Dengue virus reduces expression of low-density lipoprotein receptor-related protein 1 to facilitate replication in *Aedes aegypti*

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*Aedes aegypti* is the primary vector of a number of viruses pathogenic to humans including dengue virus (DENV). DENV infection leads to widespread transcriptomic and proteomic alterations in mosquito cells. Here we identified alterations to the mosquito cell secretome during DENV infection by performing liquid chromatography tandem mass spectrometry. We found that an extracellular fragment of low-density lipoprotein receptor-related protein 1 (LRP-1) was present during infection. Previous literature suggests that LRP-1 regulates cholesterol homeostasis. Therefore, we hypothesized that DENV modifies LRP-1 protein expression to maintain host-derived intracellular cholesterol, which would facilitate virus replication within membrane-associated replication compartments. Accordingly, stimuli that are present during flavivirus infection reduced LRP-1 protein expression. We also found that dsRNA knockdown of LRP-1 increased intracellular cholesterol and DENV viral RNA. Further, depletion of intracellular lipids reduced infection. Together, these data suggest that DENV reduces LRP-1 protein expression, possibly through regulated intramembrane proteolysis (RIP), to increase intracellular cholesterol and facilitate replication in *Ae. aegypti*.

*Aedes aegypti* is the primary vector of a growing list of pathogenic viruses that cause disease in humans including dengue virus (DENV). DENV infection leads to multiple clinical manifestations ranging from a febrile illness called dengue fever to severe dengue, which can include septic shock1,2. There are no targeted antiviral therapies for DENV and use of a conventional vaccine may risk causing antibody-dependent enhancement of DENV or a related pathogenic flavivirus3. It is theoretically possible to target vector proteins or cellular pathways as a method to interfere with the flavivirus transmission cycle4–11. This strategy requires identification of proteins or cellular pathways that modify flavivirus acquisition or transmission.

Flavivirus acquisition in the mosquito begins when the mosquito engorges on the blood of an infected vertebrate host. Virions establish infection of midgut epithelial cells and infection leads to widespread transcriptomic and proteomic alterations4,12. We hypothesized that flavivirus infection alters the mosquito cell secretome – the proteins and peptides that are secreted into the extracellular milieu and play important pathological and physiological roles. Characterizing the secretome can identify key proteins and cellular pathways that are modified during infection and reveal new therapeutic targets4–15.

Here we find that DENV infection of *Ae. aegypti* cells leads to accumulation of extracellular fragments of low-density lipoprotein receptor-related protein 1 (LRP-1). LRP-1 belongs to a group of cell surface proteins that can undergo regulated intramembrane proteolysis (RIP) upon ligand binding in a Notch-like fashion. LRP-1 proteolysis leads to the release of both intracellular and extracellular domains. The intracellular domain mediates signal transduction. The role of the soluble extracellular domain is less clear16.

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Previous literature suggests that LRP-1 is a multifunctional cell surface receptor with diverse physiological roles ranging from cellular uptake of lipoproteins and other cargo to promoting cholesterol export and inhibiting accumulation of intracellular cholesterol\textsuperscript{12–24}. LRP-1 deficiency has led to intracellular lipid accumulation in a number of experimental models\textsuperscript{17–24}. In the context of cytomegalovirus (CMV) infection, LRP-1 inhibits virion infectivity by depleting intracellular cholesterol, which limits the amount of cholesterol in the viral envelope\textsuperscript{25}. We hypothesized that DENV infection modifies LRP-1 protein expression, which increases host-derived intracellular cholesterol - a requirement for synthesis of intracellular membrane replication compartments.

Materials and Methods

Cell culture and virus production. Two Aedes spp. cell lines were used in this study, Aag2 and C6/36 cells. The Ae. aegypti cell line, Aag2 (ATCC, VA), was used for in vitro studies. Aag2 cells were grown at 28°C with 5% CO2 in Schneider’s Drosophila Medium for transfection studies and DMEM high glucose media for the remaining studies. Both types of media were supplemented with 10% heat-inactivated fetal bovine serum (Gemini, CA), 1% penicillin-streptomycin, and 1% tryptose phosphate broth (Sigma, MO). Lipid-depleted serum was made by incubating serum with fumed silica overnight followed by removal of silica-lipid complexes by centrifugation\textsuperscript{26}. Lipid-depleted serum was added to cell culture components to make lipid-depleted media. In addition, the Ae. albopictus cell line, C6/36 (obtained from Erol Fikrig), was used to grow DENV stocks using the same media. The dengue strain used was DENV-2 New Guinea C. C6/36 cells were infected at an MOI of 1.0 virus stock was stored at −80°C until use.

