Supplemental Data
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Composition and Three-Dimensional Architecture of the Dengue Virus Replication and Assembly Sites

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Supplemental Experimental Procedures

Plasmid Constructs

Standard molecular biology techniques were used for cloning (Sambrook, 2001). The structures of all plasmids were determined by nucleotide sequence and restriction analysis. The full length DENV2 NGC strain cDNA (a kind gift of Andrew Davidson, School of Medical Sciences, Bristol, UK) served as a template for polymerase chain reaction (PCR) amplification of individual DENV genes. For expression of DENV-glutathione-S-transferase (GST)-fusion proteins, DNA fragments encoding for the following amino acids (aa) of the respective DENV proteins were generated by PCR and inserted into the bacterial expression vector pGEX-6P-1 (Amersham Bioscience) in frame with the GST sequence: prM, aa 1 to aa 166; NS1, aa 1 to aa 352, and NS2B, aa 1 to aa 130. PCR fragments encoding for the amino acids of prM (sense primer 5´-ATATTGGATCCTTCCATTTAACCACACGTAACGGAG-3´, antisense primer 5´-ATAAGATCTTTATGTCATTGAAGGAGCGACAGCTGTC-3´), the NS1 protein (sense primer 5´-ATATTGGATCCGATAGTGGTTGCGTTG-3´, antisense primer 5´-ATAAGATCTTTAGGCTGTGACCAAGGA-3´), and NS2B (sense primer 5´-ATATTGGATCCAGCTGGCCACTAAATG-3´, antisense primer 5´-ATAAGATCTTTACCGTGGTTTCTTCACT-3´) were inserted into pGEX-6P-1 (Amersham Bioscience, Freiburg, Germany) via the *BamH*I and *Bgl*II restriction site (underlined).

Expression of DENV prM, NS1-, and NS2B-GST Fusion Proteins and Generation of Antisera

For the expression of fragments or full length versions of Glutathione-S-Transferase (GST)-prM, NS1, and NS2B fusion proteins the bacterial expression vector pGEX-6P-1 was used. The constructs were expressed using the *Escherichia coli* strain BL21 codon plus (DE3)-RIL (Stratagene, Amsterdam, Netherlands). After induction with 1mM isopropyl-β-D-thiogalactoside (IPTG; AppliChem, Darmstadt, Germany) the cells were grown for 4-6 h at 30°C and afterwards pelleted and ruptured by sonication in phosphate-buffered saline (PBS) supplemented with

protease inhibitors. Pellets obtained after centrifugation (4,000 x g) were treated with different concentrations of urea (3-9 M), and after centrifugation supernatants were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie blue. GST-fusion proteins were excised and eluted from the gel slice by using an electro elution chamber (Schleicher & Schuell, Dassel, Germany) as described in the manual. Antisera were raised in New Zealand White rabbits immunized over a period of 4 months with the purified GST fusion proteins (Eurogentec, Seraing, Belgium).

For immunofluorescence studies the monospecific polyclonal antisera were purified by affinity purification as described previously (Miller et al., 2006).

Immunofluorescence Microscopy

Monolayers were grown on glass coverslips and, following infection, fixed at the indicated time in 4% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100 in PBS, incubated with primary antibodies for 1h in PBS containing 3% bovine serum albumine (BSA), and washed 3 times for 5 min with PBS. Secondary antibody incubation was performed for 45min in PBS containing 3% bovine serum albumine, after which the cells were washed as described above and mounted in Mobiglow (MoBiTec, Göttingen, Germany). Fluorescence images were taken with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) and transferred to a computer workstation running LSM5 Image Browser 3.2 software (Carl Zeiss). Images were merged with Adobe Photoshop CS3 software.

