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DENGUE VIRUS UTILIZES NBR1 DURING INDUCED LIPOPHAGY AND FOR VIRAL EGRESS

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Dedicated to my family who have been there for me through my entire life and supportive throughout my scientific career.

To my loving and inspiring partner Michelle Cully who has been a foundation and a source of motivation these past six years.

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Abstract

Dengue virus (DENV) infection induces a proviral lipophagy to mobilize lipids from the lipid droplet. This study demonstrates that the selective autophagy receptor Neighbor of BRCA 1 (NBR1) is recruited to lipid droplets after DENV infection. Silencing NBR1 does not alter the induction of autophagy in DENV-infected cells, but specifically inhibits the DENV NS4A & NS4B-induced depletion of lipid droplets. Silencing NBR1 does not impact RNA replication nor viral assembly but does inhibit release of infectious particles via a non-canonical secretion method. Exogenous addition of free fatty acids allows DENV to leave via this uncharacterized method in NBR1 silenced cells. This uncharacterized secretion method possibly allows DENV to escape the cell in an extracellular vesicle protecting it from antibody neutralization. The data from this study suggests that NBR1 and DENV induced lipophagy play a critical role in the autophagic secretion of DENV.

Chapter 1: Introduction

Dengue Virus

Dengue virus (DENV), a member of the *Flaviviridae* family, is a single-stranded positive-sense RNA virus. Its 10.7kb genome consists of 10 genes: seven nonstructural genes (Nonstructural protein 1 (NS1), NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and 3 structural genes (Envelope (E), Membrane (M), and Capsid (C))[1]. The viral capsid protein and the RNA genome form a complex referred to as the nucleocapsid, the Membrane and Envelope protein along with the host derived membrane make up the viral envelope [2,3]. There are four distinct serotypes of DENV: DENV-1, -2, -3, and -4, with all four serotypes capable of causing disease in humans [4]. Recently a newly discovered fifth serotype, DENV-5, was isolated in Malaysia [5]. As the most common arbovirus it is primarily transmitted by the *Aedes aegypti* mosquito, but other *Aedes* mosquito species, such as *Aedes albopictus*, can act as secondary vectors [6]. Dengue outbreaks are cyclic with large outbreaks occurring every 3-5 years [7].

As an emerging global pathogen, DENV infects almost 400 million people every year manifesting as disease in 96 million cases [8]. Approximately 3.6 billion people currently live in areas at risk for dengue infection and about 75% of DENV infections occur in tropical and subtropical regions such as South America, Africa, and South/Southeast Asia [8–10]. These infections result in approximately 500,000 hospitalizations every year due to diseases such as dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) resulting in 22,000 annual global deaths [9]. 2-7 days after an infected mosquito bites a human, DENV may cause DF in the host. Afterwards it may progress to DHF which if left untreated may advance to DSS as an uncontrolled cytokine

storm occurs [3]. Despite such a fatal disease progression, there are currently no clinically approved therapeutics for DENV infection, although there is a tetravalent vaccine licensed in endemic countries that protects against ~2/3rds of disease [11,12].

In vitro DENV infects a wide variety of cell types including hepatocytes such as Huh7 and HepG2 cells, fibroblasts, endothelial cells such as HUVEC, monocytes/macrophages like U937 and THP-1 cells, fibroblasts, lymphoblasts, and cells derived from the mosquito midgut such as C6/36 cells [13–17]. Despite this wide tropism in cell culture, DENV primarily infects monocytes, macrophages, and dendritic cells *in vivo* for dissemination [18]. Infection of these cells may be enhanced in the presence of non-neutralizing antibodies or a sub-neutralizing concentration of antibodies via a process called antibody-dependent enhancement of infection. These non-neutralizing and sub-neutralizing antibodies can be generated due to a primary DENV infection, which then enhances secondary infection with DENV of a different serotype. These antibodies bind to the dengue virions without neutralizing them, allowing them to bind to the FCγ receptor enhancing infection of macrophages, monocytes, and dendritic cells [19,20]. However, some of DENV's pathology may be explained by infecting cells other than those of the myeloid lineage. For example, it has been proposed that the hemorrhaging that occurs during DHF may be caused by DENV infecting endothelial cells and viral NS1 causing damage leading to vascular leakage[14]. DENV infection of the hepatocytes may induce jaundice or other liver damage mediated disease[21].

For DENV to infect cells it must first bind to a cell surface receptor via the viral Envelope protein and be endocytosed into the cell endosome within the cytoplasm. The specific cell surface receptor used by DENV to bind to the cell has not been identified yet,

though several attempts have been made to do so. Due to the wide tissue tropism of DENV and the fact the virus is capable of infecting both vertebrates and mosquitos, the receptor must either be a ubiquitous one or DENV can utilize multiple different receptors to bind to the surface of the cell. Many candidate proteins have been proposed to act as a cell surface receptor including heparan sulfate, CD14, and HSP70/90 in mammalian hosts and prohibitin in insect hosts [22,23]. DENV E protein's Domain III is the putative receptor binding domain due to several different experiments demonstrating that the domain is necessary for viral adsorption and entry into both mammalian and mosquito cells [24–26]. After binding to the cell surface, the virus is internalized via either clathrin-mediated endocytosis or clathrin-independent pathways depending on the cell type and virus strain [15,27]. The virus is taken into the acidic endosome and then a conformational change of the E protein causes the viral envelope to fuse with the endosomal membrane releasing the capsid and RNA genome into the cytoplasm. The genomic RNA is then translated by the host ribosomes as a single polyprotein before NS3, with NS2B acting as a cofactor; cleaves the polyprotein into the 3 structural proteins and 7 non-structural proteins. NS5 acts as an RNA dependent RNA polymerase to replicate the viral RNA within the endoplasmic reticulum (ER) derived replication compartments. The RNA copies can either be used as mRNA to produce more of the structural proteins or as genome copies for the viral progeny [3,28]. The genomic RNA is then packaged into the newly translated and assembled capsids and the nascent immature virions enter the ER. As the virions move through the secretory pathway, they enter into the Trans-Golgi-Network where the cellular endoprotease furin cleaves the viral premembrane protein (prM) into the mature M protein [15,29]. The mature virions then exit the cell to go on and infect other

cells. Despite its relatively small genome, the DENV life cycle is complex as its RNA genome and various proteins manipulate several different cell pathways to establish infection.

