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Interplay between autophagy and Sindbis virus in cells derived from key arbovirus vectors, *Aedes albopictus* and *Aedes aegypti* mosquitoes

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ABSTRACT

Aedes albopictus and *Aedes aegypti* are two species of *Aedes* mosquitoes which transmit multiple arboviruses causing serious diseases in human. Intriguingly, infection of arbovirus in both *Aedes* mosquitoes does not cause dramatic pathology, indicating that both mosquitoes have evolved mechanisms to tolerate persistent infection and restrict viral replication to nonpathogenic levels. Therefore, understanding how these mosquitoes interact with viruses would help to find targets for controlling the related mosquito-borne diseases. Autophagy is a conserved cellular recycling process functioning in maintenance of cellular homeostasis and recirculation of cytoplasmic materials under stressful conditions. Autophagy also acts as a cellular defense mechanism against viral infection. It is known that autophagy plays important roles in the replication of several *Aedes* mosquito-borne viruses in mammalian systems. However, little information is available regarding the role of autophagy in replication of those viruses in their primary vector, *Aedes* mosquitoes. This study found that interaction between autophagy and replication of Sindbis virus (SINV) occurred in *Aedes albopictus* C6/36 cells and *Ae. aegypti* Aag2 cells. Moreover, it discovered that the patterns of interaction between autophagy and SINV replication are different in C6/36 cells and Aag2 cells. It was shown that replication of SINV induced complete autophagy in C6/36 cells but suppressed autophagy in Aag2 cells. Moreover, induction of autophagy by rapamycin treatment restricted SINV replication in C6/36 cells but promoted SINV replication in Aag2 cells. Consistent with this, suppression of autophagy by down regulation of Atg8 promoted SINV replication in C6/36 cells but restricted SINV replication in Aag2 cells. It was also found that, in both C6/36 and Aag2 cells, interaction between autophagy and SINV replication occurred after viral entry and prior to viral assembly. Collectively, this work demonstrated that SINV replication manipulated autophagy in *Aedes* mosquito cells and provided strong evidence of the role autophagy played in viral replication in *Aedes* mosquitoes. The findings have laid a foundation to elucidate the correlation between autophagy and arbovirus replication in *Aedes* mosquitoes and could help to understand the difference in viral transmission capacity of the two *Aedes* mosquitoes, *Ae. albopictus* and *Ae. aegypti*.

1. Introduction

As competent vectors, mosquitoes are very permissive to systemic and persistent arbovirus infection. Arboviruses causing infection of hundreds of millions of people each year are generally maintained in a cycle between mosquitoes and vertebrate animals [1,2]. *Aedes albopictus* (*Ae. albopictus*) and *Aedes aegypti* (*Ae. aegypti*) mosquitoes are important vectors of human pathogens which transmit multiple viruses causing serious diseases in human including dengue virus (DENV), chikungunya virus (CHIKV), West Nile virus (WNV) and Zika virus (ZIKV) [1,3–6]. Intriguingly, persistent viral propagation in both *Aedes* mosquitoes does not cause dramatic pathological sequelae, indicating that both mosquitoes have evolved mechanisms to tolerate persistent infection and developed efficient antiviral strategies to restrict viral replication to nonpathogenic levels [7,8]. Therefore, understanding of how these mosquitoes interact with the viruses would help to find targets for the control interventions.

Autophagy is an evolutionarily conserved cellular recycling process removing superfluous proteins and damaged organelles which is critical for the organism to adapt to changing nutrient conditions and to maintain cellular homeostasis [9,10]. Autophagy also acts as a cellular defense mechanism to prevent infection by certain pathogenic viruses

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[11,12]. Many studies have found that multiple viruses exploited autophagy during their replication in mammalian system. It is reported that dengue virus-2 (DENV-2) infection can trigger an autophagic process in Huh7 and mouse MEF cells [13]. Interactions between hepatitis C virus (HCV) and autophagy or autophagy related proteins (ATGs) have been studied in Huh7 and Huh7.5 cells [14,15]. Coxsackievirus B3 (CVB3) infection in HeLa or HEK293A cells has been proved to promote accumulation of autophagosomes which enhanced the efficiency of viral replication in turn [16]. It was reported that classical swine fever virus (CSFV) triggered a complete autophagic response in porcine kidney cell line PK-15 and porcine macrophage cell line 3D4/2, and CSFV needed to trigger a functional autophagy pathway to enhance virus replication and maturity in host cells [17]. Interaction between influenza A virus and autophagy has been studied in A549, MLE-12, MDAMC and HaCat cells. It was found that influenza A virus infection inhibits autophagy via blocking autophagosome fusion with lysosome by viral matrix protein 2, but the inhibition does not influence viral replication [18]. Human parainfluenza virus type 3 (HPIV3) is demonstrated to induce incomplete autophagy in MK2 and HeLa cells by blocking autophagosome-lysosome fusion, resulting in increased virus production [19].

Several arboviruses transmitted by *Ae. albopictus* and *Ae. aegypti* mosquitoes have shown to interact with autophagy in mammalian systems as well. DENV is one important arbovirus transmitted to vertebrate hosts via *Ae. albopictus* or *Ae. aegypti* mosquitoes. It was shown that DENV induced and required autophagy for robust viral replication by altering cellular lipid metabolism [20]. ZIKV is another arbovirus commonly transmitted through the bites of infected *Aedes* mosquitoes [21]. It is reported that ZIKV infection of skin fibroblasts leads to the formation of autophagosomes which associates with enhanced viral replication [22]. A recent report shows that NS4A and NS4B proteins of ZIKV cooperate to upregulate autophagy for viral replication via suppressing host Akt-mTOR signaling in neural stem cells [23]. Interaction between arbovirus WNV and autophagy was also studied, while the role of autophagy in WNV infection remains controversial [24,25]. The published data regarding the role of autophagy in arboviruses infection in mammalian systems highlight the significance of studying interaction between autophagy and arboviruses in mosquito systems. Though induced autophagy in arbovirus infection was observed in mosquito cells, the impact of autophagy on arbovirus infection remains unclear [26].

Sindbis virus (SINV) is a member of the family Alphaviridae and is transmitted between vertebrate animal populations in nature by mosquitoes [27]. This research utilized SINV to study the impact of autophagy on SINV infection in cells derived from *Ae. albopictus* and *Ae. Aegypti* mosquitoes. The paper reported the role of autophagy in viral replication in *Aedes* mosquito cells. The findings in this report have laid a foundation to understand the correlation to understand the correlation between autophagy and viral replication in *Aedes* mosquitoes.