LC + MS/MS. Cell free supernatants were taken from mock and DENV2-infected Aag2 cells at 1 and 7 dpi and submitted to the Interdisciplinary Center for Biotechnology Research at the University of Florida for liquid chromatography tandem mass spectrometry (LC + MS/MS). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot. Mascot was set up to search the Aedes aegypti_201505 database (selected for Ae. aegypti, unknown version, 37,800 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Gln- > pyro-Glu of the n-terminus, deamidated of asparagine and glutamine and oxidation of methionine were specified in Mascot containing at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm. Scaffold (version Scaffold_4.4.8, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.

qRT-PCR-based assays. RNA from Aag2 cells was isolated using RNeasy kit (Qiagen, CA) following the manufacturer’s instructions and RNA was kept at −20°C until testing. The qRT-PCR assay was done using the QuantFast kit according to the manufacturer’s instructions (Qiagen, CA). Oligos for the qRT-PCR reactions were: DENV-2 envelope (E) protein: F: 5′-CATTCCTAAGTGAGATCTCTTTTGCA-3′ R: 5′-CAGATCTCTGATGAATAACCAACG-3′; Ae. aegypti Actin: F: 5′-GAACACCCGCTGCTGACA-3′, R: 5′-TGCGTCATCTTCTCACGGTTAG-3′. Ae. aegypti LRP-1: 5′-3′ F: 5′-GAAGAATTCTCGAGTTGTGTACTACCCAAGC-3′; R: 5′-GGATCCCATATAGCGATTGTAGCGGTTTTTTG-3′. Ae. aegypti LRP-1 qRT-PCR-based assays were performed using the QuantiFast kit according to the manufacturer’s instructions (Qiagen, CA). Oligos for the qRT-PCR reactions were: DENV-2 envelope (E) protein: F: 5′-CATTCCTAAGTGAGATCTCTTTTGCA-3′ R: 5′-CAGATCTCTGATGAATAACCAACG-3′; Ae. aegypti Actin: F: 5′-GAACACCCGCTGCTGACA-3′, R: 5′-TGCGTCATCTTCTCACGGTTAG-3′. Ae. aegypti LRP-1: 5′-3′ F: 5′-GAAGAATTCTCGAGTTGTGTACTACCCAAGC-3′; R: 5′-GGATCCCATATAGCGATTGTAGCGGTTTTTTG-3′. Viral RNA or LRP-1 expression was normalized to either total cellular RNA or actin expression. Each sample was tested in duplicate.

dsRNA production and transfection. For gene knockdown, dsRNA was produced from approximately 500bp coding regions of either Ae. aegypti LRP-1 (VectorBase: AEEL007041, GenBank: EAT41289) or green fluorescent protein (GFP) as a control. Briefly, PCR was used to produce a DNA template with T7 overhangs that was then used with the Ambion Megascript kit according to manufacturer’s instructions to produce the dsRNA molecules. Oligos used for making dsRNA are as follows: GFP: F: 5′-TAATACGACTCACTATAGGAGATATTGACACCACTTACGGGAAAGC-3′ R: 5′-TAATACGACTCACTATAGGAGAGACCACTTACGGGACCTATCGACTACGACTA

Figure 1. Immunocytochemistry of DENV2-infected Aag2 cells. Aag2 cells were grown in glass chambers and were inoculated with virus free (Mock) or DENV2-infected cell culture media and then fixed 7 dpi. Fixed cells were incubated with anti-DENV E antibody followed by a secondary antibody containing a fluorophore (green). Cell nuclei were counterstained with DAPI. Images were taken at 100x magnification.
| Identifier   | Comment                                                                 |
|--------------|-------------------------------------------------------------------------|
| AAEL009437   | N/A                                                                     |
| AAEL012951   | transformation/transcription domain-associated protein                 |
| AAEL007132   | dynein heavy chain                                                      |
| AAEL007041   | low density lipoprotein receptor (ldl)                                  |
| AAEL012437   | N/A                                                                     |
| AAEL00981    | N/A                                                                     |
| AAEL007158   | mmp-1 protein (novel nuclear protein 1) (nmp-52)                        |
| AAEL010871   | N/A                                                                     |
| AAEL00430    | N/A                                                                     |
| AAEL008895   | peroxisome biogenesis factor 1 (peroxin-1)                             |
| AAEL004699   | N/A                                                                     |
| AAEL009431   | mitochondrial ribosomal protein, L10, putative                         |
| AAEL011239   | short-chain dehydrogenase                                              |
| AAEL005240   | rabaptin-5, putative                                                   |
| AAEL013174   | N/A                                                                     |
| AAEL013060   | N/A                                                                     |
| AAEL008220   | N/A                                                                     |
| AAEL017221   | odorant receptor                                                       |
| gi|167515086 | sodium-dependent nutrient amino acid transporter 8                     |
| AAEL007980   | N/A                                                                     |
| AAEL011526   | GPCR Methuselah Family                                                 |
| AAEL003575   | N/A                                                                     |
| AAEL00589    | serine/threonine protein kinase                                        |
| AAEL010607   | galactose-1-phosphate uridylyltransferase                              |
| AAEL011693   | mitotic control protein dx3                                            |
| AAEL010459   | N/A                                                                     |
| AAEL011810   | N/A                                                                     |
| AAEL009382   | lysine-specific demethylase NO66                                       |
| AAEL006340   | N/A                                                                     |
| AAEL006645   | N/A                                                                     |
| AAEL009477   | N/A                                                                     |
| AAEL015352   | sashchin, putative                                                     |
| AAEL006765   | N/A                                                                     |
| AAEL003020   | tail less (tl)                                                         |
| AAEL012825   | bifunctional purine biosynthesis protein                               |
| AAEL005319   | myosin light chain kinase                                              |
| AAEL001796   | nuclear hormone receptor (HR78)                                        |
| AAEL006708   | hedgehog                                                               |
| AAEL010693   | N/A                                                                     |
| AAEL004064   | meiotic checkpoint regulator cut4                                      |
| AAEL002508   | 26S protease regulatory subunit 6a                                      |
| AAEL001716   | THO complex subunit 2 (Tho2)                                           |
| AAEL002544   | N/A                                                                     |
| AAEL006352   | N/A                                                                     |
| AAEL002628   | N/A                                                                     |
| AAEL004600   | N/A                                                                     |
| AAEL003168   | N/A                                                                     |
| AAEL011965   | nuclear lamin L1 alpha, putative                                       |
| AAEL014144   | rapsynoid                                                              |
| AAEL006247   | N/A                                                                     |
| AAEL001442   | map-kinase activating death domain protein                             |
| AAEL012744   | N/A                                                                     |
| AAEL013473   | cAMP-specific 3,5-cyclic phosphodiesterase                            |
| AAEL001596   | myosin                                                                  |
| AAEL004376   | N/A                                                                     |
| AAEL013466   | ankyrin 2,3/unc44                                                      |