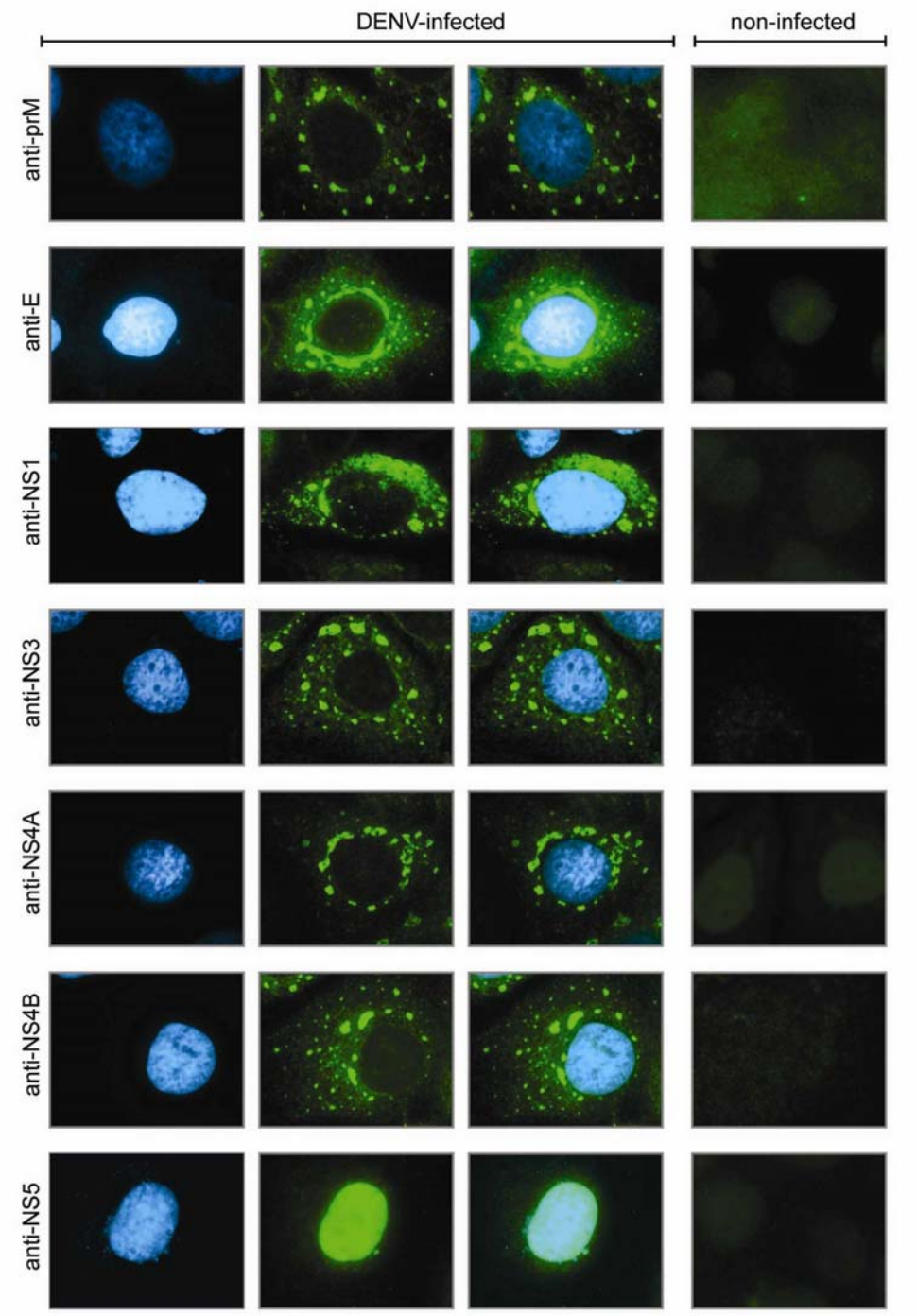

Figure S1. Localization of DENV Proteins in Infected Cells

Huh-7 cells were infected with DENV2 (column 1 to 3 from the left) or left uninfected (right column). The cells were fixed at 24 hr p.i. with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and processed for immunolabeling. DENV antigens were detected by using monospecific rabbit polyclonal antibodies directed against the DENV antigens indicated on the left. Nuclear DNA was stained with DAPI (4',6'-diamidino-2-phenylindole dihydrochloride). The three columns of DENV infected cells show (from left to right) DAPI, IF stain and merge, respectively.