Like all viruses, DENV relies on several host cellular factors to establish a productive infection. Other investigators have reported that DENV interacts with the epigenetic regulator DIDO1 and with various histones via NS1 and the Capsid protein respectively to enhance DENV replication [3,30,31]. DENV NS1 interacts with Toll-Like Receptor 4 (TLR4) to upregulate secretion of inflammatory cytokines IL-6, TNF-α, IL-1β, and IL-8 [32]. NS1 is also capable of interacting with the host chaperone glucose-regulated protein 78 (GRP78) to mediate secretion of NS1 [33]. The DENV E protein interacts with the host transcription factor TAL-1 to inhibit host transcription [34]. DENV NS3 interacts with Fatty Acid Synthase to relocalize it to viral replication sites and upregulate synthesis of fatty acids [35]. DENV NS3 also interacts with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to inhibit its glycolytic activity and translocate it to the endoplasmic reticulum [36]. DENV causes an upregulation of cyclin-dependent kinase 8 (CDK8) and relies on CDK9 to increase the production of various metabolic enzymes, including those critical for virally induced lipophagy to mediate a proviral effect [37]. The DENV NS5 protein is responsible for hijacking snRNPS and RMB10 to alter the splicing process [38,39]. To interfere with the RIG-I dependent immune response, DENV NS4B elongates the mitochondria by inhibiting the mitochondrial fission factor DRP1[29,40]. To translate its genome, DENV's 5' m^7G-cap binds to the Eukaryotic Initiation Factor 4E (eIF4E) and the 3' Untranslated Region (UTR) binds to the host Poly(A)-binding protein (PABP) allowing the viral RNA to attach to the cellular ribosomes for translation [28,41,42].

In addition to these various host factors, DENV manipulates the cellular autophagy pathway to support its infection. Several independent studies have demonstrated that the autophagy pathway is critical for robust DENV production. DENV infection induces a selective autophagy that targets lipid droplets to stimulate lipid metabolism [43]. Related viruses also manipulate lipid droplets and cellular lipid metabolism. HCV protein NS5A induces an accumulation of lipid droplets within the infected cell by inhibiting AMPK phosphorylation [44]. HCV utilizes the lipid droplets (LD) as scaffolding via the Core protein for virion assembly [45,46] . Extracellular HCV exists at two different buoyancies: a less infectious higher density and a more infectious lower density [47,48]. The lower density infectious particles are lipidated HCV virions [49]. Zika virus (ZIKV) infectious particles are enriched in sphingolipids [50].

Autophagy

Autophagy is a highly conserved catabolic cellular housekeeping process in which intracellular contents are digested by the degradative organelle called the lysosome. This cellular process represents a major contributor to the metabolism and homeostasis of the cell [51]. Autophagy can be divided into two broad categories: Bulk autophagy and selective autophagy. Bulk or non-selective autophagy consists of the non-specific capture of cytosolic cargo, such as organelles or macromolecular complexes, for autophagosomal degradation; this form of autophagy is often triggered by starvation. The cell recycles cellular components to make up for the lack of nutrients. In addition to generalized autophagy, selective autophagy is the specific targeting of cargo such as misfolded proteins, damaged organelles, and nutrient stores to the lysosome. There are three major kinds of autophagy: microautophagy, chaperone-mediated autophagy, and

macroautophagy [52]. Microautophagy represents the process by which the cell directly degrades the autophagic target. The cargo is directly captured by either a lysosome or a late endosome and then degraded in the endolysosome [53]. During chaperone-mediated autophagy proteins with the KFERQ motif are carried to the lysosome by a variety of chaperone proteins such as HSC70, CHIP, and HSP40 [54]. Macroautophagy (hereafter referred to as simply autophagy) consists of the capture of cargo into Microtubule-associated proteins 1A/1B light chain 3B (LC3-II) covered double-membraned organelles known as autophagosomes. The autophagosome then fuses with the lysosome forming the autolysosome degrading the contents [51,52,55]. Selective autophagy is categorized by the type of cargo it targets for degradation. For example mitophagy targets mitochondria, pexophagy targets peroxisomes, and lipophagy targets lipid droplets [51,56]. Autophagy also contributes to host defense against invading pathogens such as viruses and bacteria [57],[58].

Viruses and Autophagy

Xenophagy consists of the autophagic degradation of pathogens such as bacteria or viruses. This process most often occurs in macrophages, an immune phagocyte. This process is critical in the innate immune response against *Mycobacterium tuberculosis* and *Salmonella typhimurium* [59,60]. Overexpression of the xenophagy receptors has a negative effect on vaccinia virus [61]. The Sindbis viral capsid is targeted for autophagic degradation by an autophagy adaptor protein called p62 [57]. Many viruses inhibit other forms of autophagy to better support their replication and infection [62]. Coronavirus proteins bind to beclin-1 and STING1 to prevent autophagosome and lysosome fusion [63]. Influenza A virus's M2 protein inhibits autophagosome maturation leading to cell apoptosis and increased viral dissemination [64,65]. Human parainfluenza virus also inhibits autolysosome

formation to enhance viral egress [66]. Autophagy plays a critical role in adaptive immunity; many viruses inhibit the autophagy pathway to evade the cellular immune response [67]. The herpes simplex virus 1 (HSV-1) protein ICP34.5 impairs autophagy in CD4 T cells and dendritic cells [68,69]. HIV-1 activates mTORC to inhibit autophagy and downregulate antigen presentation in infected dendritic cells [70]. Epstein-Barr virus's (EBV) nuclear antigen 1 (EBNA1) localizes to the cellular nucleus to limit the autophagy pathway's access to it, impairing antigen presentation in dendritic cells [71]. In addition to these anti-pathogen roles, autophagy has been implicated in several proviral contexts.