Continued
| Identifier | Comment                                      |
|------------|----------------------------------------------|
| AAEL009159 | nervous wreck, putative                      |
| AAEL009847 | microtubule-associated protein               |
| AAEL001582 | kinesin                                      |
| AAEL007079 | N/A                                          |
| AAEL006958 | cell adhesion molecule                       |
| AAEL004299 | angiotensin-converting-related enzyme         |
| AAEL002896 | N/A                                          |
| AAEL014296 | major sperm protein                          |
| AAEL008559 | glutaminase                                   |
| AAEL012158 | WD repeat-containing protein 48              |
| AAEL003008 | N/A                                          |
| AAEL007857 | N/A                                          |
| AAEL008610 | myosin vii                                   |
| AAEL005681 | GPCR Histamine Family                        |
| AAEL006187 | translational activator gcn1                 |
| AAEL009878 | N/A                                          |
| AAEL009019 | N/A                                          |
| AAEL012072 | histone H3                                   |
| AAEL009762 | cytochrome P450                               |
| AAEL003641 | sodium/chloride dependent amino acid transporter |
| AAEL001598 | N/A                                          |
| AAEL006806 | N/A                                          |
| AAEL011187 | U520                                         |
| AAEL015365 | N/A                                          |
| AAEL008591 | zinc finger protein, putative                |
| AAEL004644 | N/A                                          |
| AAEL008704 | N/A                                          |
| AAEL010473 | NAD dependent epimerase/dehydratase          |
| AAEL008365 | N/A                                          |
| AAEL014885 | N/A                                          |
| gi|70907192 | N/A                                          |
| AAEL013903 | gamma-tubulin complex component 2 (gcp-2)   |
| AAEL009480 | N/A                                          |
| AAEL009152 | N/A                                          |
| AAEL006050 | nuclear RNA export factor 2 (NXF2), putative |
| AAEL003229 | N/A                                          |
| AAEL009049 | N/A                                          |
| AAEL000489 | P21-activated kinase, pak                     |
| AAEL014315 | N/A                                          |
| AAEL010575 | dimethyladenosine transferase                |
| AAEL004049 | N/A                                          |
| AAEL017063 | gustatory receptor Gr32                      |
| AAEL007565 | leucine rich protein, putative               |
| AAEL006394 | N/A                                          |
| AAEL002814 | N/A                                          |
| AAEL015467 | segmentation protein cap'n'collar            |
| AAEL002256 | collagen alpha 1(xviii) chain                |
| AAEL012774 | protease m1 zinc metalloprotease             |
| AAEL002850 | patched 1                                    |
| AAEL000318 | N/A                                          |
| AAEL000168 | N/A                                          |
| AAEL011566 | cell adhesion molecule                       |
| AAEL001773 | N/A                                          |
| AAEL004320 | WOC protein, putative                        |
| AAEL007400 | signal recognition particle 54 kda protein   |
| AAEL006067 | N/A                                          |
not identified in mock-infected supernatants at 1 or 7 dpi (Tables 1 and 2, Fig. 2A). Only 6 proteins were detected in mock infected samples. Approximately 80% of proteins detected in DENV-infected Aag2 cell supernatants were collected 1 and 7 dpi and submitted for liquid chromatography tandem mass spectrometry (LC-MS/MS) to detect the proteins in the secretome. We identified a total of 239 and 120 proteins in mock and DENV2-infected supernatants at 1 and 7 dpi, respectively. The majority of proteins detected during infection were unique compared to conditions according to SignalP4.1, previous literature has characterized protein-protein interactions between dynein heavy chain and myosin with flavivirus structural proteins suggesting that these proteins may end up in the supernatant. Three of the proteins (SeqIDs, AAEL013174, AAEL002544, and AAEL01773) lacked homology to any known proteins. The remaining three proteins were annotated as dynein heavy chain (AAEL007132), myosin (AAEL000596), and inorganic phosphate cotransporter, putative (AAEL001113). Although each of these proteins also lacked signal peptides according to SignalP4.1, we used these sequences for further analysis. We chose to investigate the six proteins found in DENV2-infected Aag2 cell supernatants at both 1 and 7 dpi. Three of the proteins (SeqIDs, AAEL013174, AAEL002544, and AAEL01773) lacked homology to any known protein and lacked predicted signal peptides according to SignalP4.1 and were eliminated from further analysis. The remaining three proteins were annotated as dynein heavy chain (AAEL007132), myosin (AAEL000596), and low-density lipoprotein receptor (Idl) (AAEL007041). Although each of these proteins also lacked signal peptides according to SignalP4.1, previous literature has characterized protein-protein interactions between dynein heavy chain and myosin with flavivirus structural proteins suggesting that these proteins may end up in the supernatant.