2. Materials and methods

2.1. Cells, viruses and antibodies

C6/36 cells were maintained in minimum essential medium (MEM, HyClone) supplemented with 10% fetal bovine serum (FBS, BI) at 28 °C with 5% CO_2. Aag2 cells were cultured at 28 °C in Schneider's insect medium (Sigma) supplemented with 10% fetal bovine serum (FBS, BI). BHK-21 cells were cultured in Dulbecco's modified Eagle medium (DMEM, HyClone) containing 10% fetal bovine serum (FBS, BI) at 37 °C with 5% CO_2. SINV TE12 strain and recombinant virus SINV-GFP were prepared as previously described [28]. Briefly, capped transcripts of SINV RNA were transcribed using MEGAscript SP6 kits (Applied Biosystems Ambion) and m7G(5′)ppp(5′)G Cap Analog (Applied Biosystems Ambion). Aliquots of each transcript reaction were transfected into BHK-21 cells using Lipofectamine 2000 (Invitrogen). After 3 days, virus-containing medium was harvested, aliquoted and stored at −80 °C. UV inactivated SINV was obtained by irradiating SINV-GFP with 1 J/cm^2 UV light for 10 min at room temperature. The inactivation of SINV-GFP was confirmed by inoculation of monolayer of BHK-21 cells and the inactivated viral particles were expected to be able to enter the cells in this study according to a previous report [29]. Plasmid containing SINV replicon sequence (plasmid-SINV^{rep}-GFP) was constructed by removing SINV coding sequence for structural proteins from the SINV-GFP clone. Capped RNA transcribed from plasmid SINV^{rep}-GFP using MEGAscript SP6 kits (Applied Biosystems Ambion) and m7G(5′)ppp(5′)G Cap Analog (Applied Biosystems Ambion) was used in the study as a SINV replicon (SINV^{rep}-GFP). The products were transfected into C6/36 cells using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Antibodies used in the study included mouse anti-Actin antibody (Proteintech, Rosemont, IL, USA), rabbit anti-Atg8 antibody (an antibody against GABARAP, MBL, Tokyo, Japan), rabbit anti-p62 (MBL, Tokyo, Japan) and mouse anti-GFP antibody (Santa Cruz Biotechnology, Dallas, TX, USA).

2.2. Viral infection and titration

Cells were infected with virus at indicated multiplicity of infection (MOI). After 2 h absorption in serum-free medium, the medium was removed. Then, cells were washed three times with PBS and maintained (under the conditions described above) until harvesting. Cells treated under same condition without virus were used as controls. Viral titration was performed according 50% tissue culture infectious doses ($TCID_{50}$) assay in BHK-21 cells. The $TCID_{50}$ of each sample was converted to PFU/mL by multiplying by 0.69 [30].

2.3. Chemical treatment

For chemical treatment, C6/36 cells were treated with rapamycin (Rapa, MCE) or chloroquine (CQ, Sigma-Aldrich) as described at a final concentration of 500 nM or 300 μM, respectively. Rapa was diluted in Dimethyl Sulfoxide (DMSO, BBI Life Sciences) and CQ was diluted in ddH_2O.

2.4. Generation of siRNA

The siRNA used in this study were synthesized by Sangon Biological Co., Ltd. (Shanghai, China). The sequences are listed in Supplementary Table S1.

2.5. Plasmid and siRNA transfection

Cells were transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, cells were grown in 12-well plates to 70%–80% confluence before transfection. A total of 1 μg plasmid DNA or 200 pmol siRNA in 50 μL of opti-MEM (Hyclone) was mixed with 2 μL of lipofectamine in 50 μL of opti-MEM and the mixture was added to the cells after incubation at room temperature for 30 min. Cells were then incubated at 28 °C for the time period as indicated in the figure legends.

2.6. Immunoblotting analysis

Prepared cell lysates were mixed with 5 × SDS loading buffer, incubated at 100 °C for 5 min, then subjected to SDS-PAGE (8 to 15% polyacrylamide). Proteins were transferred to Immobilon-P membrane (Merck millipore). The membrane was blocked in Tris-buffered saline (TBS) with 5% nonfat dry milk and 0.1% Tween 20 for 1 h and then incubated with primary antibody for 1 h. After washed three times with TBST, the membrane was incubated with corresponding secondary antibodies conjugated to HRP for 1 h. Then, the membrane was washed three times with TBST buffer and bands were visualized by LAS 4000

(Fujifilm) after incubating the membrane with Western Bright Sirius HRP substrate (Advansta). Quantification was performed by using Quantity one software (version 4.6.2).

2.7. Transmission electron microscopy

C6/36 cells infected by SINV for 48 h at a MOI of 2 or treated with CQ for 48 h were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in PBS (1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4, pH 7.4) for 2 h at room temperature. The cells were harvested and fixed with 2.5% glutaraldehyde on ice for 2 h followed by post fixation in 2% osmium tetroxide, and then cells were dehydrated with sequential washes in 50%, 70%, 90%, 95%, and 100% ethanol. After dehydration in a graded ethanol series, cells were embedded in epoxy resin. Next, ultrathin sections were obtained and mounted on mesh copper grids, stained with 2% uranyl acetate in acetone followed by bismuth subnitrate. Then, cell images were taken with a JEM-1400 plus electron microscope operated at 100 kV (Joel Ltd., Tokyo, Japan).

2.8. Confocal fluorescence microscopy

Cells were inoculated onto a circular cover glass in cell culture plate and treated as indicated. Then, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed three times with PBS. Then, Hoechst 33342 (Sigma-Aldrich) was used to counterstain cell nuclei. Cell images were taken with a scanning confocal fluorescence microscope (SP8, Leica, Germany). ImageJ 1.51 k was used as a tool for fluorescent spots counting in cells [31]. Fluorescent spots were counted in ROIs of each C6/36 cell.

2.9. Data analysis

The pictures were edited with Adobe Photoshop CC 2017 (Adobe, San Jose, California, USA). GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) was used for plotting graphs. All data was presented as Means ± SD of triplicate experiments. Group comparisons were performed by *t*-test or one-way ANOVA with the Duncan test with SPSS 19 system software (SPSS Inc. Chicago, IL, USA). A value of *P* less than 0.05 was considered to be significantly different.

3. Results

3.1. SINV infection triggered accumulation of autophagic vacuoles in *Ae. albopictus* C6/36 cells

To investigate whether autophagy plays a role in viral infection in *Ae. albopictus* mosquito, the impact of SINV infection on autophagy in *Ae. albopictus* C6/36 cells was determined first. As preliminary data, the effect of SINV infection on formation of autophagic vacuoles, a characteristic event in autophagy process, was obtained by analyzing the number of GFP-AaAtg8 puncta which can be used to monitor autophagy in C6/36 cells [32]. When infecting C6/36 cells, SINV significantly increased the numbers of GFP-AaAtg8 puncta at 36 h post infection (Fig. 1A), indicating that SINV infection induced accumulation of autophagic vacuoles in *Ae. albopictus* C6/36 cells. To further confirm the above result, samples prepared from mock treated, chloroquine (CQ) treated or SINV infected C6/36 cells were subjected to transmission electron microscopy to directly visualize the autophagic vacuoles in the samples. CQ is a lysosomotrophic agent that inhibits protonation and prevents the acidification of intracellular organelles and can cause accumulation of autophagosomes and early autolysosomes by blocking late stage of autophagy. Indeed, a significant increase of autophagic vacuoles including single membrane autolysosomes and double membrane autophagosomes were observed in CQ treated C6/36 cells, compared to mock treated C6/36 cells (Fig. 1B). Importantly, in SINV-infected C6/36 cells, several similar single-membrane autolysosomes and double-membrane autophagosomes were observed (Fig. 1B). Taken together, the data demonstrated that SINV infection triggered accumulation of autophagic vacuoles in C6/36 cells.

