Co-localization of constituents of the dengue virus translation and replication machinery with amphisomes

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Infections with dengue virus (DENV) are a significant public health concern in tropical and subtropical regions. However, little detail is known about how DENV interacts with the host-cell machinery to facilitate its translation and replication. In DENV-infected HepG2 cells, an increase in the level of LC3-II (microtubule-associated protein 1 light chain 3 form II), the autophagosomal membrane-bound form of LC3, was observed, and LC3 was found to co-localize with dsRNA and DENV NS1 protein, as well as ribosomal protein L28, indicating the presence of at least some of the DENV translation/replication machinery on autophagic vacuoles. Inhibition of fusion of autophagic vacuoles with lysosomes resulted in an increase in both intracellular and extracellular virus, and co-localization observed between mannose-6-phosphate receptor (MPR) and dsRNA and between MPR and LC3 identified the autophagic vacuoles as amphisomes. Amphisomes are formed as a result of fusion between endosomal and autophagic vacuoles, and as such provide a direct link between virus entry and subsequent replication and translation.

INTRODUCTION

With an estimated 100 million infections per year worldwide, dengue virus (DENV), which is spread to humans by the bite of female Aedes mosquitoes, represents a significant public health threat in tropical and subtropical countries (Guzman & Kouri, 2002). The DENV complex comprises four antigenically distinct viruses termed DENV serotypes 1, 2, 3 and 4 (DENV-1 to -4), all of which can cause a wide spectrum of disease presentation, from a relatively mild febrile disease to a life-threatening haemorrhagic syndrome (Malavige et al., 2004). DENV is an enveloped, positive-sense, single-stranded RNA virus of approximately 11 kb that encodes three structural proteins (core, pre-membrane and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame (Chang, 1997). Entry of DENV into a susceptible cell occurs primarily through receptor-mediated endocytosis via clathrin-coated pits (Krishnan et al., 2007) and the virus is then trafficked to endosomes (Krishnan et al., 2007; van der Schaar et al., 2007) and fuses with the endosomal membranes (Heinz et al., 2004) through a pH-dependent conformational change in the envelope protein (Modis et al., 2004; Mukhopadhyay et al., 2005). The fate of the released nucleocapsid is largely obscure, although it is known that, after uncoating of the virus genome, the host-cell translational machinery is utilized to synthesize a polyprotein precursor that is processed co- and post-translationally by a virus-encoded protease (NS3) and host signallases (Cahour et al., 1992). Translation and subsequent replication of the DENV genome occur in tight association with intracellular membranous structures that are believed to be endoplasmic reticulum (ER)-derived (Clyde et al., 2006; Miller & Krijnse-Locker, 2008; Salonen et al., 2005), although the exact origin of these membranes remains unclear.

Autophagy is a lysosomal degradation pathway involved in the cellular turnover of macromolecules and organelles. It is conserved among eukaryotes and has been shown to be important for cellular development and remodelling (Levine & Klionsky, 2002; Meijer & Codogno, 2006), as well as being involved in a wide range of disease processes including cancer, neurodegeneration, innate and adaptive immunity, heart disease, liver disease and ageing (Lerena et al., 2008; Mizushima et al., 2008). Autophagy begins with the sequestration of an area of the cytoplasm within a crescent-shaped membrane called the isolation membrane, which probably arises from a pre-existing body (Hanavi et al., 2007; Kim et al., 2007; Levine & Klionsky, 2004). This membrane has been suggested to originate from the ER (Dunn, 1990a) and/or from the trans-Golgi network (Kihara et al., 2001). The isolation membrane then expands and matures into a large, characteristically double-membraned vesicle with a diameter of 500–1000 nm called an autophagosome (Dunn, 1990a). Among the key regulators...
of this process are mTOR (a kinase target of rapamycin) and the beclin1–class III PI3K complex (Xie & Klionsky, 2007). The execution phase of autophagy is mediated primarily through two covalent conjugation pathways: the covalent linkage of Atg5 and Atg12 (Mizushima et al., 1998, 2002; Ohsumi, 2001) and the covalent lipidation of Atg8 (called microtubule-associated protein 1 light chain 3, or LC3, in mammalian cells) by phosphatidylethanolamine (Kabeya et al., 2000; Ohsumi, 2001). Lipidated LC3 eventually associates with the autophagy membranes (Kabeya et al., 2000) and as such is the only creditable marker of autophagosomes in mammalian cells (Bampton et al., 2005; Kimura et al., 2007). Fusion of the autophagosome with endosomes forms structures called amphisomes (Gordon & Seglen, 1988), whilst subsequent fusion with lysosomes forms autophagolysosomes (Dunn, 1990b).

