoff, as expression of a mutated nsP2 that fails to induce shutoff (nsP2_{R606A}) [9] did not increase mortality (Fig 4E). Strikingly, depletion of NDP52 resulted in increased amount of nuclear nsP2 (Fig 4D) and an increased ratio of nuclear/cytosolic nsP2 (Fig 4F). NDP52 depletion led to a reduction in the total amount of puromycin-labeled proteins either upon nsP2 transfection (supplementary Fig S5H online) or upon CHIKV infection (Fig 4G), suggesting that NDP52-nsP2 interaction limits cell shutoff induced by nsP2. Moreover, nsP2-mediated cell mortality in NDP52-depleted cells was increased (Fig 4H) and expression of NDP52 in nsP2-transfected cells showed a dose-dependent decrease of the toxicity of ectopically expressed nsP2 (supplementary Fig S5I online), indicating that nsP2-induced cell death is modulated by NDP52. Together, these results indicate that NDP52-nsP2 interaction in the cytoplasm restricts the amount of nuclear nsP2 and correlates with a reduced cell shutoff that limits nsP2-induced cell death.

NDP52-nsP2 interaction is species specific

Autophagy plays an antiviral role in CHIKV-infected mouse cells [27] and a proviral role in CHIKV-infected human cells [28]. To better understand these apparently conflicting results, we investigated the role of mouse NDP52 (mNDP52). In contrast to what was observed in human cells, we observed no differences in virus-induced cell death and viral production in murine embryonic fibroblast (MEF) cells depleted for NDP52 versus control MEFs (Figs 5A,B). Sequence alignment revealed that mNDP52 exhibits 31% homology with human NDP52 (hNDP52) and lacks part of the corresponding hNDP52 C-terminal region (supplementary Fig S6A online). No interaction between nsP2 and mNDP52 was observed by yeast two-hybrid assay (Fig 5C).

To further investigate the effect of mNDP52 and hNDP52 on CHIKV infection, hNDP52 was expressed in mouse cells. hNDP52 co-immunoprecipitated with nsP2 in mouse cells (Fig 5D), yet its expression did not increase viral production (Fig 5E). It has recently been shown that hNDP52 binds LC3-C (supplementary Fig S6B online) [34] that is expressed in human but not in mouse cells, in contrast to LC3-A and LC3-B. Co-expression of hNDP52 and human LC3-C (hLC3-C) in mouse cells led to a strong increase in viral production (6.1 ± 0.25 -fold, $P \leq 0.001$), as compared to control cells or cells expressing either hNDP52 or hLC3-C (Fig 5E; supplementary Fig S6C online), without affecting mortality (Fig 5F). Consistent with this latter observation, nsP2 did not modulate host protein synthesis in MEFs (supplementary Fig S6D), and therefore did not affect cell survival, as it did in human cells.

Consistent with an absence of a proviral role of the autophagy machinery in mouse cells, we observed an increase in virus-induced cell death (2.5 ± 0.1 -fold, $P \leq 0.01$) (Fig 5G) and viral production (18.4 ± 3.1 -fold, $P \leq 0.01$) (Fig 5H) in *atg5*^{-/-} MEFs, as compared to wt MEFs. As in human cells (Fig 2), mouse p62 immunoprecipitated capsid (Fig 5I) and depletion of p62 in mouse cells significantly increased virus-induced cell death (1.6 ± 0.03 -fold, $P \leq 0.01$) and viral production (5.5 ± 0.2 -fold, $P \leq 0.01$) (Figs 5J,K). Together, these results show that, in contrast to human cells, autophagy is antiviral in mouse cells and indicates that CHIKV species specificity is linked, at least in part, to the ability of NDP52 to bind both nsP2 and LC3-C, leading to a proviral effect. Recently, it has been shown that mNDP52 targets *Mycobacterium tuberculosis* to autophagic degradation, showing that mNDP52 might act as a canonical autophagy receptor in mouse cells [35]. These data indicate that the ability of hNDP52 to bind both hLC3-C and nsP2 is implicated in its species-specific proviral effect on CHIKV. These results reconcile the two apparently conflicting reports, which showed that autophagy plays an antiviral role in mouse cells [27] but a proviral role in human cells [28]. Moreover, these results suggest that the lower permissiveness to CHIKV of the mouse relative to humans [29] might result, at least in part, from the absence of the proviral role of NDP52 in the mouse.