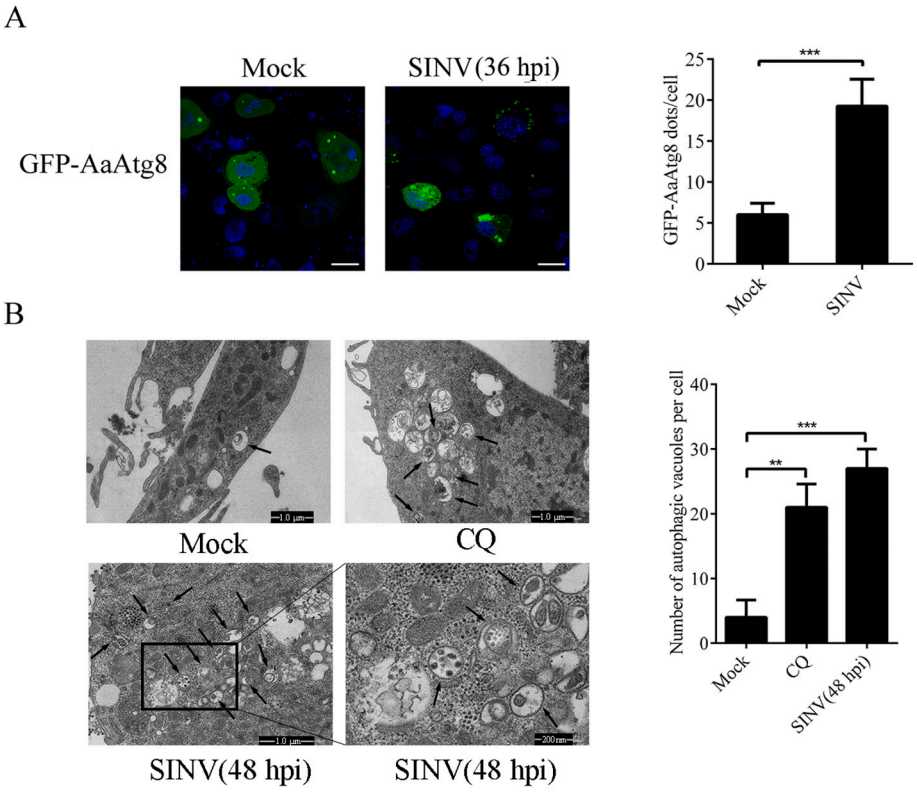


Fig. 1. SINV infection triggered the accumulation of autophagic vacuoles in *Ae. albopictus* C6/36 cells. (A) C6/36 cells were mock infected or infected with SINV at a MOI of 2 after overexpression of GFP-AaAtg8 for 12 h. At 36 h post infection, C6/36 cells were observed and cell images were taken under fluorescence microscopy. Hoechst 33342 was used to stain nuclear DNA. Scale bar: 10 μm. The numbers of GFP-AaAtg8 puncta in the samples were counted respectively. (B) Mock-treated, CQ-treated, or SINV-infected C6/36 cells were processed and autophagic vacuoles were observed via electron microscopy. Black arrows indicate autophagic vacuoles. The numbers of autophagic vacuoles in the samples were counted respectively. The data were presented as Means ± SD for three biological replicates, and statistical significance was calculated by *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2. SINV infection induced complete autophagy in C6/36 cells

Accumulation of autophagic vacuoles could be a result of blocked basal level autophagy, or, induced complete or incomplete autophagic flux. To ascertain the cause of observed accumulation of autophagic vacuoles induced by SINV infection in C6/36 cells, the effect of SINV infection on the levels of AaAtg8 and p62 were assayed by immunoblotting analysis. Accumulation of AaAtg8-II is a marker of complete autophagy or incomplete autophagy in C6/36 cells [32,33], and degradation of p62 is an known indicator of complete autophagy [34]. Immunoblotting analysis showed that both higher level of AaAtg8-II and lower level of p62 were induced by SINV infection in C6/36 cells (Fig. 2A), revealing that SINV infection promoted complete autophagic flux. To further confirm that SINV infection induced complete autophagy in C6/36 cells, another widely used method to distinguish incomplete and complete autophagy via monitoring the number of