Recently, it has been shown that autophagy is induced upon DENV-2 infection of Huh7 cells (Lee et al., 2008). Biochemical inhibition of autophagy resulted in a reduction in the number of virus progeny produced, and infection was significantly inhibited in Atg5-knockout MEF cells, suggesting that DENV subverts the autophagic process (Lee et al., 2008). However, the details of how DENV affects the autophagic process remain unknown. Given that double-membrane vesicle structures have been associated with DENV replication (Miller & Krijnse-Locker, 2008) and that such double-membrane structures are a classic hallmark of autophagosomes (Dunn, 1990a), it is possible that, similar to poliovirus (Jackson et al., 2005), equine arteritis virus (Pedersen et al., 1999), coronavirus and mouse hepatitis virus (Lee & Iwasaki, 2008), DENV uses autophagic membranes as sites for virus replication.

**METHODS**

**Cells, viruses and infection.** The human hepatoma cell line HepG2 (ATCC HB-8065) was cultivated at 37 °C under 10% CO2 in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) (DMEM/FBS) and 100 U penicillin/streptomycin ml⁻¹ (PAA). For characterization of autophagy, HepG2 cells were seeded onto glass coverslips and grown for 24 h under standard conditions after which the growth medium was replaced with complete growth medium or growth medium supplemented with either 100 nM rapamycin (Sigma-Aldrich) or 100 nM rapamycin and 10 mM 3-methyladenine (3-MA; Sigma-Aldrich).

DENV-2 strain 16681 was propagated in the *Aedes albopictus*-derived cell line C6/36 (ATCC CRL-1660). The virus was partially purified by centrifugation to remove cell debris and stored at −80 °C.

For infection of HepG2 cells, cells were grown to subconfluency and pre-treated for 3 h with 10 mM 3-MA in DMEM/FBS or for 1 h with 100 nM rapamycin or 30 mM L-asparagine (L-Asn; Sigma-Aldrich) in DMEM/FBS, or left untreated, and were then infected with DENV-2 at 10 p.f.u. per cell for 2 h in DMEM with or without an autophagy modulator as appropriate. After 2 h, normal growth medium (with or without autophagy modulator as appropriate) was added and cells were incubated under normal conditions until harvesting of the cells or medium. Virus titres were determined by standard plaque assay as described previously (Sithisarn et al., 2003) and intracellular virus levels were determined as described elsewhere (Thepparit & Smith, 2004).

**Indirect immunofluorescence.** Approximately 3 × 10⁴ HepG2 cells were seeded and grown on 1 cm² coverslips under standard conditions for 24 h, followed directly by infection with DENV-2 at 10 p.f.u. per cell for 2 h or pre-incubated for 3 h with 100 nM rapamycin or 30 mM L-asparagine (L-Asn; Sigma-Aldrich) in DMEM/FBS or for 1 h with 100 nM rapamycin or 30 mM L-Asn in DMEM/FBS before virus infection at 10 p.f.u. per cell. At various time points, cells were washed twice with PBS and then fixed in 100% ice-cold methanol for 20 min. Cells were subsequently permeabilized with 0.3% Triton X-100 in PBS for 10 min and then washed with