### Table 1. LC + MS/MS hits specific to DENV2-infected Aag2 cell supernatant 1 dpi.

| Identifier | Comment |
|------------|---------|
| AAEL012483 | N/A     |
| AAEL014664 | AMP dependent coa ligase |
| AAEL003829 | dynamin |
| AAEL001113 | inorganic phosphate cotransporter, putative |
| AAEL013085 | N/A     |
| AAEL011231 | N/A     |
| AAEL010765 | N/A     |
| AAEL010308 | N/A     |

TAGGGAGAatcgacgtttcggacgaagacgcggacc-3': 5'-TAATACGACTCACTATAGGGAGATTAATGCTAGGTGAGT GTTCGTgttggtac-3'. The dsRNA molecules were transfected into cells using Lipofectamine 2000 (Thermo Fisher) according to manufacturer's instructions.

**Intracellular cholesterol content.** Total intracellular cholesterol was measured using the Amplex Red Cholesterol Assay Kit (Invitrogen). Aag2 cells were lysed in PBS containing 1% Triton X-100 and protease inhibitor cocktail. 2 µg of total protein was used for each sample. The reactions were brought up to a total reaction volume of 50 ul using 1x reaction buffer from the kit and applied to individual wells on a 96-well microplate. 50 µl of Amplex Red reagent/HRP/cholesterol oxidase/cholesterol esterase working solution was added to each well and the plate was incubated at 37°C for 30 minutes protected from light. The fluorescence was measured using a Tecan Infinite M200 Pro microplate reader using excitation at 550 nm and emission detection at 590 nm.

**Western blot.** Aag2 cells were lysed using RIPA buffer containing a protease inhibitor cocktail (Sigma). Protein concentration was determined using a BCA assay. 9 µg of total protein from each sample was separated by a SDS-PAGE gel (5–9%) and transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk at room temperature for 1 hour prior to overnight incubation at 4°C with primary antibody anti-LRP1 (cat. no. ab 92544, 1:20000, Abcam) and anti-actin antibody (cat. No. SC-1615, 1:1000, Santa Cruz Biotechnology) as a loading control. The membranes were washed with PBST and incubated at room temperature for 1 hour with anti-rabbit IgG-HRP (cat. no. 70745, 1:2000, Cell Signaling Technology) and anti-goat IgG-HRP linked (cat. no. ab6741, 1:2000, Abcam) secondary antibodies. Signal was detected using a ChemiDoc Touch Imaging System by Biorad.

**Focus-forming unit assay.** Aag2 cells were fed with complete or lipid-depleted media for 7 days and then inoculated with 50 focus-forming units of DENV2 in complete media. Unbound virus was removed after one hour and complete media was added. Confluent monolayers were fixed with 4% paraformaldehyde and stained with 1:200 anti-DENV2 envelope antibody (3H5-1, EMD Millipore) 3 days post-infection and the total number of flavivirus positive foci were counted per well. Flavivirus positive foci were revealed using a 1:2000 horseradish peroxidase-conjugated secondary antibody and a Vectastain ABC kit immunocytochemistry kit. Digital images were taken using an Evos Core inverted microscope.