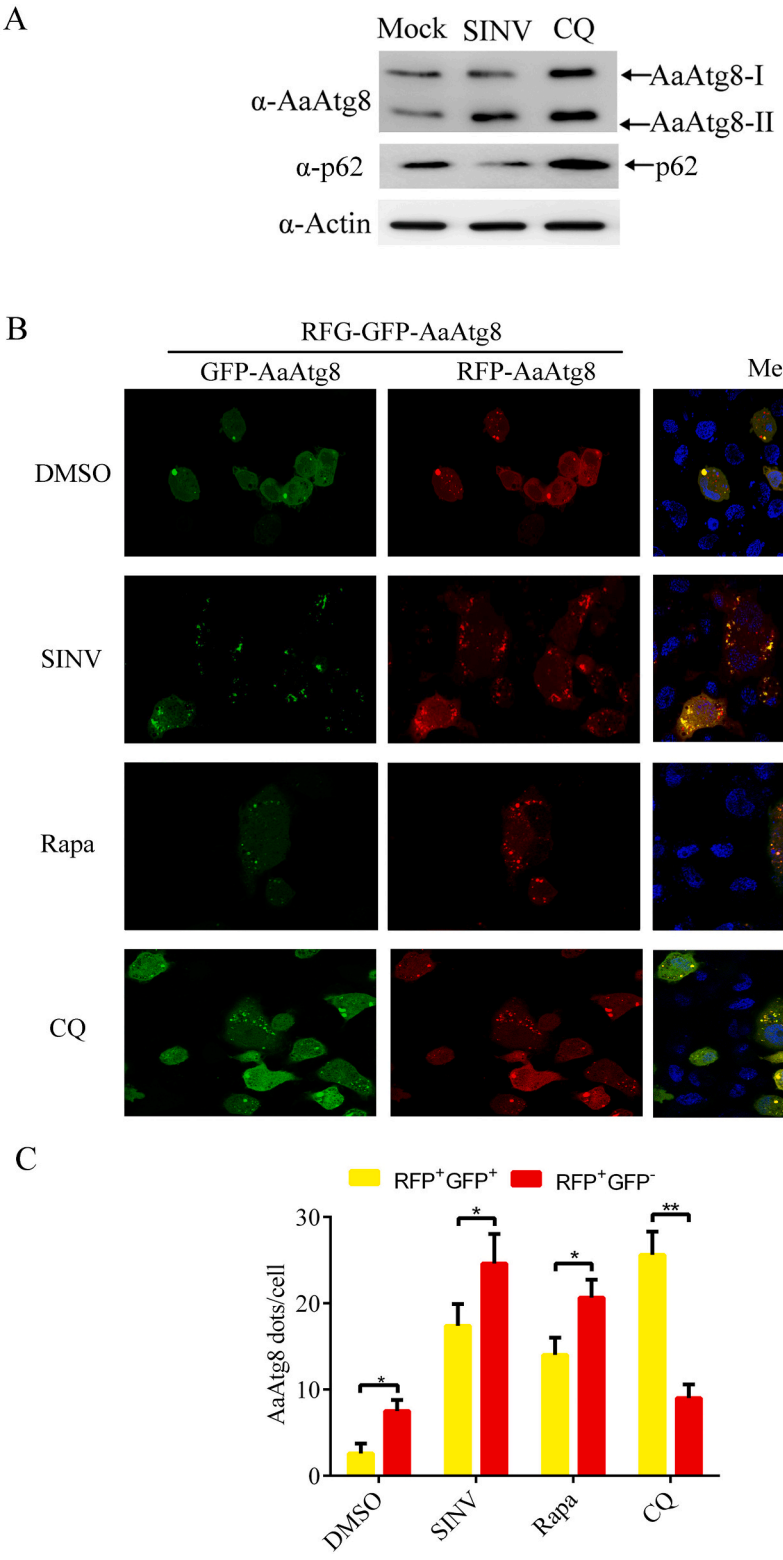


Fig. 2. SINV infection induced complete autophagy in C6/36 cells. (A) C6/36 cells were mock treated, infected with SINV at a MOI of 2 or treated with CQ. Cell lysates were prepared at 36 h post treatment and were subjected to immunoblotting analysis with antibodies against Atg8, p62 and Actin (loading control), respectively. (B-C) C6/36 cells were transfected with plasmid expressing RFP-GFP-AaAtg8 for 12 h and then treated with SINV infection at a MOI of 2 for 36 h, Rapa for 6 h and CQ for 36 h, respectively. DMSO treated C6/36 cells were used as a control. Cells were observed and cell images were taken under confocal fluorescence microscope. Hoechst 33342 was used to stain nuclear DNA. Scale bar: 10 μm. The average number of AaAtg8-labeled vacuoles observed in at least 30 cells was calculated. The data were presented as Means ± SD for three biological replicates, and statistical significance was calculated by *t*-test, $*P < 0.05$, $**P < 0.01$.

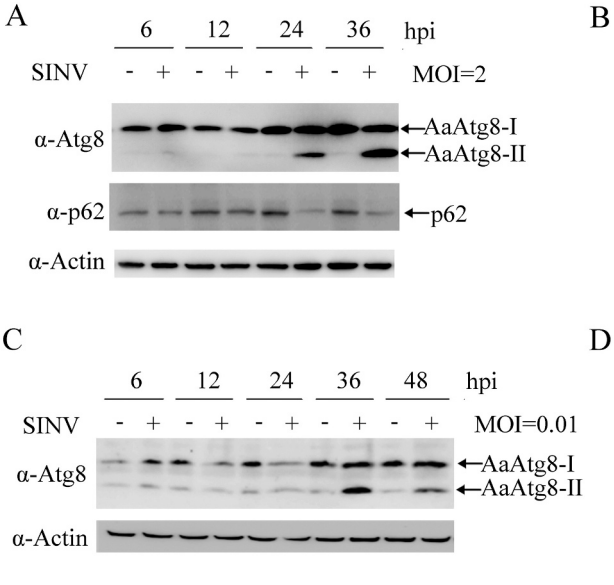
yellow and red puncta generated by RFP-GFP-tagged Atg8 was conducted. It is known that GFP fluorescence is quenched in low pH condition. Thus, RFP-GFP-AaAtg8 puncta associated with autolysosome formed by fusion of autophagosome and lysosome in complete autophagy would be observed as red signals from fluorescence of RFP due to the low pH condition. However, RFP-GFP-Atg8 associated with autophagosome not fused with lysosome in incomplete autophagy would be observed as yellow signals generated by merged fluorescence of RFP and GFP [35]. RFP-GFP-AaAtg8 overexpressed C6/36 cells were infected by SINV or treated respectively with rapamycin (Rapa) which can induce complete autophagy or CQ which blocks the fusion of autophagosome with lysosome and then RFP-GFP-AaAtg8 puncta were detected by confocal fluorescence microscopy. DMSO treated C6/36 cells were used as control. Compared to DMSO treatment, Rapa or CQ treatment increased total number of red puncta and yellow puncta. Moreover, consistent with the respective effect of Rapa or CQ on autophagy pathway, the number of yellow puncta was higher than that of red puncta in Rapa treated C6/36 cells, but lower in CQ treated C6/36 cells, indicating complete autophagy induction by Rapa and inhibition of basal level autophagy by CQ treatment. In SINV infected C6/36 cells, increased total number of red puncta and yellow puncta was observed. Importantly, SINV infection led to more accumulation of red puncta than yellow puncta, indicating that SINV-infection induced autophagy flux as well as fusion of autophagosome with lysosome in C6/36 cells (Fig. 2B-C). Taken together, the results demonstrated that SINV infection induced complete autophagy in C6/36 cells.

3.3. SINV induced autophagy was correlated with SINV replication in C6/36 cells

Since SINV infection induced complete autophagy in C6/36 cells, the correlation between autophagy and SINV replication was studied. First, C6/36 cells were infected with SINV at a MOI of 2 and the levels of autophagy and titers of extracellular infectious SINV viral particles were assayed at certain time points post infection. During 6 to 36 h post infection, AaAtg8-II levels were notably increased by SINV infection from 24 h post infection and accumulated to higher levels at 36 h post infection, suggesting a cumulative increase in autophagy from 24 to 36 h post infection. Moreover, during the same time period, levels of p62 were drastically decreased by SINV infection, confirming that SINV infection increased autophagy from 24 to 36 h post infection in C6/36 cells (Fig. 3A). Meanwhile, the titers of extracellular viral particles were increased with infection time extended. At 24 h post infection, the titer of extracellular viral particles was above 10^7 PFU/mL (Fig. 3B). The above data suggesting that the autophagy induced by SINV infection was correlated with SINV replication in C6/36 cells. To confirm it, C6/36 cells were infected with SINV at a low MOI of 0.01. When infecting C6/36 cells at a low MOI of 0.01, SINV infection influenced the autophagy in the pattern similar to that of SINV infection at a MOI of 2 (Fig. 3C-D). However, the time point observed notable increase in the level of AaAtg8-II was 36 h post infection when the titer of extracellular viral particle was above 10^7 PFU/mL, which was longer than 24 h post infection in the case of infection at a MOI of 2. Altogether, the results indicated that SINV induced autophagy was correlated with SINV replication in C6/36 cells.