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Fig. 1. Induction of autophagy in HepG2 cell. HepG2 cells were grown on glass coverslips and then incubated in complete medium (control) or in the presence of rapamycin or rapamycin and 3-MA for 15 min and subsequently incubated with appropriate primary and secondary antibodies to detect LC3 (red) and LAMP1 (green). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative, non-contrast-adjusted merged images are shown.
0.03% Triton X-100 in PBS or permeabilized, washed and then blocked for 1 h at room temperature with 5% FBS in 0.03% Triton X-100 in PBS (for anti-NS1 antibody). The cells were then incubated with two or three primary antibodies at 4°C overnight. Following incubation, cells were washed four times with 0.03% Triton X-100 in PBS and incubated with two or three appropriate secondary antibodies for 1 h at room temperature. Subsequently, coverslips were washed with 0.03% Triton X-100 in PBS six times and then mounted onto glass slides. The cells were observed under a confocal microscope.

The primary antibodies used were a rabbit polyclonal anti-MAP-LC3 antibody (Santa Cruz Biotechnology), a goat polyclonal anti-MAP-LC3 antibody (Santa Cruz Biotechnology), a mouse monoclonal anti-CD107a (LAMP1) antibody (BD Transduction), a rabbit polyclonal anti-LAMP1 antibody (Abcam), a mouse monoclonal anti-DENV NS1 antibody (Puttikhunt et al., 2003), a mouse monoclonal anti-dsRNA antibody (J2; English and Scientific Consulting), a goat polyclonal anti-ribosomal protein L28 antibody (Santa Cruz Biotechnology) and a rabbit polyclonal anti-mannose-6-phosphate receptor antibody (Abcam).

Secondary antibodies used as appropriate were rhodamine Red X-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG.

**Fig. 2.** Induction of autophagy in response to DENV-2 infection. HepG2 cells were grown on glass coverslips and either mock-infected or infected with DENV-2 either directly (a, b) or in the presence of rapamycin (c), 3-MA (d) or L-Asn (e). Cells were examined simultaneously for the localization of LC3 (far red) and LAMP1 (green) and separately for NS1 (red) (a, c–e) or simultaneously for LC3 (blue), LAMP1 (green) and NS1 (red) (b), in uninfected or infected cells as indicated. Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined. Images were merged for LC3 and LAMP1, with the NS1 signal from the same field shown merged with the bright-field view.
Fluorescence confocal microscope imaging and analysis.
Fluorescently labelled samples were observed under an Olympus FluoView 1000 microscope equipped with Olympus FluoView software version 1.6. For samples stained with two primary antibodies, images were captured in the red (rhodamine-labelled secondary antibodies), far-red (Cy5-labelled secondary antibodies) or green (FITC-labelled secondary antibodies) channels. For samples stained with three primary antibodies, images were captured in the green (FITC-labelled secondary antibodies), red (Alexa Fluor 594-labelled secondary antibodies) and far-red (Cy5-labelled secondary antibodies) channels. Final images were a non-contrast-adjusted merge of two or three channels. Where three channels were merged, far-red images were shown as blue. Some images had a bright-field image included in the final merge. At least 15 fields from each coverslip were examined and a minimum of two independent experiments was undertaken for each condition. Representative images of selected fields are shown.

Image analysis was undertaken using the ImageJ analysis program (Abramoff et al., 2004) using the PSC co-localization plug-in (French et al., 2008) to calculate co-localization. At least 20 cells were analysed for each condition. Results are presented in terms of Pearson correlation coefficients, which represent the linear relationship of the signal intensity from the green and red channels of the analysed image. The program allowed masking of areas to be excluded from the analysis, and uninfected cells were masked prior to analysis. Statistical analysis of significance between datasets was undertaken by a paired sample test using SPSS with a value of $P<0.05$ for significance.