Finally, we investigated whether the ability of nsP2 to bind hNDP52 is common to Old World alphaviruses. We showed by

Fig 5 | NDP52 promotes CHIKV infection in a species-specific manner. MEF cells were treated with CTRL or NDP52 siRNA, then infected for 24 h. Cell mortality (that is, fold change relative to mock-infected cells) (A) and viral production (B) were measured. Yeast cells expressing Gal4 DNA BD fused alone (empty vector) or to nsP2 were cotransformed with a plasmid encoding the Gal4 AD fused to hNDP52 or mouse NDP52 (C). MEF cells were infected for 3 h and transfected with empty plasmid (CTRL) or hNDP52-3XFLAG. Immunoprecipitation experiments were performed 24 h p.i. using antibodies to FLAG or nonspecific IgG controls. Immunoprecipitated proteins were revealed with anti-nsP2 antibody (D). MEF cells were infected for 3 h and transfected with empty plasmid (CTRL), hNDP52, hLC3-C or hNDP52 and hLC3-C. Viral production (E) and cell mortality (F) were assessed 24 h post transfection. Isogenic wt or Atg5^{-/-} MEFs were infected for 24 h and cell mortality (that is, fold change relative to mock-infected cells) (G) and viral production (H) were assessed. MEF cells were infected and immunoprecipitation experiments were performed 24 h p.i. using antibodies to p62 or nonspecific IgG controls. Immunoprecipitated proteins were revealed with anti-capsid antibody (I). MEF cells were treated with CTRL or p62 siRNA, then infected for 24 h. Cell mortality (that is, fold change relative to mock-infected cells) (J) and viral production were assessed. Whole-cell lysates of siRNA-treated cells were immunoblotted for p62 or actin (K). Images and graphs shown are representative of at least three independent experiments and data presented in graphs correspond to mean ± s.d. (n = 3). Scale bar, 10 μm. AD, transactivation domain; BD, binding domain; CHIKV, Chikungunya virus; CTRL, control; hLC3-C, human LC3-C; hNDP52, human NDP52; IP, immunoprecipitation; MEF, mouse embryonic fibroblast; mNDP52, mouse NDP52; NDP52, nuclear dot protein 52 kDa; nsP, nonstructural protein; p.i., post infection; siRNA, short interfering RNA; WB, western blot.

yeast two-hybrid assay that hNDP52 binds nsP2 of SINV and SFV (supplementary Fig S6E online), indicating that the proviral role of hNDP52 likely applies to Old World alphaviruses and suggesting a putative general involvement of hNDP52-LC3-C in alphaviral replication.

CONCLUSION

Here we provide insight into the mechanisms by which autophagy receptors target CHIKV. We have shown that the autophagy machinery globally prevents CHIKV-induced cell death and that p62 and NDP52 play anti- and pro-viral roles in human cells, respectively (supplementary Fig S7 online). We also reveal a novel mechanism by which NDP52 promotes viral infection in a species-specific manner. The absence of NDP52–nsP2 interaction in mouse cells might account for the lower permissiveness of mice to CHIKV relative to humans [29]. These results shed light on the cell biology of CHIKV, pave the way for humanized hNDP52/LC3-C mouse model to study CHIKV infection and for the development of antiviral strategies against alphaviruses.

METHODS

Infection. HeLa cells were infected at a multiplicity of infection (MOI) of 5, which results in a synchronized infection of 100% of cells during 15 or 24 h (supplementary Fig S8 online). All other cell lines were infected at the same MOI as HeLa cells.