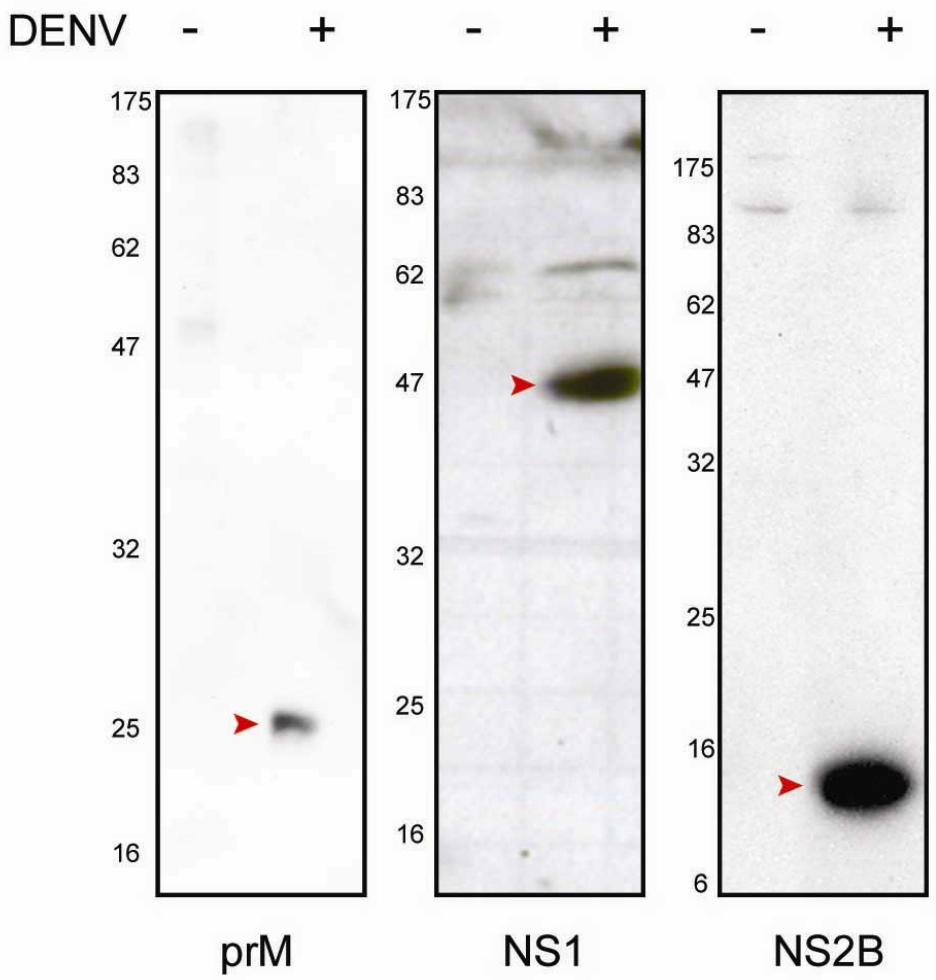

Figure S2. Characterization of DENV-Specific Antisera by Western Blotting

To investigate the localization of DENV NS-proteins in infected cells we generated rabbit polyclonal antisera monospecific for the individual non-structural proteins (production and characterization of antibodies directed against DENV-2 E, NS3, NS4A, NS4B, and NS5 was described elsewhere (Miller et al., 2007; Miller et al., 2006). After immunization of New Zealand White rabbits with recombinant purified DENV prM-, NS1- and NS2B-GST fusion proteins obtained antisera were characterized by Western blot analysis of lysates of DENV-infected Huh-7 cells. For this purpose, lysates of cells either infected with DENV (+) or mock-infected (-) were loaded onto a 12% polyacrylamide-SDS gel, and proteins were electrophoretically separated. After transfer of proteins to nitrocellulose membrane blots were incubated in blocking solution (5% milk powder and 0.5% Tween 20 in PBS) overnight at 4°C. Incubation with the primary antibody was performed in blocking solution for 1h at room temperature. Blots were washed 3 times for 10 min in washing solution (0.5% Tween 20 in PBS), incubated for 1h with the secondary

antibody in blocking solution, and washed again in the same way. Antibody-protein complexes were detected using the ECL plus western blotting detection system (Amersham Bioscience).

All antisera reacted with proteins of the expected sizes in DENV-infected, but not in mock-infected cells (red arrowheads). PrM was detected as a protein with an apparent molecular weight of approximately 26kDa. The NS1-specific antiserum detected an about 46 kDa protein corresponding to the expected size. In addition two NS1-reactive proteins with a higher molecular weight were observed. They may represent different glycosylated forms or homo- and hexameric forms of the protein as described elsewhere (Crooks et al., 1994; Flamand et al., 1999; Winkler et al., 1989; Winkler et al., 1988). NS2B was detected as a dominant 14 kDa species corresponding to the expected molecular weight (Falgout et al., 1993). These results confirmed the specificity of the rabbit antisera that were used throughout this study. Attempts to raise NS2A-specific antisera were not successful, probably due to poor immunogenicity and high hydrophobicity.