Western blot analysis. Total proteins from mock-infected or DENV-infected HepG2 cells were extracted and separated by PAGE before transfer to a solid support. Membranes were blocked with 5 % skimmed milk in TBS for 2 h at room temperature, followed by incubation with antibodies against LC3 in 5 % BSA in TBS or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 5 % skimmed milk in TBS at 4 °C overnight. The membranes were then incubated with appropriate secondary antibodies in 5 % skimmed milk in TBS at room temperature for 1 h. The antibodies used were a 1:3000 dilution for rabbit polyclonal anti-LC3 antibody (Novus Biological) and a 1:800 dilution for mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology), followed by a 1:3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) or a 1:4000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Sigma Chemical Co.). Signals were developed using an ECL-Plus Western Blotting Analysis kit (GE Healthcare).

Statistical analysis. Virus production data were analysed using the GraphPad Prism program (GraphPad Software). Statistical analysis of significance was undertaken by a paired sample test using SPSS with a value of $P<0.05$ for significance.

RESULTS

Autophagy in HepG2 cells
Lee et al. (2008) showed that autophagy is induced in response to DENV infection, but the study was undertaken in Huh7 cells. This study therefore sought initially to establish that autophagy could be induced in HepG2 cells and that this pathway was susceptible to manipulation in these cells. To establish that the autophagy pathway is viable in HepG2 cells, the localization of LC3 (a specific marker of autophagic vacuoles) and LAMP1 (a marker of endosomal and lysosomal membranes) was examined in control cells, in cells treated with the autophagy inducer rapamycin (Noda & Ohsumi, 1998) and in cells treated with DENV-2 infection and effects of autophagy modulation. (a) Western blot analysis of LC3 and GAPDH expression in mock-infected or DENV-2-infected HepG2 cells on days 1–3 p.i. (b) Extracellular virus production of HepG2 cells infected with DENV-2 in the presence or absence of 3-MA, rapamycin or l-Asn. Virus yield is plotted as log virus titre ± SD. Data were derived from six independent replicates, with duplicate assays of each replicate. The statistical significance of virus output compared with the control is shown. *a, $P<0.001$; *b, $P=0.012$. (c) Intracellular virus production of HepG2 cells infected with DENV-2 in the presence or absence of 3-MA or l-Asn. Virus yield is plotted as log virus titre ± SD. Data were derived from six independent replicates, with duplicate assays of each replicate. The statistical significance of virus yield compared with the control is shown. *a, $P<0.001$; *b, $P=0.001$. 

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with both rapamycin and the PI3k kinase class III inhibitor 3-MA, which inhibits autophagy (Seglen & Gordon, 1982). Little if any co-localization was seen in control cells, whilst co-localization was observed in rapamycin-treated cells by 15 min after the addition of rapamycin. Co-localization of LAMP1 and LC3 in response to rapamycin treatment was completely abolished by 3-MA treatment (Fig. 1).

**Induction of autophagy in response to DENV infection**

To confirm that autophagy was induced in response to DENV-2 infection using strain 16681 as opposed to strain PL0146 as used by Lee et al. (2008), HepG2 cells were infected with DENV-2 strain 16681 at 10 p.f.u. per cell in parallel with samples treated with rapamycin, 3-MA and L-Asn, which inhibits fusion of lysosomes with autophagosomes and amphisomes (Gordon & Seglen, 1988). An m.o.i. of 10 had been determined separately to give infection rates of greater than 90 % at 24 h post-infection (p.i.) (data not shown). At 24 h p.i., samples were simultaneously stained with antibodies directed against LC3, LAMP1 and NS1. The co-localization of LC3 and LAMP1 was examined only in cells positive for NS1 staining to ensure that only DENV-infected cells were analysed.