Many viruses require induction of autophagy to efficiently replicate and infect cells. Measles virus induces multiple waves of autophagy to enhance replication [72,73]. Hepatitis C Virus (HCV) induces an incomplete autophagy via the unfolded protein response (UPR) which is necessary to initiate HCV replication [74,75]. Parvovirus B19 causes autophagy in infected cells to prevent cell death [76]. As autophagy plays a major role in cellular metabolism, viruses commonly hijack various autophagy pathways to more efficiently replicate within infected cells [77–80]. Lipophagy especially is an important process for energy production and lipid metabolism is a frequent target for flavivirus manipulation[81].

DENV and Lipophagy

Multiple studies have demonstrated that DENV induces a proviral autophagy in infected cells, as inhibition of autophagy significantly reduces the amount of infectious virus released by the cell [77,80,82–84]. Heaton and Randall demonstrated that DENV induces a selective autophagy known as lipophagy, wherein LD are targeted to the autophagosomes. Infection with DENV induces an increase in the number of LC3 puncta per cell and a decrease in the lipid droplet abundance. Knockdown of critical components

of the autophagy pathway or treatment with autophagy inhibitor 3-methyladenine caused a significant reduction in DENV production. Exogenous addition of free fatty acids (FFA) in the form of oleic acid rescued the production of infectious DENV [43]. This study presented the LD as a critical target of DENV manipulation of the cellular metabolic pathways.

Lipid droplets are dynamic energetically dense organelles consisting of triglycerides and cholesterol esters. They are a major form of energy and lipid storage in the cell and are a major site of apolipoprotein degradation [85]. Lipophagy induces the release of free fatty acids which are trafficked to the mitochondria to undergo β-oxidation, resulting in increased ATP production by the infected cell [86,87]. Jordan and Randall identified that the AMPK-mTORC signaling axis is critical for DENV to induce lipophagy in infected cells [82]. Zhang et al demonstrated that the viral proteins NS4A and NS4B are sufficient to translocate the lipid droplet-associated AUP1 to the autophagosome to induce lipophagy. The investigators screened for mono-ubiquitinated lipid droplet proteins in DENV infected cells and identified Ancient Ubiquitous Protein 1 (AUP1). Additional experiments revealed that non-ubiquitinated AUP1 is necessary for robust DENV infection and for DENV induced autophagy and lipophagy. Due to the association of viral NS4A with AUP1, the investigators demonstrated that DENV NS4A and NS4B expression was sufficient for induction of lipophagy [83]. The mechanisms of DENV induced lipophagy has become a prominent topic for those investigating the interactions between a pathogen and host metabolic networks. Lipid droplets are a common target for flaviviruses, contributing to a wide range of roles including metabolism, assembly, and

viral spread [81,88–90]. Despite the lipid droplets' significance in flavivirus infection, the mechanism of how it is specifically targeted during infection remains to be elucidated.

Selective Autophagy Receptors

The induction of selective autophagy often involves the ligation of poly-ubiquitin chains onto the cytosolic surface of the cargo. An adaptor protein known as an autophagy receptor can bind these poly-ubiquitin chains via a carboxy-terminal ubiquitin-associated domain (UBA) and additionally bind to the LC3 on the surface of the developing autophagosome via a LC3-interacting region (LIR). These interactions form a bridge between the cargo and the autophagosome effectively targeting the cargo for lysosomal degradation [51,87,91]. Several different autophagy receptors have been identified and characterized, such as p62, optineurin, NDP52, TAX1BP1, and NBR1 [92–94]. Different receptors often target different cargo to the autophagosome for lysosomal degradation and have even been shown to contribute to other processes in the cell [95,96]. P62/SQSTSM1 is the first vertebrate autophagy receptor to be identified when it was demonstrated to target protein aggregates to the autophagosome [92,97]. Optineurin targets damaged mitochondria to the autophagosome during mitophagy [94]. NBR1 is perhaps the oldest autophagy receptor with homologs found in *Arabidopsis* and yeast (as Atg19 and Atg34) [96]. It was first described as cooperating with p62 during aggrephagy; later it was identified as the specific autophagy receptor for targeting peroxisomes for autophagic degradation (pexophagy) [93,98].

In addition to the UBA domain and its 2 LIRs, NBR1 contains several motifs that contribute to its overall function.

The amino terminal Phox/Bem1P (PB1) domain allows NBR1 to interact with other proteins that contain a PB1 domain, such as p62 [99]. The adaptor protein also has a 50 amino acid stretch containing two structural zinc ions referred to as the ZZ domain [100]. In yeast, the NBR1 ZZ domain functions to transport the cytosolic aminopeptidases Ape4, Ape2, Lap2, by binding to, but in mammalian NBR1, this domain's function is still uncharacterized [96,100]. There are also 2 coiled coil domains in NBR1 referred to as CC1 and CC2. CC1 mediates binding to other NBR1 molecules allowing it to oligomerize. CC2 contains one of the two LIR motifs to mediate binding to LC3, the domain also mediates interaction with the focal-adhesion kinase interacting protein of 200KDa (FIP200). The second LIR motif (LIR2) sits within amino acids 540 and 636, and while it is capable of binding to LC3, it does not bind as strongly as LIR1 in the second coiled coil domain [93]. NBR1 has a domain that consists of four tryptophans known as FW which interact with MAP1B and TAX1BP1. An amphipathic helix (AH) domain which folds into an α-helix when it comes into contact with phosphatidylinositol-phosphates (PIP) also found within the NBR1 protein. This AH domain appears to be necessary for NBR1 localization to lysosomes and membranes [98,101].