3.4. Autophagy inhibited SINV replication in C6/36 cells

To characterize the impact of autophagy on SINV infection in C6/36 cells, the effect of autophagy inhibition on SINV replication was investigated first. To do this, an siRNA knockdown experiment was performed to specifically downregulate endogenous AaAtg8 to inhibit autophagy and the effect on SINV replication was studied. As shown, C6/36 cells transfected with small interfering RNA siAaAtg8-3 significantly decreased the level of endogenous AaAtg8 from 16 to 72 h post transfection (Fig. 4A). Previous data showed that the level of AaAtg8-II was greatly increased when the titer of extracellular infectious SINV viral particles was above 10^7 PFU/mL (Fig. 3). Here, when AaAtg8 was knocked down, C6/36 cells were impaired to accumulate AaAtg8-II even when the titer of extracellular infectious SINV viral particles was above 10^7 PFU/mL, indicating that siAaAtg8-3 successfully inhibited autophagy by downregulating endogenous AaAtg8 expression. Importantly, inhibition of autophagy via knockdown of AaAtg8 significantly increased the titer of extracellular infectious SINV viral particles, suggesting that autophagy might suppress SINV replication in C6/36 cells (Fig. 4B-C). Next, the effect of autophagy induction on SINV replication was studied by performing SINV infection on mock, DMSO or Rapa pretreated C6/36 cells. At 24 h post infection, Rapa treatment increased the autophagy flux, as indicated by the increased level of AaAtg8-II (Fig. 4D). Importantly, increased autophagy in C6/36 cells triggered by Rapa treatment decreased the titer of extracellular infectious SINV viral particles (Fig. 4E). Taken together, the data indicated that autophagy inhibited SINV replication in *Ae. albopictus* C6/36 cells.



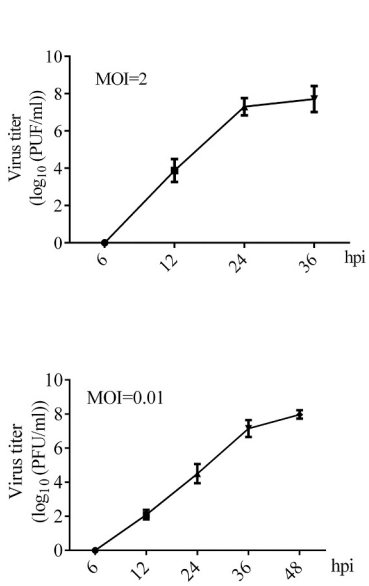


Fig. 3. Correlation between autophagy and SINV replication in C6/36 cells. C6/36 cells were mock-infected or infected with SINV at a MOI of 2 and 0.01, respectively. At the indicated time points post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against Atg8, p62 and Actin (loading control), respectively (A) or with antibodies against Atg8 and Actin (loading control), respectively (C), and the titers of extracellular infectious SINV particles were titrated (B and D).

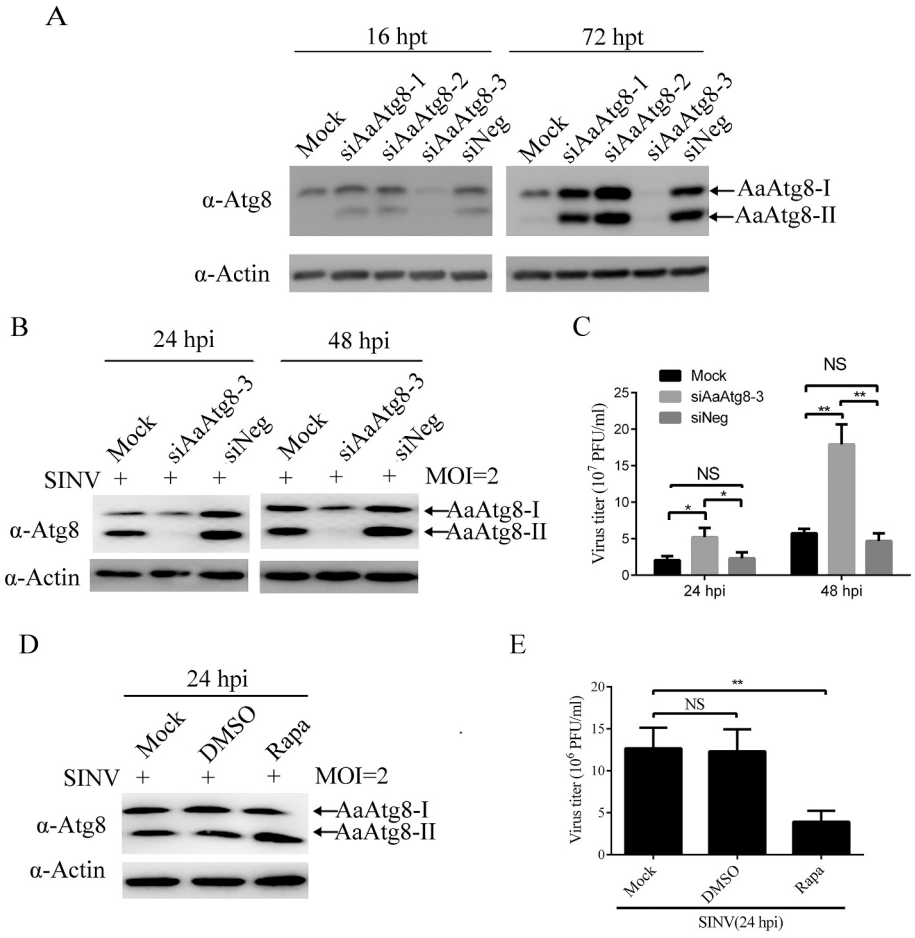


Fig. 4. Autophagy inhibited SINV replication in C6/36 cells. (A) C6/36 cells were transfected with siRNAs targeting AaAtg8 for 16 h or 72 h and cell lysates were subjected to immunoblotting analysis with antibodies against Atg8 and Actin (loading control), respectively. Nonspecific siRNA was used as a negative control (siNeg). (B-C) C6/36 cells transfected with siAaAtg8-3 or siNeg for 16 h were infected by SINV at a MOI of 2. At 24 or 48 h post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against Atg8 and Actin (loading control), respectively (B), and the titers of extracellular infectious SINV particles were titrated (C). (D-E) C6/36 cells pretreated with Rapa for 2 h were subjected to SINV adsorption for 2 h at a MOI of 2. The cells were further cultured in Rapa containing medium. Mock or DMSO treated cells were used as controls. At 24 h post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against Atg8 and Actin (loading control), respectively (D), and the titers of extracellular infectious SINV particles were titrated (E). The data were presented as Means ± SD for three biological replicates, and statistical significance was calculated by *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, NS: not significant.