**Results**

**Alterations of the Aag2 secretome during DENV infection.** We hypothesized that DENV infection would alter the secretome of mosquito cells. To test this hypothesis, we first confirmed previous results that the Ae. aegypti Aag2 cell line was permissive to DENV2 infection by inoculating cells with or without 1 × 10^6 genome equivalents of DENV2 New Guinea Strain C. Cells were fixed 7 days post infection (dpi) and stained with an anti-DENV2 envelope monoclonal antibody and secondary antibody conjugated with Alexa Fluor 488. Aag2 cells were highly permissible to infection and DENV2 envelope protein was observed in almost every cell (Fig. 1). Aag2 cells were then inoculated with approximately 1 × 10^6 genomic equivalents of DENV2 for 1 hr, unbound virus was removed, and fresh media was added. Mock-infected controls were included. Cell-free supernatants were collected 1 and 7 dpi and submitted for liquid chromatography tandem mass spectrometry (LC-MS/MS) to detect the proteins in the secretome. We identified a total of 239 and 120 proteins in mock and DENV2-infected supernatants at 1 dpi, respectively. We identified a total of 183 and 136 proteins in mock and DENV2-infected supernatants at 7 dpi, respectively. The majority of proteins detected during infection were unique compared to mock infected samples. Approximately 80% of proteins detected in DENV-infected Aag2 cell supernatants were not identified in mock-infected supernatants at 1 or 7 dpi (Tables 1 and 2, Fig. 2A). Only 6 proteins were detected in DENV2-infected Aag2 cell supernatants but not mock-infected supernatants at both 1 and 7 dpi (Table 3, Fig. 2A).
| Identifier  | Comment                                      |
|-------------|----------------------------------------------|
| AAEL00845   | N/A                                          |
| AAEL001454  | N/A                                          |
| AAEL004179  | N/A                                          |
| AAEL007225  | dynein heavy chain                           |
| AAEL011083  | N/A                                          |
| AAEL007132  | dynein heavy chain                           |
| AAEL013943  | mediator complex, 100kD-subunit, putative    |
| AAEL015065  | spectrin                                      |
| AAEL013690  | DNA mismatch repair protein pms2             |
| AAEL006309  | N/A                                          |
| AAEL006374  | N/A                                          |
| AAEL006954  | N/A                                          |
| AAEL008154  | N/A                                          |
| AAEL000510  | N/A                                          |
| AAEL011103  | centromere/kinetochore protein zw10          |
| AAEL010342  | racGTPase-activating protein                 |
| AAEL007099  | environmental stress-induced protein, putative |
| AAEL011433  | small nuclear ribonucleoprotein sm d3        |
| AAEL009220  | N/A                                          |
| AAEL001581  | N/A                                          |
| AAEL008608  | N/A                                          |
| AAEL008318  | cadherin, putative                           |
| gi|167651374 | phosphoglycerate kinase, partial             |
| AAEL011178  | posterior sex combs protein                  |
| AAEL005881  | autocrine motility factor receptor, amfr     |
| AAEL002101  | N/A                                          |
| AAEL012446  | Inhibitor of Apoptosis (IAP) containing Baculoviral IAP Repeate(s) |
| AAEL012338  | N/A                                          |
| AAEL003044  | fasciclin-1 precursor                        |
| AAEL013593  | N/A                                          |
| AAEL009660  | N/A                                          |
| AAEL009839  | nuclear cap-binding protein subunit 1        |
| AAEL001921  | N/A                                          |
| AAEL001201  | N/A                                          |
| AAEL009308  | WD-repeat protein                            |
| AAEL012587  | N/A                                          |
| AAEL013227  | PIWI                                         |
| AAEL002782  | N/A                                          |
| AAEL001357  | N/A                                          |
| AAEL006790  | N/A                                          |
| AAEL002250  | terminal deoxycytidyl transferase rev1       |
| AAEL004834  | PHD finger protein                            |
| AAEL008670  | protein tara                                  |
| AAEL006082  | transcription initiation factor TFIIA subunit 1 |
| AAEL010336  | zinc phosphodiesterase                       |
| AAEL005211  | microtubule associated-protein orbit         |
| AAEL000668  | N/A                                          |
| AAEL002551  | DNA topoisomerase type 1                     |
| AAEL005284  | receptor tyrosine phosphatase type r2a        |
| AAEL003739  | M-type 9 protein, putative                   |
| AAEL008357  | zinc finger protein                           |
| AAEL006339  | peptidylglycine alpha-amidating monooxygenase C-terminal protein-1 |
| AAEL002670  | AMP dependent ligase                         |
| AAEL006808  | N/A                                          |
| AAEL002070  | N/A                                          |
|             | Continued                                    |
| Identifier   | Comment                                                                 |
|--------------|-------------------------------------------------------------------------|
| AAEL013110   | N/A                                                                     |
| AAEL005381   | dissatisfaction (Dsf)                                                   |
| AAEL001438   | N/A                                                                     |
| AAEL011052   | N/A                                                                     |
| AAEL004017   | DNA polymerase v                                                        |
| AAEL015631   | asparagine synthetase                                                   |
| AAEL014138   | serine protease inhibitor (serpin)                                      |
| AAEL005450   | N/A                                                                     |
| AAEL003105   | supervillin                                                             |
| AAEL000007   | N/A                                                                     |
| AAEL00596    | myosin                                                                  |
| AAEL010061   | N/A                                                                     |
| AAEL007111   | N/A                                                                     |
| AAEL001411   | myosin heavy chain, nonmuscle or smooth muscle                          |
| AAEL012331   | N/A                                                                     |
| AAEL005484   | sorting nexin                                                           |
| AAEL013534   | tgf-beta resistance-associated protein trag                            |
| AAEL005180   | N/A                                                                     |
| AAEL014995   | ornithine decarboxylase                                                  |
| AAEL013559   | uncoordinated protein                                                   |
| AAEL013561   | N/A                                                                     |
| AAEL004839   | cyclin t                                                                |
| AAEL013635   | N/A                                                                     |
| AAEL005949   | beta-1,2-n-acetylglicosaminyltransferase ii                             |
| AAEL004136   | N/A                                                                     |
| AAEL009164   | tRNA delta(2)-isopentenylyphosphosphate transferase                    |
| AAEL004219   | zap GTPase-activating protein                                           |
| AAEL010940   | N/A                                                                     |
| AAEL010916   | N/A                                                                     |
| AAEL009673   | GPCR Gonadotropin Releasing Hormone Family                              |
| AAEL010161   | N/A                                                                     |
| AAEL007041   | low density lipoprotein receptor (ldl)                                  |
| AAEL013999   | N/A                                                                     |
| AAEL007447   | N/A                                                                     |
| gi|90655947    | female specific 1 protein                                               |
| AAEL011551   | N/A                                                                     |
| AAEL009958   | N/A                                                                     |
| AAEL005101   | carboxy/choline esterase alpha esterase                                 |
| AAEL008943   | chromatin assembly factor 1, p180-subunit, putative                     |
| AAEL012223   | zinc finger protein                                                     |
| AAEL008462   | api5 protein                                                            |
| AAEL003939   | eukaryotic translation initiation factor 2-alpha kinase 3              |
| AAEL010341   | Ruvb-like helicase 2                                                   |
| AAEL000912   | N/A                                                                     |
| AAEL004854   | N/A                                                                     |
| AAEL001375   | Y-box binding protein                                                   |
| AAEL005461   | N/A                                                                     |
| AAEL002357   | N/A                                                                     |
| AAEL008358   | ablim                                                                   |
| AAEL002833   | cathepsin l                                                             |
| AAEL012412   | slit protein                                                            |
| AAEL007820   | N/A                                                                     |
| AAEL008423   | N/A                                                                     |
| AAEL011791   | N/A                                                                     |
| AAEL008653   | engulfment and cell motility protein                                    |
| AAEL004917   | N/A                                                                     |
| AAEL001649   | leucine aminopeptidase                                                  |