The results showed an increased co-localization between LC3 and LAMP1 in response to DENV-2 infection [mean Pearson correlation coefficient 0.34, 95 % confidence interval (CI) 0.31–0.37] compared with mock-infected cells (mean Pearson correlation coefficient 0.14, 95 % CI 0.13–0.15; P<0.001; Fig. 2a). The level of co-localization between LC3 and LAMP1 in infected cells was significantly increased in the presence of the autophagy inducer rapamycin (mean Pearson correlation coefficient 0.55, 95 % CI 0.52–0.58; P<0.001; Fig. 2c) and abolished in the presence of 3-MA (Fig. 2d). An increase in the co-localization of LC3 and LAMP1 over and above that seen in infected cells was observed in cells infected in the presence of L-Asn (mean Pearson correlation coefficient 0.46, CI 0.43–0.49; P<0.001; Fig. 2e). Western blotting demonstrated the formation of the autophagy-associated form of LC3, LC3-II, in response to DENV infection (Fig. 3a). Virus yield at 24 h p.i. as determined by standard plaque assay was significantly reduced in response to 3-MA treatment (P<0.001) and increased by the presence of both rapamycin (P=0.012) and L-Asn (P=0.012), with all experiments undertaken six times independently, with duplicate assays of titre (Fig. 3b). The increase in virus yield seen in the presence of L-Asn suggested that fusion with lysosomes resulted in a degree of virus degradation. The yield of intracellular virus, determined as described elsewhere (Thepparit & Smith, 2004), was similarly increased in the presence of L-Asn (P<0.001) and decreased in the presence of 3-MA (P=0.001) (Fig. 3c).

**Fig. 4.** Localization of the DENV replication complex. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 either directly or in the presence of 3-MA or L-Asn, and examined for the localization of LC3 (red) and either dsRNA (green) (a) or NS1 (green) (b). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative, non-contrast-adjusted merged images are shown.

**Autophagy and DENV replication and translation**

To investigate whether parts of the replication/translation machinery of DENV co-localized with LC3, DENV-2-infected cells were examined for the localization of DENV
NS1 protein and dsRNA in relation to LC3. The location of dsRNA was detected using the antibody J2, which has previously been established as being able to detect flavivirus dsRNA (Weber et al., 2006), whilst NS1 protein was detected using a well-characterized monoclonal antibody (Puttikhunt et al., 2003).

At 24 h p.i., significant levels of co-localization were observed between dsRNA and LC3 (mean Pearson correlation coefficient 0.35, 95 % CI 0.28–0.42), which was largely eliminated by treatment with 3-MA, whilst L-Asn treatment significantly increased the co-localization between dsRNA and LC3 (mean Pearson correlation coefficient 0.45, 95 % CI 0.39–0.51; \( P=0.001 \); Fig. 4a). NS1 protein similarly co-localized with LC3 in DENV-2-infected cells (mean Pearson correlation coefficient 0.44, 95 % CI 0.39–0.49), and co-localization was again largely eliminated by treatment with 3-MA and increased when DENV-2-infected cells were treated with L-Asn (mean Pearson correlation coefficient 0.51, 95 % CI 0.46–0.56; Fig. 4b), again suggesting degradation of virus or viral proteins upon fusion of the autophagic vacuole with lysosomes. NS1 protein also co-localized with both LC3 and LAMP1 (Fig. 2b).

We further investigated the co-localization of ribosomal proteins with the presence of dsRNA to determine whether there was translational capacity at the site of RNA replication and found significant co-localization between dsRNA and ribosomal proteins using an antibody directed against ribosomal protein L28 (Fig. 5).

As a marker of both endosomes and lysosomes, LAMP1 co-localization is unable to discriminate between the formation of autophagolysosomes (fusion of autophagosomes with lysosomes) and amphisomes (fusion of autophagosomes with endosomes). We therefore investigated whether the endosomal marker mannose-6-phosphate receptor (MPR) co-localized with dsRNA. High levels of MPR signal were observed in mock-infected cells, and co-localization between MPR and LC3 was more evident than co-localization between LC3 and LAMP1 in mock-infected cells. This may reflect either a higher level of MPR in HepG2 cells or may simply result from differences in antibody avidity. However, clear co-localization between MPR and dsRNA (Fig. 6a) was observed, as well as increased co-localization between MPR and LC3 in infected cells (Fig. 6b), suggesting amphisomes as the site of DENV-2 replication and translation.

To confirm amphisomes as a site of at least part of the DENV replication complex, triple staining using antibodies directed against MPR, LC3 and dsRNA was undertaken in infected cells. The results (Fig. 7) showed co-localization of these three markers.