CHIKV replication and production. CHIKV replication was determined by measuring the GFP mean intensity per live cell in mock-infected and infected cells, and by real-time qRT-PCR. GFP mean intensity was analyzed by fluorescence-activated cell sorting (FACS) with a FACSCalibur instrument (BD Biosciences) and the results were analysed with Cell Quest Pro software (Cell Quest). CHIKV production was determined by titration of the supernatant by tissue cytopathic infectious dose 50 (TCID₅₀) on Vero cells. Viral titers are expressed as TCID₅₀ per cell [29].

Cell viability assays. Viability of cells was determined using the Scepter handled automated Cell Counter (Ref no. PHCC00000 from Millipore) with 60 μm Scepter tips (Ref no. PHCC60050 from Millipore).

Ethics statement. This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals, as provided by the French Ministry of Agriculture and of the European Union.

Statistical analysis. Student's *t*-tests were performed for all experiments. The level of significance is shown in each figure (**P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We are grateful to S. Higgs for providing CHIKV cDNA clones and to N. Mizushima for providing Atg5^{-/-} and Atg5^{+/+} MEFs. We thank V. Sancho-Shimizu, C. Prehault, M. Hamon, L. Radoshevich, P. Desprès, A. Merits, V. Meas-Yedid Hardy, E. Meurs and A. Dufour for their generous gift of reagents or technical advices. We also thank members of the Biology of Infection Unit, F. Rey, P. Cossart and P. Codogno, for their helpful discussions and critical reading of the manuscript. This work was supported by Institut Pasteur, FRM, Ville de Paris, Fondation BNP-Paribas, ICRES FP7, LabEx IBEID and the Programme Interdisciplinaire CNRS Maladies Infectieuses Emergentes. D.J. was supported by a doctoral fellowship from the Axa Research Fund and M.B. by a doctoral fellowship from Région Ile-de-France.

Author contributions: D.J., S.M., M.C.P., M.L., P.O.V., T.C. and M. Lecuit planned the experiments; D.J., S.M., M.B., N.G., M.L., M.L.H., N.C., M.C.P., P.O.V., T.C. performed the experiments, D.J., S.M., M.L., Y.J., M.C.P., P.P., F.T., C.Z., P.O.V., T.C. and M. Lecuit analysed the experiments; D.J., S.M., T.C. and M. Lecuit designed the overall research; D.J., S.M., T.C. and M. Lecuit wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Bodenmann P, Genton B (2006) Chikungunya: an epidemic in real time. *Lancet* **368**: 258
2. Kuhn RJ (2007) Togaviridae: The viruses and their replication. *Fields' Virol* pp 1001–1022
3. Grimley PM, Berezesky IK, Friedman RM (1968) Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. *J Virol* **2**: 1326–1338
4. Froshauer S, Kartenbeck J, Helenius A (1988) Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J Cell Biol* **107**: 2075–2086
5. Spuul P, Balistreri G, Kaariainen L, Ahola T (2010) Phosphatidylinositol 3-kinase-, actin-, and microtubule-dependent transport of Semliki Forest Virus replication complexes from the plasma membrane to modified lysosomes. *J Virol* **84**: 7543–7557
6. Kujala P, Ikaheimonen A, Ehsani N, Vihinen H, Auvinen P, Kaariainen L (2001) Biogenesis of the Semliki Forest virus RNA replication complex. *J Virol* **75**: 3873–3884