NBR1 has been described as targeting many different types of cargo to the autophagy pathway in mammals; including midbody remnants, focal adhesions, and the MHC class I receptor [102–105]. NBR1 also has a LIR dependent role in skeletal remodeling in conjunction with p62 [106]. In plants and fungi NBR1 has a role in targeting even more intracellular cargo for degradation including viral capsids and viral RNA silencing suppressors [96,107,108]. NBR1 has been identified as being closely associated with lipid droplets and exosomes [109,110]. Some groups have identified non-autophagy roles for NBR1

such as T cell differentiation and as an inhibitor of tyrosine kinase degradation [101,111]. NBR1 contributes to many different cellular processes making it an attractive target for viral hijacking. Interactions between autophagy receptors and viruses are a key area of research.

Several studies have been done on how autophagy receptors target pathogens for autophagic degradation. NDP52 binds to ubiquitinated *Salmonella* to target them to the autophagosome as an innate immune response [112]. P62 and NDP52 cooperate to induce autophagic degradation of *Shigella, Listeria,* and *E. coli* [113,114]. Optineurin is phosphorylated to target *Salmonella* to the autophagosome [115]. NBR1 is a critical component for xenophagy of *M. tuberculosis* [116]. Autophagy receptors have also been shown to target viruses to the autophagosome as well as bacteria. P62 protects the central nervous system against Sindbis virus [57]. Optineurin targets HSV-1 for degradation in neuronal cells [117]. Vaccinia must subvert p62-mediated xenophagy to establish a successful infection [61]. Many investigations into how viruses interact with autophagy receptors have focused on viral inhibition of autophagy receptor mediated antiviral effects[118]. Autophagy and autophagy receptors contribute to many different cellular pathways; how viruses exploit autophagy receptors to hijack these pathways to replicate more efficiently is not as well studied.

Secretory Autophagy

In addition to a role in degradation, autophagy also functions in exocytosis, termed secretory autophagy [119,120]. In this unconventional secretion method, proteins that lack the N terminal sequence that is usually necessary for secretion into the extracellular space can leave the cell via autophagy-mediated means. This process shares many aspects

with the well characterized degradative autophagy, cargo is captured in the membranous autophagosomes but instead of lysosomal degradation, they are secreted from the cell. After the cargo is captured within a secretory autophagosome, the vesicle may fuse directly with the plasma membrane to expel its contents out into the extracellular space. It may also fuse with a multivesicular body (MVB) to form an amphisome which subsequently fuses with the plasma membrane to release exosomes containing the autophagic cargo from the cell. Alternatively, pathogens may use autophagy dependent pathways that differ from the ones outlined above. Bacteria can utilize autophagy machinery such as Beclin1, p62, and ATG1 to form an ejectosome for the purposes of exiting the cell. The enterovirus poliovirus is captured in LC3+ phosphatidyl serine rich double membraned vesicles that proceed to fuse with the plasma membrane releasing poliovirus-containing vesicles. Degradative autophagy can also result in the secretion of cargo as following degradation, the autolysosome may fuse with the plasma membrane releasing its contents into the extracellular space [119]. Secretory autophagy represents another pathway the cell uses to eliminate a number of different cytosolic proteins and cargo using expulsion as opposed to degradation.

There are four major identified current functions of secretory autophagy. First: the unconventional secretion of cytosolic proteins that need to leave the cell to complete their function. IL-1β is a pro-inflammatory cytokine that lacks the N-terminal sequence for leaving the cell and is secreted upon activation of the inflammasome, and is one of the earliest identified examples of a protein that leaves the cell via secretory autophagy [121,122]. HMGB1 is a damage-associated molecular pattern (DAMP) with pro-inflammatory effects after its release from cells. Both of these cytosolic proteins have their secretion enhanced

when autophagy is enhanced and inhibited when critical components of the autophagy pathway are knocked out [123]. Another function of secretory autophagy is to remove protein aggregates. Amyloid β and α-synuclein are two proteins that have been shown to aggregate intracellularly during Alzheimer's disease and Parkinson's disease, respectively. Inhibition of degradative autophagy increases the amount of α-synuclein secreted into the extracellular space [124]. Mice deficient in autophagy have an increased intracellular concentration of amyloid β [119,121,122,125]. Secretory autophagy also plays a role in the cellular release of organelles. Induction of autophagy in developing reticulocytes was demonstrated to remove the mitochondria [126]. Caspase-activation causes human endothelial cells to secrete their autophagic vacuoles [127]. Finally, secretory autophagy has been shown to contribute to the transmission of several pathogens both bacterial and viral.

There are multiple forms of extracellular vesicles that can leave the cell to deliver intracellular contents, such as cell messengers or intracellular pathogens, to other cells. These secreted structures are defined by their membranous lipid bilayer that surrounds an aqueous core. These extracellular vesicles are further differentiated by their size and biogenesis. Apoptotic bodies are large vesicles ranging in size from 1-5 μm released from apoptotic cells. Microvesicles are smaller vesicles (150 nm to 1 μm) that are released from direct outward budding of the membrane. Their release relies on several intracellular changes in calcium levels, lipid components, and protein composition to alter the membrane curvature. Activated neutrophils release microvesicles to signal to platelets [128,129]. Exosomes are even smaller with a diameter ranging from 30 nm to 150 nm with a density in sucrose of 1.13 g/mL to 1.19 g/mL and are generated when MVBs fuse with

the plasma membrane to release vesicles into the extracellular vesicles [130,131]. MVBs, also known as multivesicular endosomes (MVEs) are normally targeted for degradation, but various machineries involved in the regulation of intracellular cargo elimination can adjust this balance to lean more towards secretion instead of degradation. MHC class II is trafficked to the MVE in dendritic cells and then either targeted for lysosomal degradation via ubiquitin and endosomal sorting complex required for transport (ESCRT) machinery dependent pathways and targeted for exosome secretion via ubiquitin and ESCRT machinery independent pathways [132,133].