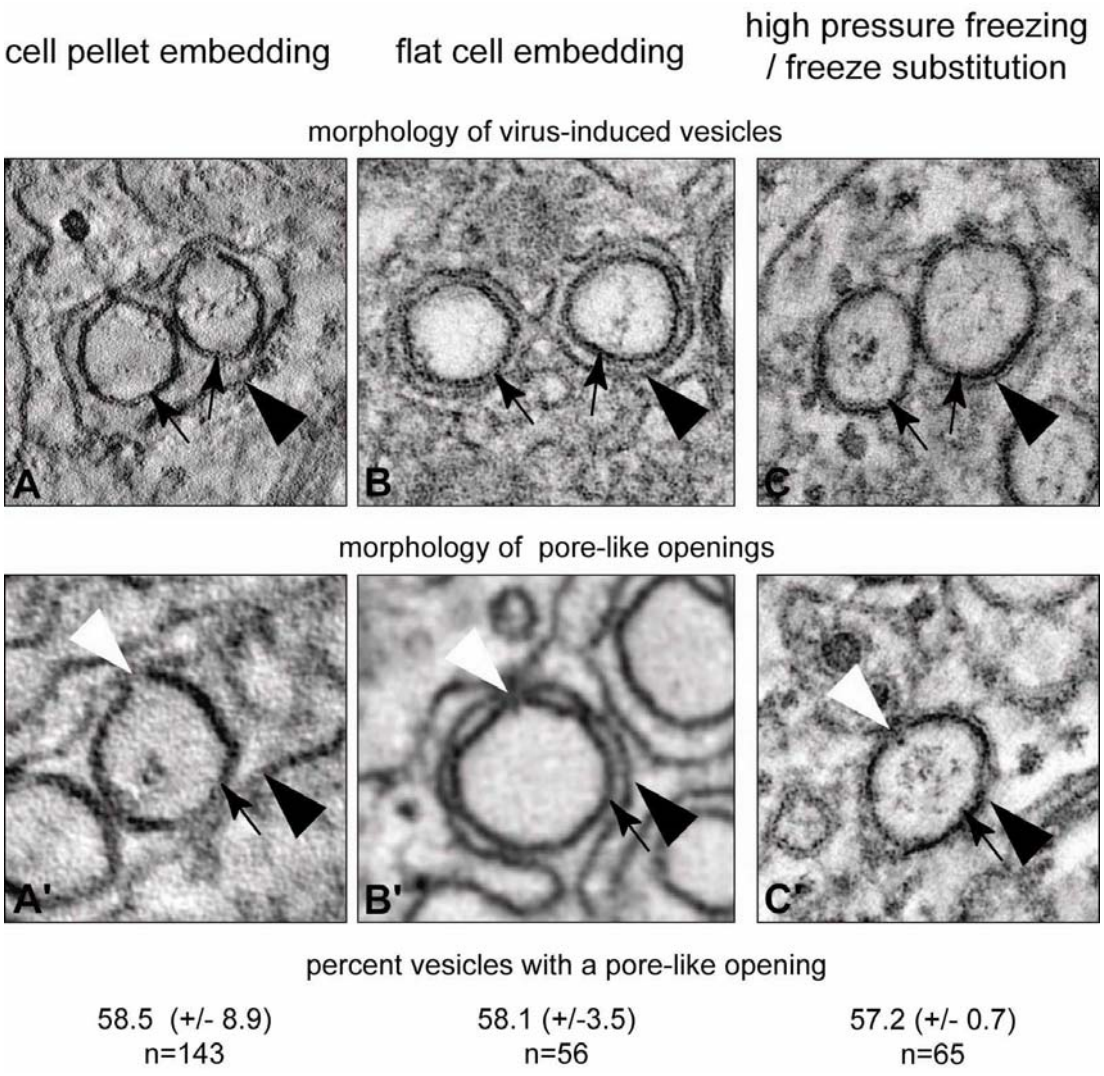

Figure S3. Overall Appearance of Virus-Induced Vesicles Is Influenced by Sample Preparation Technique, but Visibility of Membrane Continuities or Vesicle Pores Is Consistent