Continued
during shedding of progeny virions or through secretion of exosomes. We therefore chose to further investigate the role of AAEL007041 in DENV infection.

DENV infection reduces LRP-1 protein expression. Despite its annotation, BLAST analysis of AAEL007041 revealed that this protein is homologous to low-density lipoprotein receptor-related protein 1 (LRP-1) rather than low-density lipoprotein receptor (LDLR). LRP-1 belongs to a group of cell surface proteins that can undergo regulated intramembrane proteolysis (RIP) upon ligand binding in a Notch-like fashion. LRP-1 proteolysis leads to the release of both intracellular and extracellular domains. Additionally, LRP-1 has been identified as a factor that reduces accumulation of intracellular cholesterol. We hypothesized that DENV infection modifies LRP-1 protein expression, which would impact intracellular cholesterol levels. We first confirmed that the two LRP-1 peptides we detected by LC-MS/MS resided in the approximately 500 kDa extracellular domain. Both peptides (i.e., CVGIDNFLMYSIGHQLK and ADYDGRNRV) were located in the 500 kDa extracellular domain of LRP-1. We then determined if LRP-1 expression is altered at the transcriptional level. DENV infection did not alter LRP-1 gene expression 1 or 7 dpi (Fig. 2B,C). Our result is consistent with previously published RNASeq data that determined the impact of DENV infection on gene expression in Ae. aegypti carcass, midgut, and salivary gland tissue. In contrast, Western blot analysis using a monoclonal antibody against an evolutionarily conserved epitope in the 85 kDa intracellular LRP-1 domain revealed that DENV2 infection reduced LRP-1 protein expression (Fig. 2D). These results indicate that DENV infection reduces LRP-1 protein expression while increasing peptides that correspond to LRP-1’s extracellular domain.