Secretory autophagy presents an unconventional method for proteins and other cytosolic objects to leave the cell and enter the extracellular space; this pathway represents a valuable method for pathogens to egress without affecting overall membrane permeabilization. *Mycobacterium marinum* has been shown to utilize an incomplete autophagy to enhance cell-cell transmission while *Brucella* uses the autophagic vacuole to increase cell-cell infection [134,135]. In addition to bacteria, many viruses such as flaviviruses, picornaviruses and others hijack autophagy dependent secretion methods to facilitate egress. Cell lysis was generally thought to be necessary for the non-enveloped poliovirus to egress, but investigators observed that poliovirus could exit the cell in a non-lytic manner using autophagy derived vesicles [79,136,137]. Coxsackie B Virus, another picornavirus, has been found in LC3+ extracellular vesicles [138]. Morbillivirus induced autophagy via cell-cell fusion to enhance viral spread [139]. Influenza A Virus uses its M2 protein to relocalize the LC3 protein to the plasma membrane to induce a filamentous budding the virus utilizes to spread infection [65]. Flaviviruses such as ZIKV and DENV also use autophagy assisted egress pathways to enhance infection and egress[78,111].

HCV is capable of packaging its genomic RNA into exosomes which can enter other cells to spread the virus and/or trigger the innate immune response [140]. Some investigators have hypothesized that the binding of the HCV core protein to the lipid droplet acts as a way for the virus to egress from the cell in addition to utilizing the LD as an assembly scaffold [81].

DENV utilizes autophagy to release the virus in LC3+ dependent vesicles that can protect the virus from antibody neutralization. Wu et al determined that inhibition of ATG5 expression or via drug inhibition severely reduces DENV's ability to spread infection in close contact culture. Microscopy staining contrasting infected donor cells and uninfected recipient cells demonstrated vesicles leaving the infected cells. These vesicles contain lipid droplets and autophagic machinery such as LC3 and the DENV E protein, providing evidence that DENV autophagic targeting of the lipid droplet may assist in this non-canonical secretion method. Inhibition of autophagy enhanced antibody neutralization of the virus. The investigators concluded that DENV utilizes multiple secretion pathways for viral egress and at least one of which is autophagy mediated [78]. Mateo et al also demonstrated that treating cells with the autophagy inhibitor Spautin-1 also significantly impaired DENV's ability to secrete from the cell after it is processed in the golgi. DENV RNA is still observed to leave the infected cells that have been treated with Spautin-1 suggesting the potential existence of multiple pathways the virus may use to exit the cell with a major secretion pathway being dependent on autophagy [84]. This virion secretion method represents a largely uncharacterized egress pathway, and its examination may illuminate a critical step in dengue virus infection.

ZIKV utilizes Lyn-kinase to cause the release of ZIKV virions encapsulated in LC3+ membranous vesicles that when run on a discontinuous sucrose gradient sediment to higher buoyancy fraction than the standard free-floating virions. The investigators determined that DENV infection activated the Src family kinases (SFKs), Src, Fyn, and Lyn. Several components of the secretory pathway and vesicular transport co-immunoprecipitated with these SFKs. Inhibiting Lyn kinase expression via siRNA or CRISPR/Cas9 significantly reduces post-golgi ZIKV secretion. Characterization of this Lyn dependent secretion reveals that Lyn kinase dependent ZIKV secretion is in LC3+ vesicles that sediment at a lower density on sucrose gradient than the more well known non vesicle-enclosed vesicles [111]. These vesicles aid the virus in crossing the placental barrier *in vivo* providing more evidence that flaviviruses utilize secretory autophagy to spread their infection [88].

Goals of this thesis

This study investigates the mechanism of how dengue virus specifically targets the lipid droplet for autophagic degradation and how this lipophagy may aid in DENV cellular egress. Tristan Jordan showed that DENV induced lipophagy is likely a ubiquitin-mediated autophagy as after infection, ubiquitin chains are ligated to the cytosolic surface of the lipid droplet. He also demonstrated that the selective autophagy receptor NBR1 and not other autophagy receptors are recruited to the surface of the lipid droplet after infection with DENV. Data in this dissertation will show that knocking down NBR1 does not prevent DENV from inducing autophagy but does prevent the autophagic flux of the lipid droplets. With this new development, the next step was to identify which step in the DENV life cycle requires the targeting of the lipid droplet for lipophagic degradation. The

data will show that NBR1 is not necessary for replication of the DENV RNA genome nor for the assembly of infectious viral particles. NBR1 does however play a significant role in the egress of dengue virus. To investigate how DENV induced lipophagy contributes to viral release, the experiments will demonstrate that DENV requires NBR1 to leave the cell by utilizing a non-canonical autophagy assisted secretion method and the exogenous addition of free fatty acids bypasses this requirement for NBR1. Afterwards this dissertation will show data on various attempts to further characterize this NBR1-dependent secretion route and the development of the hypothesis on the link between DENV induced lipophagy and the autophagic secretion of the virus.