3.5. Interaction between autophagy and SINV infection occurred in the stage after viral entry and prior to viral assembly in C6/36 cells

To further explore the interaction between the autophagy machinery and SINV replication, correlation between autophagy and the events in SINV infection was studied by utilizing SINV-GFP (a recombinant SINV carrying a GFP reporter whose expression could reflect SINV replication) and SINV^{rep}-GFP (a replicon of SINV-GFP lacking coding sequences of structural proteins which was unable to produce progeny particles but could be used to monitor expression of nonstructural proteins and RNA replication of SINV via GFP expression) (Fig. 5A) [36]. First, C6/36 cells were infected with infectious or ultraviolet (UV)-inactivated SINV-GFP at a MOI of 2 or 10 respectively, and autophagy induction was analyzed. When infecting C6/36 cells, only live SINV-GFP virus triggered autophagy as indicated by the increased level of AaAtg8-II whereas UV-inactivated SINV-GFP virus did not cause change in the level of AaAtg8-II even when infection was performed at a high MOI of 10 (Fig. 5B). Thus, events in viral entry including attachment and endocytosis did not induce autophagy. Next, C6/36 cells were transfected with SINV replicon (SINV^{rep}-GFP) and the effect of the replication of the replicon on autophagy was analyzed. Mock treated C6/36 cells and C6/36 cells transfected with plasmid expressing GFP-Flag were used as negative controls. The immunoblotting analysis showed that SINV^{rep}-GFP increased level of AaAtg8-II, indicating that SINV^{rep}-GFP successfully induced autophagy (Fig. 5C). The above data indicated that expression of nonstructural proteins and/or processes involved in RNA production in SINV infection were sufficient to induce autophagy in C6/36 cells. Together, the data in Fig. 5B and Fig. 5C indicated that induction of autophagy by SINV infection in C6/36 cells occurred in the stage after viral entry and prior to viral assembly. It was shown in Fig. 4C that knockdown of AaAtg8 significantly increased the production of extracellular infectious SINV viral particles. To further confirm that interaction between autophagy indeed occurred in the stage after viral entry and prior to viral assembly, autophagy was blocked via AaAtg8 knockdown and the effect on replication of SINV replicon (SINV^{rep}-GFP) was tested. As shown, AaAtg8 knockdown increased replication of SINV replicon, indicated by the increased level of GFP in immunoblotting and fluorescent microscopy observation (Fig. 5D-E). All the data in Fig. 4 and Fig. 5 indicated that both autophagy induction by SINV infection and suppression of SINV replication by autophagy occurred in the stage after viral entry and prior to viral assembly in C6/36 cells.

3.6. Interaction between autophagy and SINV replication in Ae. aegypti Aag2 cells

Ae. albopictus belongs to the same subgenus (Stegomyia) as *Ae. Aegypti*. Both *Ae. albopictus* and *Ae. aegypti* mosquitoes carry and transmit multiple viruses causing serious diseases in human. Thus, it is important to know how autophagy affects SINV replication in vivo, in *Ae. aegypti* mosquitoes. To reveal that, the effect of SINV infection on autophagy was examined in Aag2 cells derived from *Ae. aegypti* mosquitoes. Surprisingly, SINV infection led to inhibition instead of induction of autophagy in Aag2 cells, indicated by decreased AeAtg8-II levels observed in SINV infected cells compared to mock infected Aag2 cells. This was confirmed by higher levels of p62 in SINV infected Aag2 cells compared to mock infected Aag2 cells (Fig. 6A). Moreover, a notable decrease of AeAtg8-II levels occurred from 24 to 48 h post infection during which time the titer of extracellular infectious viral particle was above 10^5 (Fig. 6B), suggesting that autophagy inhibition was correlated with SINV replication in Aag2 cells.