Diverse stimuli reduce LRP-1 protein expression in mosquito cells. Previous research identified a number of physiologic stimuli that can reduce LRP-protein expression, and this process often occurs through RIP. Both reactive oxygen species and cholesterol depletion can reduce LRP-1 protein expression. Flavivirus infection is known to lead to the production of reactive oxygen species and it modifies intracellular cholesterol. We determined if reactive oxygen species and lipid depletion could reduce LRP-1 protein expression in Aag2 cells. Hydrogen peroxide was used as a prototypical reactive oxygen species that is induced during DENV infection. A six-hour treatment with 1 mM hydrogen peroxide reduced LRP-1 protein expression (Fig. 3A). We also tested if treatment of Aag2 cells with lipid-depleted media reduced intracellular cholesterol concentration after 1, 2, and 3 days. Intracellular cholesterol concentration was significantly reduced 2 and 3 days post treatment (Fig. 3B). A three-day treatment with lipid-depleted media reduced LRP-1 expression (Fig. 3C). These data support that cellular stimuli that occur during flavivirus infection can reduce LRP-1 protein expression. We then determined if DENV infection creates a cellular environment that would mimic lipid depletion by quantifying the cholesterol content in uninfected and infected cells each day during a 1-week time course. We found that DENV infection limited intracellular cholesterol accumulation similar to our treatment with...
lipid-depleted media (Figs 3C and 4). These data reveal that DENV infection reduces cholesterol accumulation in mosquito cells, which is a stimulus that can reduce LRP-1 protein expression. LRP-1 decreases intracellular cholesterol content and inhibits DENV replication. One function of LRP-1 is to limit the concentration of intracellular cholesterol18,21,23–25. We hypothesized DENV reduces LRP-1 protein expression to maintain intracellular cholesterol levels, which facilitates virus replication. In order to test this hypothesis, we transfected Aag2 cells with control or LRP-1 dsRNA and confirmed gene knockdown by qRT-PCR (Fig. 5A). We then transfected Aag2 cells with control or LRP-1 dsRNA, and then inoculated cells with DENV2. We assessed intracellular cholesterol content and total viral RNA 24 hours post infection (hpi). LRP-1 knockdown significantly increased intracellular cholesterol levels (Fig. 5B). Further, LRP-1 knockdown significantly increased total viral RNA levels (Fig. 5C). These data suggest that LRP-1 knockdown promotes intracellular cholesterol accumulation and that this correlates with increased virus replication.

We confirmed that intracellular cholesterol is important for DENV infection by growing Aag2 cells in both complete and lipid-depleted media for 7 days. Cells grown in lipid-depleted media had significantly less intracellular cholesterol (Fig. 6A). Both control and lipid-depleted cells were inoculated with DENV in complete media,

| Identifier     | Comment                  |
|----------------|--------------------------|
| AAEL007132     | dynein heavy chain       |
| AAEL000596     | myosin                   |
| AAEL007041     | low density lipoprotein receptor (ldl) |
| AAEL013174     | N/A                      |
| AAEL002544     | N/A                      |
| AAEL001773     | N/A                      |

Table 3. LC + MS/MS hits specific to DENV2-infected AaG2 cell supernatants 1 dpi and 7 dpi.
unbound virus was removed, and complete media was replaced onto the cells. A focus forming unit assay was performed 3 dpi and a statistically significant decrease in virus replication was observed on the lipid-depleted cells (Fig. 6B,C). These data confirm that intracellular cholesterol facilitates DENV infection.

Aag2 cells grow in lipid-depleted media. Our data suggest that DENV reduces LRP-1 protein expression in order to maintain intracellular cholesterol to facilitate its replication; however, we also found that Aag2 cells reduce LRP-1 protein expression when treated with lipid-depleted media (Fig. 3B,C). It is possible lipid depletion is also harmful to the cell, and that both the virus and the cell benefit by reducing LRP-1 protein expression. To test this hypothesis, we determined the growth kinetics of uninfected and infected Aag2 cells in the presence of complete and lipid-depleted media. We found that Aag2 cells grown in lipid-depleted media had a marginal growth disadvantage that was not statistically significant and that the growth kinetics did not change during infection (Fig. 7A,B). These data suggest that a reduction in host-derived intracellular lipids do not significantly impact cell growth.

Discussion

DENV is one of the most important vector-borne human diseases, and has the potential to spread due to changes in climate and movement of humans and animals. Few effective options are available to combat the spread of this disease. New or improved control strategies are needed. There is increasing interest in the molecular mechanisms of DENV replication in its mosquito vector as a prophylactic or therapeutic target because of proof-of-principle studies showing that it is theoretically possible to target vector proteins or pathways that interfere with acquisition and transmission of vector-borne diseases.