Materials and Methods

Cells and Virus

HepG2 cells, Huh7.5 cells, hepatocyte derived carcinoma cell lines from ATCC and Dr. Charles Rice respectively, and HEK293T cells from ATCC were maintained in Dulbecco's modified Eagle medium-high glucose supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids (NEAA), and 1% penicillin-streptomycin (P/S). Infectious DENV-2 clone 16681 from Dr. Claire Huang was used to infect cells. Virus was propagated according to a protocol published by Chu et al in c6/36 *Aedes albopictus* cells obtained from ATCC as described below [141]. Cells were infected in DMEM-high glucose supplemented with either 2% or 5% Fetal bovine serum 0.1 mM nonessential amino acids, and 1% Penicillin-streptomycin.

DENV Propagation

DENV strain 16681 serotype 2 was propagated in C6/36 cells. C6/36 cells were grown until they formed a confluent monolayer in a 15 cm petri dish and then they were infected with DENV at a multiplicity of infection (MOI) 0.02 in 2 mL serum free RPMI 1640 medium at 30 °C with 5% CO_2 for two hours. During DENV infection, cells were gently shaken at 15 min intervals. Afterwards, the infectious medium in C6/36 cells was replaced with a low serum RPMI 1640 medium containing 2% FBS, 1× NEAA, 1% P/S, and 1mM sodium pyruvate, and incubated at 30 °C with 5% CO_2. The culture medium was collected 4 days after infection and cell debris were removed by centrifugation with 9,000× g for 30 min at 4 °C. The supernatant of the culture medium was aliquoted in 2 mL aliquots and stored at −80 °C before later being thawed and having the amount of infectious DENV particles measured via TCID50.

Antibodies

The antibodies used in this study include rabbit anti-DENV NS4A (1:1000, GeneTex), mouse anti-DENV E (1:1000, D1-4G2-4-15), rabbit anti-DENV NS3 (1:5000, Jossman), rabbit anti-LC3 (1:1000, MBL), rabbit anti-NBR1 (1:500, Cell Signal) rabbit anti-p62(1:500, Sigma), rabbit anti-Optineurin (1:500, abcam), rabbit anti-myc (Cell Signal, 1:1000), rabbit anti-K63 ubiquitin (EMDMillipore, 1:500), and secondary antibodies donkey anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen), goat anti-mouse Alexa Fluor 594 (1:1000, Invitrogen), goat anti-mouse Alexa Fluor 568 (1:1000, Invitrogen), and goat anti-mouse Alexa Fluor 647 (1:1000, Invitrogen), donkey anti-rabbit Alexa Fluor 350 (Invitrogen 1:600) for immunofluorescence. For western blotting, mouse anti-DENV E (1:1000, D1-4G2-4-15) mouse anti-NBR1 (1:1000, Santa Cruz), HRP conjugated mouse anti-actin (1:10,000, Santa Cruz), and secondary goat HRP conjugated anti mouse IgG (1:10,000 abcam).

siRNA transfection

siRNAs used in this study include Trilencer-27 Universal scrambled Negative control siRNA (SR30004, Origene), NBR1 silencer select siRNA (Ambion, Cat# 4392420, s57874 (siNBR1-1) or s227479 (siNBR1-2)). Huh7.5 cells were reverse transfected using Lipofectamine RNAiMAX for 72 hours before infection or collection of the lysate for Western Blotting. The siRNA was reconstituted to a concentration of 10 μM in RNAse free water. Then 9 μL of lipofectamine RNAiMAX per well to be transfected was mixed with 150 μL of Opti-Mem Reduced Serum media per well. In a separate tube for each siRNA to be used 6 μL (60 pmol) per well is added into 150 μL of Opti-Mem Reduced Serum media per well. The Lipofectamine mixture is added to the siRNA mixture in a 1:1

volume, incubated at room temperature for 5 minutes and then 250µL of the Lipofectamine/siRNA mixture is added to each well in a 6 well plate before 0.5e6 Huh7.5 cells is added on top of the siRNA mixture. When performing a reverse transfection in 96 well plates or 24 well plates, the volumes are scaled down by a factor of 6 and 3 respectively. After the cells are seeded onto the lipofectamine/siRNA mixture, DMEM with 10% FBS 0.1 mM NEAA, and 1% P/S is added to the wells to bring the volume up to the recommended amount depending on the type of well. 6-wells are brought up to 3 mL, 24-wells are brought up to 1 mL, and 96-wells are brought up to 100 µL. The cells are then left to incubate at 37°C overnight and then the media is refreshed with fresh media without lipofectamine.

Western Blot

Cells were lysed with 1% Igepal lysis buffer for 10 minutes on ice. Lysates were lifted off the plate via cell scraper and then frozen at -80°C. To isolate protein, lysate is sheared up and down 10 times by 25-gauge syringe and then centrifuged at 14,000 rpm/18,800 x g for 30 minutes at 4°C. The supernatant is then transferred to another tube. Protein concentration is measured via spectrophotometer using the Bradford assay. To perform the Bradford Assay a standard curve is generated using different amounts of Bovine Serum Antigen (BSA): 0 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, and 4 mg/mL. The different amounts of BSA are brought up to 15 µL with water, mixed with 5 µL of 1% Igepal solution, 200 µL of BioRad Protein Assay Dye, and 780 µL of water, vortexed, and then incubated for 5 minutes before being measured on a ThermoFisher BioMate3 spectrophotometer to generate the standard curve. Then 5 µL of the protein samples are then mixed with 200 µL of BioRad Protein Assay Dye and 795 µL of water, vortexed, and