7. Frolova EI, Gorchakov R, Pereboeva L, Atasheva S, Frolov I (2010) Functional Sindbis virus replicative complexes are formed at the plasma membrane. *J Virol* **84**: 11679–11695
8. Garmashova N, Gorchakov R, Volkova E, Paessler S, Frolova E, Frolov I (2007) The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol* **81**: 2472–2484
9. Bourai M et al (2012) Mapping of Chikungunya virus interactions with host proteins identified nsP2 as a highly connected viral component. *J Virol* **86**: 3121–3134
10. Tamm K, Merits A, Sarand I (2008) Mutations in the nuclear localization signal of nsP2 influencing RNA synthesis, protein expression and cytotoxicity of Semliki Forest virus. *J Gen Virol* **89**: 676–686
11. Garmashova N, Gorchakov R, Frolova E, Frolov I (2006) Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J Virol* **80**: 5686–5696
12. Yang Z, Klionsky DJ (2010) Eaten alive: a history of macroautophagy. *Nat Cell Biol* **12**: 814–822
13. Kraft C, Peter M, Hofmann K (2010) Selective autophagy: ubiquitin-mediated recognition and beyond. *Nat Cell Biol* **12**: 836–841
14. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* **282**: 24131–24145
15. Klionsky DJ et al (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8**: 445–544
16. Thurston TL, Wandel MP, von Muhlinen N, Foeglein A, Randow F (2012) Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature* **482**: 414–418
17. Mostowy S, Sancho-Shimizu V, Hamon MA, Simeone R, Brosch R, Johansen T, Cossart P (2011) p62 and NDP52 proteins target intracytosolic Shigella and Listeria to different autophagy pathways. *J Biol Chem* **286**: 26987–26995
18. Cemma M, Kim PK, Brumell JH (2011) The ubiquitin-binding adaptor proteins p62/SQSTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target *Salmonella* to the autophagy pathway. *Autophagy* **7**: 22–26
19. Mostowy S et al (2010) Entrapment of intracytosolic bacteria by septin cage-like structures. *Cell Host Microbe* **8**: 433–444
20. Mostowy S, Cossart P (2012) Bacterial autophagy: restriction or promotion of bacterial replication? *Trends Cell Biol* **22**: 283–291
21. Levine B, Deretic V (2007) Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol* **7**: 767–777
22. Randow F, Munz C (2012) Autophagy in the regulation of pathogen replication and adaptive immunity. *Trends Immunol* **33**: 475–487
23. Eng KE, Panas MD, Murphy D, Karlsson Hedestam GB, McInerney GM (2012) Accumulation of autophagosomes in Semliki Forest virus-infected cells is dependent on expression of the viral glycoproteins. *J Virol* **86**: 5674–5685
24. Orvedahl A, MacPherson S, Sumpter R Jr., Tallozy Z, Zou Z, Levine B (2010) Autophagy protects against Sindbis virus infection of the central nervous system. *Cell Host Microbe* **7**: 115–127
25. Orvedahl A, Alexander D, Tallozy Z, Sun Q, Wei Y, Zhang W, Burns D, Leib DA, Levine B (2007) HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* **1**: 23–35
26. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, Herman B, Levine B (1998) Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol* **72**: 8586–8596
27. Joubert PE et al (2012) Chikungunya virus-induced autophagy delays caspase-dependent cell death. *J Exp Med* **209**: 1029–1047
28. Krejbich-Trotot P, Gay B, Li-Pat-Yuen G, Hoarau JJ, Jaffar-Bandjee MC, Briant L, Gasque P, Denizot M (2011) Chikungunya triggers an autophagic process which promotes viral replication. *Virol J* **8**: 432
29. Couderc T et al (2008) A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathog* **4**: e29
30. Orvedahl A et al (2011) Image-based genome-wide siRNA screen identifies selective autophagy factors. *Nature* **480**: 113–117
31. Watanabe Y, Tanaka M (2011) p62/SQSTM1 in autophagic clearance of a non-ubiquitinated substrate. *J Cell Sci* **124**: 2692–2701
32. Mai J, Sawicki SG, Sawicki DL (2009) Fate of minus-strand templates and replication complexes produced by a p23-cleavage-defective mutant of Sindbis virus. *J Virol* **83**: 8553–8564
33. Salonen A, Ahola T, Kaariainen L (2005) Viral RNA replication in association with cellular membranes. *Curr Top Microbiol Immunol* **285**: 139–173
34. von Muhlinen N, Akutsu M, Ravenhill BJ, Foeglein A, Bloor S, Rutherford TJ, Freund SM, Komander D, Randow F (2012) LC3C, bound selectively by a noncanonical LIR motif in NDP52, is required for antibacterial autophagy. *Mol Cell* **48**: 329–342
35. Watson RO, Manzanillo PS, Cox JS (2012) Extracellular *M. tuberculosis* DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* **150**: 803–815