**DISCUSSION**

Whilst entry of DENV into target cells by receptor-mediated endocytosis into clathrin-coated pits and subsequent pH-dependent fusion of the virus structural envelope protein with membranes of the late endosomes

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**Fig. 5.** Co-localization of ribosomes with DENV dsRNA. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 and examined for the localization of L28 (red) and dsRNA (green). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative single-channel and merged non-contrast-adjusted images are shown.
has been well documented (Allison et al., 1995; Mukhopadhyay et al., 2005), subsequent events are less well characterized. It is currently believed that flavivirus genomes are released into and replicate in the cytoplasm in close association with intracellular membranous structures that possibly derive from the ER (Clyde et al., 2006; Miller & Krijnse-Locker, 2008). Consistently, flavivirus infections characteristically result in significant proliferation of rough ER membranes, and the flavivirus replication complex has been partly correlated with these ER membranes (Boulton & Westaway, 1976) and with cytoplasmic vesicles and vacuoles (Mackenzie et al., 1996), and it has been proposed that flaviviruses bud from ER membranes and transit the Golgi body before release from the cell (Clyde et al., 2006; Yoshii et al., 2004).

More recently, Lee et al. (2008) showed that autophagy is induced by DENV-2 infection in Huh7 cells, and that the

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**Fig. 6.** Investigation of amphisomes as sites of DENV replication. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 and examined for the localization of MPR (red) and dsRNA (green) (a) or MPR (green) and LC3 (red) (b). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative single-channel and merged non-contrast-adjusted images are shown.
induction of autophagy serves to enhance DENV replication. Inhibition of autophagy, either by biochemical treatment with 3-MA or by knockout of Atg5 (in MEF cells) serves to reduce the levels of infectious virus produced (Lee et al., 2008). Similarly, as shown here, DENV-2 induced autophagy in HepG2 cells and biochemical inhibition of autophagy with 3-MA reduced virus yield, whilst induction of autophagy with rapamycin enhanced virus yield.

The process of autophagy initially generates double-membraned structures called autophagosomes (Dunn, 1990a), which are capable of fusing with endosomes to form amphisomes (Gordon & Seglen, 1988). Both autophagosomes and amphisomes can subsequently fuse with lysosomes to form autophagolysosomes, the primary degradative vesicle (Dunn, 1990b). Interestingly, however, the inhibition of lysosomal fusion with L-Asn served to increase both intracellular and extracellular virus yield, suggesting that lysosomal fusion of autophagic vacuoles to form autophagolysosomes has a deleterious effect on DENV replication. This consequently suggests that viable virus is produced prior to lysosomal fusion on either autophagosomes or amphisomes.

The co-localizations observed between LC3 and LAMP1, LC3 and dsRNA, LC3 and NS1, and between NS1, LC3 and LAMP1, as well as between ribosomal proteins and dsRNA, located the DENV translation/replication complex on autophagosomes, and the co-localization seen between MPR and dsRNA, MPR and LC3, and between MPR, LC3 and dsRNA would indicate that these structures are amphisomes, formed by the fusion of autophagosomes and endosomes. Given the localization of the DENV replication/translation complex on amphisomes, it is unsurprising that subsequent fusion with lysosomes (and their proteolytic contents) to form autophagolysosomes results in a decrease in the number of virus progeny.

The induction of autophagic structures and the location of part of the DENV replication/translation complex on these structures is consistent with a considerable body of work that locates the DENV replication complex on induced membranes and vacuoles (Boulton & Westaway, 1976; Mackenzie et al., 1996) and explains why modulation of autophagy serves to modulate DENV output, as seen by Lee et al. (2008) and ourselves in this study. As amphisomes are formed by the fusion of endosomes and autophagosomes (Gordon & Seglen, 1988; Gordon et al., 1992), the identification of these structures as the sites of at least part of the DENV replication/translation complex provides a basis for a unified model linking DENV entry and replication in terms of an ongoing and continual association with membranes of an endosomal–autophagic lineage.

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