Huh-7 cells were infected with DENV2 and fixed 24 hr (left and middle panel) or 26.5 hr later (right panel) with 2.5% glutaraldehyde. Cells were then (A) scraped from the tissue culture substratum and embedded as a pellet or (B) flat-embedded on the tissue culture substratum at room temperature. In (C), cells were high-pressure frozen and freeze substituted at -90° to 0°C, followed by flat embedding at room temperature. In (A) the DENV-induced vesicles appear as single-membrane vesicles in the ER lumen, whereas in (B) the outer and inner membranes are more tightly apposed. In (C), the tight space between outer and inner membrane appears to be filled with an electron dense “glue”. Black arrowheads indicate outer ER membrane, small arrows indicate inner vesicle membrane. The appearance of pore-like vesicle openings and the diffuse electron density on their outside also depends on the sample preparation technique (A’-C’, white arrowheads), but membrane continuities of outer ER and inner vesicle membranes are seen at similar ratios independently of the sample preparation technique (bottom panel).

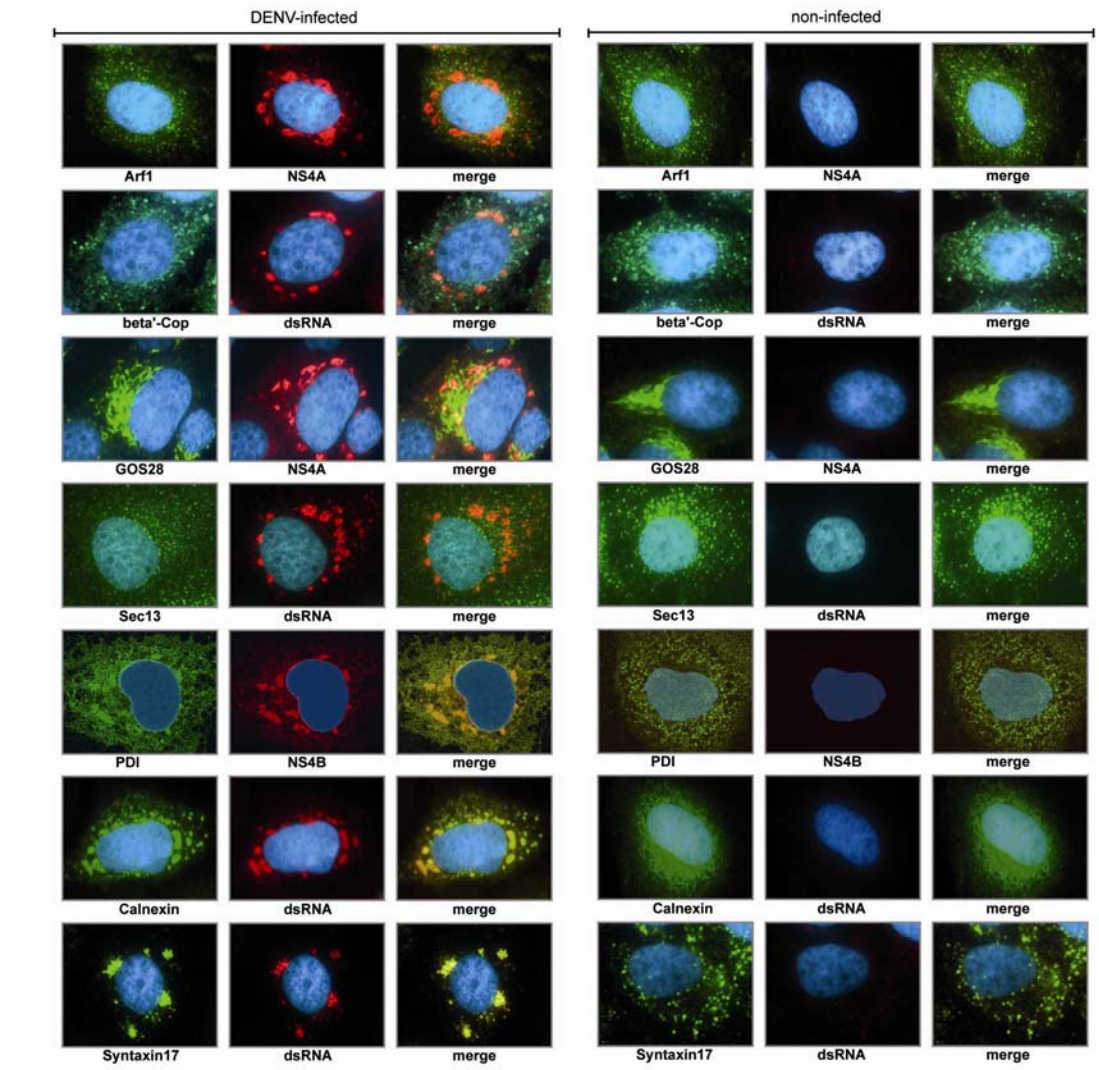

Figure S4. Colocalization of Cellular Marker Proteins with DENV dsRNA or Proteins by Immunfluorescence Microscopy