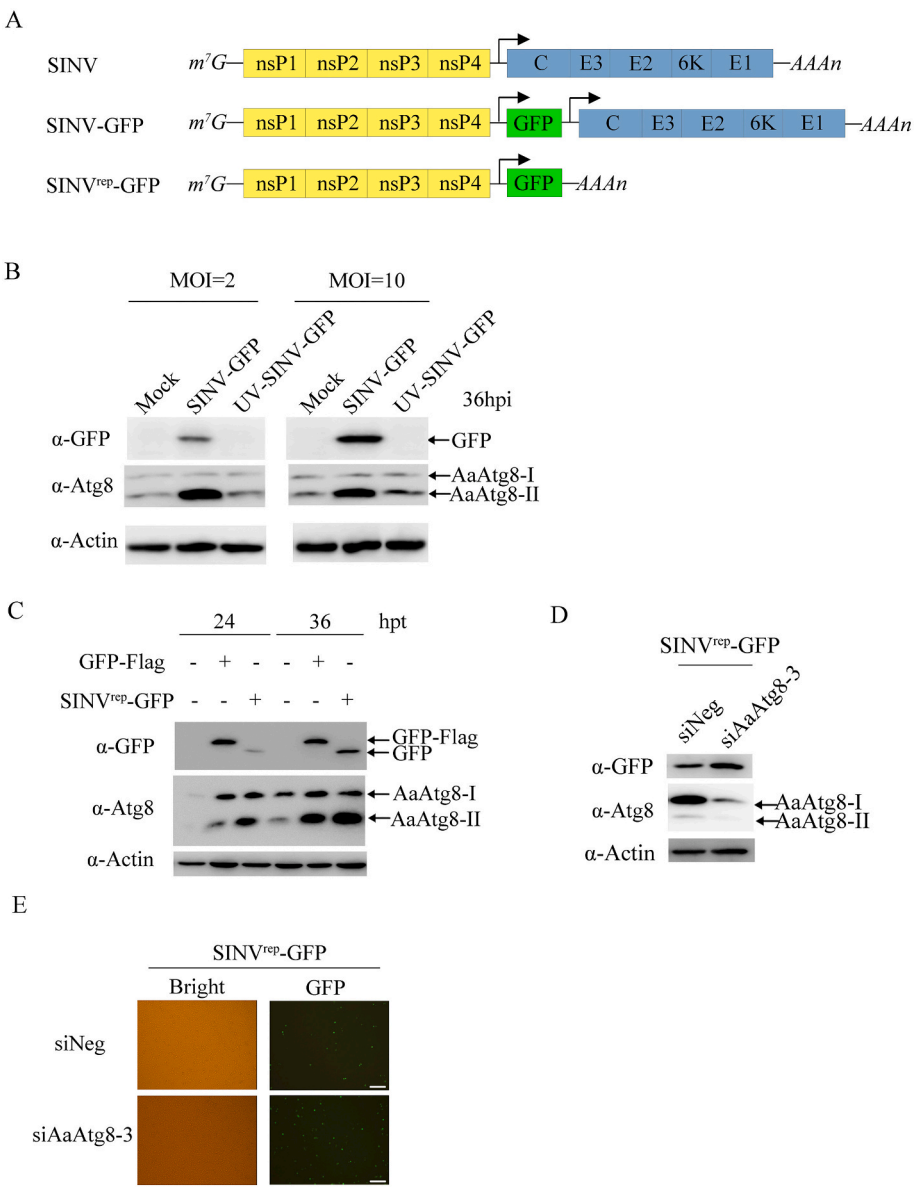


Fig. 5. Events in SINV infection correlated with autophagy in C6/36 cells. (A) Genome structure of SINV, SINV-GFP and SINV^{rep}-GFP. Arrows indicate subgenomic promoters. (B) C6/36 cells were mock-infected, infected with SINV-GFP or UV-inactivated SINV-GFP (UV SINV-GFP) for 36 h at a MOI of 2 or 10. At 36 h post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against GFP, Atg8 and Actin (loading control), respectively. (C) C6/36 cells were transfected with SINV replicon (SINV^{rep}-GFP). At the indicated time points post transfection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against GFP, Atg8 and Actin (loading control), respectively. Mock treated Aag2 cells and Aag2 cells transfected with plasmid expressing GFP-Flag were used as controls. (D-E) C6/36 cells transfected with siAaAtg8-3 or siNeg for 16 h were transfected with SINV replicon (SINV^{rep}-GFP) for 24 h. Cell lysates were prepared and subjected to immunoblotting analysis with antibodies against GFP, Atg8 and Actin (loading control), respectively (D), and cell images were taken using a fluorescence microscope (E). Scale bar: 100 μm. GFP-Flag: C-terminally Flag tagged GFP expressed by plasmid expressing GFP-Flag; GFP: GFP expressed by SINV replicon.

To study how autophagy affects SINV replication in Aag2 cells, Aag2 cells were infected by SINV when autophagy was inhibited via knockdown of AeAtg8 using small interference RNA siAaAtg8. As shown, siAaAtg8-3 successfully down regulated levels of AeAtg8 in Aag2 cells (Fig. 6C). Importantly, knockdown of AeAtg8 before SINV infection resulted in a drastic decrease in the level of AeAtg8 and the production of extracellular infectious SINV viral particles (Fig. 6D-E). The above data indicated that SINV replication led to inhibition of autophagy and inhibition of autophagy suppressed SINV replication in Aag2 cells, suggesting that autophagy was in favor of SINV replication in *Ae. aegypti* Aag2 cells, which was opposite to the case in *Ae. albopictus* C6/36 cells. To further confirm that, autophagy was induced in Aag2 cells via Rapa treatment and the effect on SINV replication was assayed. Indeed, Rapa treatment increased the extracellular viral production (Fig. 6F-G), indicating that autophagy facilitated SINV replication in *Ae. aegypti* Aag2 cells.

To verify events in SINV infection which cause inhibition of autophagy, Aag2 cells were infected by infectious SINV-GFP or UV-inactivated SINV-GFP at a MOI of 2 or 10. In both cases, live SINV-GFP virus inhibited autophagy as indicated by decreased levels of AeAtg8-II whereas UV-inactivated SINV-GFP virus did not change the levels of AeAtg8-II (Fig. 6H). Thus, attachment and endocytosis of inactivated viral particles did not inhibit autophagy in Aag2 cells, indicating entry of SINV particles was not the event causing autophagy inhibition. Then, Aag2 cells were transfected with a SINV replicon (SINV^{rep}-GFP) and the effect of the replication of the replicon on autophagy was analyzed. Mock treated Aag2 cells or Aag2 cells transfected with plasmid expressing C-terminally Flag tagged GFP were used as negative controls. It was observed that the levels of AeAtg8-II were decreased along with replication of the replicon (Fig. 6I). All the results above demonstrated that both autophagy inhibition by SINV infection and suppression of SINV replication by autophagy inhibition occurred in the stage after viral entry and prior to viral assembly in *Ae. aegypti* Aag2 cells.

4. Discussion

This study focused on the role of autophagy in SINV replication in *Ae. albopictus* C6/36 cells and *Ae. aegypti* Aag2 cells. It is known that a complex interplay between autophagy and microbial adaptations against autophagy governs the net outcome of host-microbe encounters and diverse effects of autophagy on the replication of different viruses