left to incubate for 5 minutes at room temperature before being measured via the spectrophotometer. 10-15 µg of protein is boiled at 95-100°C with a Sodium dodecyl sulfate (SDS) buffer for 5 minutes and then placed on ice. Samples are loaded onto a 4-20% electrophoresis Mini-Protean TGX Precast gel and run at 80 V for 80 minutes or until the dye reaches the bottom 2 cm of the gel. The 5x running buffer stock was made by mixing 140g of glycine, 30g of Tris base and 5g of SDS in 1 L of water, the 5x buffer was then diluted 5-fold in water. The transfer was set up in a transfer cassette in the following order: Black side of the cassette, two sponges, two pieces of Whatman paper, gel, PVDF membrane, two pieces of Whatman paper, two sponges, and the clear side of the cassette. The PVDF should be activated by wetting with methanol for 5 seconds and then kept in the transfer buffer. The gel, sponge, and Whatman paper are dunked in transfer buffer before stacking the transfer cassette. Stock 10x Transfer buffer is made by mixing 140g of Glycine and 30g of Tris Base into 1 L of water. To make a working stock of transfer buffer, mix 100 mL of 10x Transfer buffer, 200 mL of methanol, and 700 mL of water. The transfer buffer should be cooled at -20°C for an hour before setting up the transfer. The protein on the gel was wet transferred to a PVDF membrane at 100 V for 1 hour with an ice block placed in the transfer tank to function as a heat sink. The membrane is then blocked for 30 minutes in PBS with Tween (PBST) containing 5% BSA at room temperature. The membrane is then primary stained overnight at 4°C (or at room temperature if staining for NBR1) in PBST containing 2.5% BSA and secondary stained for 1 hour at room temperature in PBST. Then it is incubated with either SuperSignal West Pico Chemiluminescent or SuperSignal West Femto Chemiluminescent before being imaged in BioRad ChemiDoc MP Imaging System.

Immunofluorescence microscopy

Huh7.5 cells were seeded on poly-lysine coated coverslips in 1 mL DMEM supplemented with 10% FBS, 0.1 mM NEAA, and 1% P/S. Cells are fixed with 4% PFA for 15 minutes at 4°C. They are then blocked with 250 µL of PBS supplemented with 30% Goat serum and 0.05% saponin for at least 30 minutes, rocking at room temperature. Cells are then washed once with PBS before primary staining with antibodies diluted in 250 µL of PBS supplemented with 10% goat serum and 0.05% saponin for either 1.5 hours at room temperature or overnight at 4°C. After three washes with PBS, cells are secondary stained in 250 µL of PBS with 10% goat serum and 0.05% saponin for 1 hour at room temperature. Oil Red O staining is performed after antibody staining according to BioVision's protocol.

Oil Red O stock solution is made by dissolving 60 mg of Oil Red O in 20 mL of 100% isopropanol, mix, and let incubate at room temperature. To make the Oil Red O working solution, mix 3 parts of stock solution with 2 parts of distilled water, mix, and incubate at room temperature for 10 minutes before filtering through a 0.2 µm syringe filter. The stock solution is stable for 1 year and the working solution is stable for 2 hours. Coverslips are washed with water twice, and then incubated in 500 µL of 60% isopropanol for 5 minutes. Afterwards the cells are stained with 500 µL of the Oil Red O working solution rocking at room temperature for 10-20 minutes. The Oil Red O solution is aspirated off and the coverslip is washed with water 2-5 times or until the excess red stain is removed. The coverslips are then mounted onto microscope slides with prolong gold and DAPI or prolong gold alone if the cells were stained with the secondary antibody donkey anti-rabbit Alexa Fluor 350. The coverslips are then imaged on an Olympus DSU spinning disk

confocal microscope (Olympus Corporation of the Americas, Center Valley, PA) with an Evolve EM-CCD camera (Photometrics, Tucson, AZ) run by SlideBook v6.0 software (Intelligent Imaging Innovations, Denver, CO).

Lentivirus Particle Production

HEK293T cells in a 6 well plate are transfected with a plasmid containing a tetracycline inducible NS4A-2A-NS4B and second generation lentivirus packaging mix using the abm LentiFectin Transfection reagent according to manufacturer's protocol. A day before transfection, seed the 293T cells so that they are ~70% confluent the next day. On the day of transfection, prepare the transfection complex by diluting 1.2 µg of the NS4A-2A-NS4B and 1.2 µg of abm's second generation packaging mix in 122 µL of serum-free, antibiotic-free media. Next dilute 10 µL of LentiFectin Transfection reagent in 122 µL of serum-free, antibiotic-free media and incubate both solutions at room temperature for 5 minutes. Then mix the two solutions and incubate at room temperature for 20 minutes to generate the transfection complex. Afterwards add 550 µL of serum-free media to the transfection complex. Remove the media from the 293T cells and add the transfection complex and incubate for 5-8 hours at 37°C and add 80 µL of FBS to the cells and incubate at 37°C overnight. The next day, refresh the media with DMEM with 10% FBS, 0.1 mM NEAA, and 1% P/S and incubate at 37°C for 24 hours. The supernatants are then collected and centrifuged at 2000 g for 15 minutes at 4°C to pellet cell debris before being passed through a low protein binding Durapore 0.45 um PVDF filter. The lentiviral particles can be assessed on Lenti Go-Stix to confirm lentiviral production before being stored at -80°C.

Lentiviral Transduction

The lentiviral particles are first supplemented with 1M Hepes (20 µL/mL) and polybrene transfection reagent (0.8 µL/mL). Huh7.5 cells in a 6 well plate are transduced with 3 mLs of lentivirus in each well. The cells are spinoculated at 500g for 90 minutes and then incubated at 37°C for 4-6 hours before replacing the media with DMEM supplemented with 5% FBS, 0.1 mM NEAA, and 1% P/S. Cells are then incubated at 37°C for 48 hours before being split onto coverslips to be imaged via immunofluorescence microscopy as described above.

CRISPR/CAS9 Knockout

Lentiviral particles are generated by packaging a plasmid that codes for the CRISPR guide RNA targeting NBR1, Cas9, and a puromycin resistance gene as described above. The Lentiviral particles are then transduced into Huh7.5 cells according to the above protocol. 48 hours after the transduction, the cells are treated with 2 µg/mL puromycin to select for the transduced cells and generate a stable cell line of NBR1 knockout cells.