A number of recent studies have shown the importance of cholesterol homeostasis during DENV replication in *Ae. aegypti*. For instance, *Wolbachia* infection perturbs intracellular cholesterol and vesicular trafficking and leads to inhibition of virus replication. Further, altering cholesterol distribution by modifying expression of sterol carrier protein 2 inhibited DENV infection. Our laboratory found that human low-density lipoprotein

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**Figure 3.** Diverse cellular stimuli reduce LRP-1 protein expression. (A) Western blot analysis of LRP-1 and actin in Aag2 cell lysates treated for six-hours with 1 mM hydrogen peroxide. (B) Cholesterol content was determined in Aag2 cells treated with complete (C) and lipid-depleted (LD) media 1, 2, and 3 dpt. The experiment was performed in triplicate and Student’s t tests were performed between groups to assess statistical significance. (C) Western blot analysis of LRP-1 and actin in Aag2 cells treated for three days with lipid-depleted media.

**Figure 4.** Cholesterol content in uninfected and infected cells. Cholesterol content was determined in mock (circles) and DENV-infected (squares) Aag2 cells each day for seven days. The experiment was performed in triplicate and Student’s t tests were performed between groups to assess statistical significance.
Figure 5. LRP-1 knockdown increases intracellular cholesterol and DENV viral RNA. (A) Total cellular RNA was extracted from Aag2 cells 24 hours after being either transfected with GFP dsRNA, or transfected with LRP1 dsRNA. A no dsRNA control was included. qRT-PCR was performed to determine LRP-1 gene expression and normalized to actin gene expression. (B,C) Aag2 cells were transfected with GFP or LRP-1 dsRNA. A no dsRNA control was included. Cells were then either Mock or DENV2-infected 24 hpt. (B) Cholesterol content was determined 24 hpi. (C) qRT-PCR was performed to determine intracellular DENV2 vRNA levels 24 hpi. Each experiment was performed in triplicate and Student’s t tests were performed between groups to assess statistical significance.

Figure 6. Lipid depletion reduces DENV infection. (A) Cholesterol content was determined in Aag2 cells treated with complete (C) and lipid-depleted (LD) media 7 dpt. (B) Aag2 cells treated with complete or lipid-depleted media for 7 days were inoculated with DENV2 in complete media for 1 hr. Unbound virus was removed and complete media was added for 3 days. Cells were fixed and DENV2-positive foci were revealed used DENV2-specific antibody. Foci were quantified in each well. Mock-infected controls were included. (C) Representative 20x images of the focus forming unit assay are shown. Experiments were performed in triplicate and Student’s t tests were performed between groups to assess statistical significance.
inhibits flavivirus infection in vitro and in vivo. It is clear that DENV is dependent on cholesterol itself or lipid sorting pathways in the mosquito.

Cholesterol dependence is curious given that Ae. aegypti is a cholesterol auxotroph; although, this lipid is readily available in each blood meal. Aag2 cells and the midgut epithelium are able to take up human LDL via clathrin-mediated endocytosis. Cholesterol uptake appears to benefit oogenesis, although our current data suggests that providing cell with lipid-depleted media only marginally reduces cell growth rate. Ae. aegypti cells are able to synthesize their own lipids from protein and sugar, so host lipids are likely not required for growth of somatic cells.

The current study identified extracellular fragments of Ae. aegypti LRP-1 that were only present during DENV2 infection. We confirmed that LRP-1 protein expression is reduced during infection, and during stimuli that occur during virus infection such as exposure to reactive oxygen species and changes in cholesterol concentration. Additional research is required to determine if the level of stimulus is equal during virus infection. DENV2 infection also limited cholesterol accumulation, which either occurred by promoting shedding of intracellular cholesterol or preventing cholesterol uptake.

LRP-1 has been identified as a factor that reduces accumulation of intracellular cholesterol. We hypothesized that this function of LRP-1 would have an antiviral effect. We confirmed this hypothesis by performing RNAi and showed that LRP-1 knockdown increased both intracellular cholesterol content and DENV2 vRNA. We also showed that depletion of host lipids from Aag2 cells reduced DENV2 infection. A caveat to our study is that we have not directly linked the extracellular peptides that were identified by LC-MS/MS to RIP, which would require more research into structural alteration of Aedes aegypti LRP-1 during physiological stimuli and identification of enzymes responsible for its proteolytic cleavage. Unfortunately, the enzymes identified in vertebrate systems are not evolutionarily conserved in mosquitoes. Further research is required to understand the details of RIP in mosquito cells.

These data provide support that LRP-1 functions to reduce host-derived intracellular cholesterol content, and that DENV2 replication is enhanced when cholesterol levels are increased. Our data suggest that multiple stimuli that are present during virus infection can reduce LRP-1 protein expression and that this may be due to RIP. Future studies will reveal the molecular pathways that Ae. aegypti use to acquire and maintain host lipids and how these pathways benefit flaviviruses during acquisition and transmission.

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Author Contributions
M.O.T. performed experiments in Figures 1–3, and Figure 5. B.L.R., A.T., and T.M.C. performed experiments in Figure 4. K.M.C. performed experiments in Figure 6. M.J.C. submitted samples for LC+MS/MS, analyzed all data, and wrote the manuscript. All authors reviewed the manuscript.

Additional Information
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