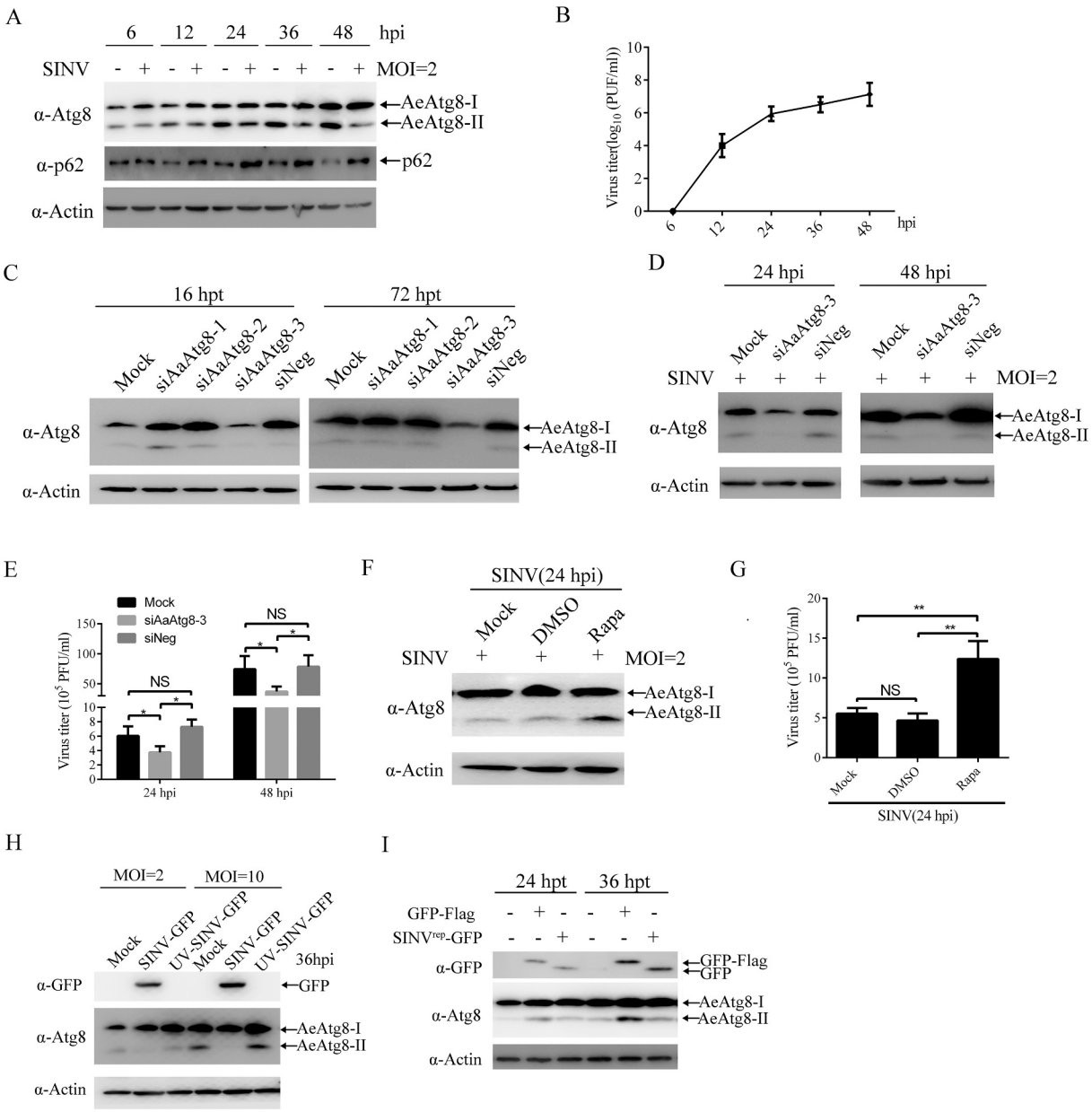


Fig. 6. Interaction between autophagy and SINV replication in *Ae. aegypti* Aag2 cells. (A and B) Aag2 cells were mock infected or infected with SINV at a MOI of 2. At the indicated time points post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against Atg8, p62 and Actin (loading control), respectively (A), and the titers of extracellular infectious SINV particles were titrated (B). (C) Aag2 cells were transfected with siRNAs targeting AeAtg8 for 16 h or 72 h and cell lysates were prepared and subjected to immunoblotting analysis with antibodies against Atg8 and Actin (loading control), respectively. Nonspecific siRNA was used as a negative control (siNeg). (D-E) Mock treated Aag2 cells, Aag2 cells transfected with siAaAtg8-3 or siNeg for 16 h were infected with SINV at a MOI of 2. At 24 or 48 h post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against Atg8 and Actin (loading control), respectively (D), and the titers of extracellular infectious SINV particles were titrated (E). (F-G) Aag2 cells pretreated with Rapa for 2 h were subjected to SINV adsorption for 2 h at a MOI of 2. Then, the cells were further cultured in Rapa containing medium. Mock or DMSO treated cells were used as controls. At 24 h post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against Atg8 and Actin (loading control), respectively (F), and the titers of extracellular infectious SINV particles were titrated (G). (H) Aag2 cells were mock-infected, infected with SINV-GFP or UV-inactivated SINV-GFP (UV SINV-GFP) for 36 h at a MOI of 2 or 10. At 36 h post infection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against GFP, Atg8 and Actin (loading control), respectively. (I) Aag2 cells were transfected with SINV replicon ($SINV^{rep}$-GFP). At the indicated time points post transfection, cell lysates were prepared and subjected to immunoblotting analysis with antibodies against GFP, Atg8 and Actin (loading control), respectively. Mock treated Aag2 cells and Aag2 cells transfected with plasmid expressing GFP-Flag were used as controls. The data were presented as Means ± SD for three biological replicates, and statistical significance was calculated by *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, NS: not significant. GFP-Flag: C-terminally Flag tagged GFP expressed by plasmid expressing GFP-Flag; GFP: GFP expressed by SINV replicon.

[37–40]. This study found that in both C6/36 and Aag2 cells, autophagy interacted with SINV infection in the stage after viral entry and prior to viral assembly. However, the patterns that autophagy interacted with SINV infection were different in C6/36 and Aag2 cells. It was found that SINV infection induced complete autophagy in *Ae. albopictus* C6/36 cells while suppressed autophagy in *Ae. aegypti* Aag2 cells. It was also shown that induction of autophagy restricted SINV replication in C6/36 cells but promoted SINV replication in Aag2 cells. Consistent with this, suppression of autophagy promoted SINV replication in C6/36 cells but restricted SINV replication in Aag2 cells. Thus, the data in the work

demonstrated that autophagy suppressed SINV replication in C6/36 cells but promoted SINV replication in Aag2 cells. These findings may help to understand the specific nature in viral transmission of *Ae. albopictus* and *Ae. aegypti* mosquitoes.

Different chemical modulators of autophagy-arbovirus-cell interface was observed in C6/36 and Aag2 cells, and the potential effect of several differences between the two cell lines including tissue origin of the cells, immunocompetency, persistent infection with certain viruses on the observation was discussed by Brackney et al., [41]. We speculate that the same range of factors could have impact on the differences in interaction between autophagy and SINV infection in C6/36 and Aag2 cells observed in this study as well. First, the two cell lines have different tissue origins, namely, C6/36 cells from larvae of *Ae. albopictus* mosquito while Aag2 cells from *Ae. aegypti* mosquito embryos. Heterogeneity in autophagic response across tissues in *C. elegans* under different conditions including stress and aging has been reported [42]. Thus, it is possible that the two cell lines derived from different tissue origins may have different autophagic response upon viral infection. Second, RNA interference is a primary antiviral immune response. Previous studies revealed that autophagy can selectively degrade components of the RNAi pathway [43,44]. C6/36 cells were found to have a dysfunctional RNA interference response [41] while Aag2 cells are RNA interference competent [45]. Whether this difference affected the pattern of interaction between autophagy and SINV infection in C6/36 and Aag2 cells requires further study. Third, Aag2 cells are persistently infected with insect-specific viruses while C6/36 cells are not [46]. The additional viruses might affect the autophagic response to SINV infection in Aag2 cells.

One of antiviral signaling pathways responding to viral infection in insect is the JAK/STAT pathway. It was reported that SINV triggered the JAK/STAT pathway in *D. melanogaster* [47]. *Ae. aegypti* was shown to use the JAK/STAT pathway to respond to infection of flaviviruses WNV, DENV, and yellow fever virus (YFV) [48]. A recent study revealed that insulin signaling is antiviral via the JAK/STAT pathway in both fly and mosquito models to against a range of flaviviruses. The study determined that insulin treatment activated Akt and reduced titers of WNV in Aag2 cells and insulin treatment activated Akt and reduced titers of WNV, ZIKV and DENV in C6/36 cells, which suggesting that the effects of insulin are independent of RNAi-mediated antiviral activity [49]. Considering the correlation between insulin and mTOR, whether the observation in this publication involved autophagy is worth further study.

The JAK/ STAT and RNAi pathways are important for controlling arboviral infection in insects. Our study investigated the role of the autophagy pathway in arboviral infection in *Ae. albopictus* and *Ae. aegypti* mosquito cells. It is necessary to study the interaction between autophagy and viral infection in *Ae. aegypti* and *Ae. albopictus* mosquito systems. Also, further study on how the JAK/ STAT, RNAi and the autophagy pathways communicate with each other in viral infection might help to provide a better picture of the antiviral immune response in *Ae. aegypti* and *Ae. albopictus* mosquitoes.

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CRediT authorship contribution statement

Qingzhen Liu: Funding acquisition; Project administration; Supervision; Writing - review & editing. **Jialu Qiao**: Investigation; Writing - original draft.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

Appendix A. Supplementary data

Supplementary material

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