Huh-7 cells were infected with DENV2 (column 1 to 3 from the left) or left uninfected (column 4 to 6 from the left), fixed at 24 hr postinfection with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS and processed for immunofluorescence. Antibodies used for the detection of cellular marker proteins are given below each image. DENV NS4A was labeled with a monospecific rabbit polyclonal antibody; ds RNA, the presumed intermediate of DENV replication was labeled with a mouse monoclonal antibody. Nuclear DNA was stained with DAPI. The cellular markers for membranes involved in Golgi/vesicular trafficking, Arf1, GOS28, sec13 and β'-Cop (beta-Cop) do not co-localize with DENV-induced structures whereas the smooth ER marker Syntaxin17 as well as the ER markers PDI and calnexin show strong colocalization in infected cells. Cross-reaction of anti-Syntaxin17 antibody with anti-dsRNA antibody could be excluded by single labelling with anti-Syntaxin17 antibody (not shown). For a summary, see Table S1.

Table S1. Colocalization of Cellular Marker Proteins with DENV-Induced Membrane Structures

Marker	Reference/ Company	Organelle	Colocalization with RC (IF)	Colocalization with RC (EM)	Experiments described in:
Arf1	Abcam, Cambridge, USA	Vesicular trafficking	no	NT	This study
Calnexin	Stressgen, Victoria, Canada; (Meacham et al., 1999)	Rough ER	yes	yes	(Miller et al., 2007)
CLIMP63	Alexis Biochemicals, Lausen, Switzerland; (Schweizer et al., 1993)	Reticular subdomain of rough ER	yes	NT	(Miller et al., 2006)
ERGIC	Alexis Biochemicals, Lausen, Switzerland; (Mitrovic et al., 2008)	ER-Golgi intermediate Compartment	no	NL	(Miller et al., 2006)
GalT	E. Berger, Zurich, Switzerland; (Steet and Kornfeld, 2006)	Trans Golgi	no	NL	(Miller et al., 2006)
GM130	Antonella de Matteis, Mario Negri Sud, Santa Maria imbargo, Italy; (Marra et al., 2001)	Cis Golgi	no	no	(Miller et al., 2006)
Golgin97	Molecular Probes, USA; (Norkin and Kuksin, 2005)	Golgi apparatus	no	NT	(Miller et al., 2006)
GOS28	Acris, Herford, Germany	Golgi SNARE protein	no	NT	This study
Lamp-1	Iowa Hybridoma Bank; (Huynh and Andrews, 2005)	Lysosomes/late endosomes	no	no	This study
Oil Red O	Sigma, Deisenhofen, Germany; (Rouille et al., 2006)	Lipid Droplets	no	NT	(Miller et al., 2006)
P230	BD Biosciences, Heidelberg, Germany; (Duran et al., 2008)	Trans Golgi	no	no	(Miller et al., 2007)
PDI	Stressgen, Victoria, Canada; (Zuber et al., 2007)	Rough ER	yes	yes	(Miller et al., 2006)
Sec13	Wanjin Hong, IMB, Singapore; (Tang et al., 1997)	COPII vesicular trafficking	no	no	This study
ß'-COP	Jeremy Simpson, EMBL, Heidelberg, Germany; (Lowe and Kreis, 1995)	COPI vesicular trafficking	no	no	This study
Syntaxin17	R. Sheller, Stanford University, USA; (Reichelt et al., 2004)	Smooth ER	yes	NT	This study

Summary of colocalization studies of cellular marker proteins with DENV-induced membrane structures in this and previous studies (references in the rightmost column) at the level of light microscopy (third column from the left; dsRNA or viral nonstructural protein labeling was used as a marker of RC) and electron microscopy (second column from the right). Antibodies to cellular proteins and their organelle specificity are indicated. Source of the antibody and further references in which the corresponding antibodies were used are given (second column from left). NT, not tested; NL, no specific labeling by EM.

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