Statistical Analysis

Data are presented as means ± standard errors of the means (SEM). To assess statistical significance, two-tailed, unpaired Welch's t tests were performed. One asterisk indicates a p value less than 0.05, two asterisks indicate a p value less than 0.01, three asterisks indicate a p value less than 0.001, and four asterisks indicate a p value less than 0.0001.

Protease Protection Assay

Whole infected supernatant and the fraction correlating to Peak 2 are either kept in a 1% Igepal solution or PBS and then are treated with either PBS or 0.2 mg/mL of Proteinase K for 30 minutes. The Proteinase K is then inactivated by mixing the sample with

cOmplete, Mini, EDTA-free protease inhibitor cocktail in a 1:1 mixture. DENV E in the sample is then probed for via western blot as described above.

RNA Extraction

To isolate the viral and host RNA within the cells, Huh7.5 is reverse transfected with either control non-targeting siRNA or one of two different siRNA targeting NBR1 in a 96-well plate. The cells are then infected with DENV and then lysed with a mixture of 130 µL of Macherey-Nagel RA1 buffer and 1.3 µL of β-mercaptoethanol per well at different timepoints after infection. The lysate is then frozen at -80°C and then thawed on ice. The lysates are then mixed with the RA4 buffer by pipetting up and down 10-15 times dissolving the lysate. The samples are then added to a Nucleospin vacuum RNA Binding plate and then bound to the silica membrane by activating the vacuum for 1 minute. The silica membrane is then desalted by adding 500 µL of RA3 to each well and the vacuum is activated and run for 3 minutes. The membrane is then washed by adding 500 µL of RA2, then 800 µL RA3, and 500 µL RA4 with the vacuum run for a minute between each step. The membrane is then dried by running the vacuum for 10 minutes. 50 µL is then added to the membrane and left to incubate for 2 minutes before eluting the RNA by running the vacuum until the membrane is dry.

Viral RNA in the supernatant is isolated using the GeneJET Viral DNA/RNA Purification Kit. 50 µL of column preparation liquid is added to the center of the spin column to prepare. Supernatant samples are lysed by mixing 200 µL of sample with 200 µL of lysis solution supplemented with carrier RNA and 50 µL of Proteinase K. The sample is then incubated at 56°C for 15 minutes in a thermomixer. The eluent is left in the thermomixer for a future step. The sample is briefly centrifuged to collect all the drops before adding

300 µL of ethanol and mixed. Next the sample is incubated for 3 minutes at room temperature before being briefly centrifuged. The lysate is then added to the spin column loaded onto a wash tube. The sample is then loaded onto the column by centrifuging the spin column for 1 minute at 6000 x g, the wash tube is then discarded and replaced. The column is then washed with 700 µL of Wash Buffer 1 supplemented with ethanol and centrifuged for 1 minute at 6000 x g. The tube is discarded and replaced with a new tube. The column is then washed with 500 µL of Wash Buffer 3 supplemented with ethanol and centrifuged for 1 minute at 6000 x g. The tube is discarded and replaced with a new tube. The column is centrifuged for 3 minutes at 16,000 x g and the wash tube is discarded as the spin column is placed into a 1.5 mL elution tube. 50 µL of the preheated elution buffer is added to the spin column, incubated for 3 minutes at room temperature, and then centrifuged for 1 minute. The purified RNA is then either measured via qPCR immediately or stored at -20°C.

50% Tissue Culture Infectious Dose (TCID50)

Huh7.5 cells were infected with DENV-2 at MOI 5 in 6 well plates for 4 hours before refreshing media and incubating at 37°C for 48 hours. The supernatant was collected and then serially diluted tenfold in Dulbecco's modified Eagle medium-high glucose (DMEM) supplemented with 2% Fetal bovine serum, 0.1 mM nonessential amino acids, and 1% Penicillin-streptomycin. The serially diluted DENV was then used to infect Huh7.5 cells in a 96 well plate. After 48 hours of infection, plates were fixed in -20°C Methanol for 5-10 minutes, washed in PBS and PBST by dunking the plate, blocked in 1% BSA for 30 minutes, incubated with 3% H2O2 for 5 minutes, washed with PBS twice and once with PBST, primary stained with 1:500 mouse anti-DENV E overnight, washed with PBS and

left in PBST for 20 minutes, secondary stain with horse HRP conjugated anti-mouse for 2 hours, washed with PBS and left in PBST for 20 minutes, and then treated with DAB until infected cells stained brown. All staining steps took place at room temperature. Wells with at least one cell stained positive for DENV E were considered positive. The infectious titer dose is calculated using the number of dilutions needed before none of the cells in the well are stained positive.

Density Gradient

2 mL of infected supernatant is loaded onto a discontinuous sucrose gradient made up of 2mL 10%, 20%, 40% and 60% or 80% sucrose fractions in an open-top polyallomer centrifuge tube. The gradient is then ultracentrifuged in a Sorvall Discovery 100SE ultracentrifuge with a SW41 swinging bucket rotor at 26,500 rpm for 1.5 hours at 4°C. Then 1 mL fractions are collected from the top of the tube. Viral RNA is extracted via GeneJet Viral DNA and RNA Purification Kit and measured via RT-qPCR as described above. Infectious particles are measured via TCID50.

IL-1β ELISA

The ELISA MAX Deluxe Set Human IL-1β kit was used to evaluate the amount of IL-1β in the cell supernatant. The day before the experiment each well in the plate was coated with 100 µL of capture antibody and incubated rocking overnight at 4°C. The day of the experiment all reagents are brought to room temperature. The plate was washed with 300 µL 4 times with Wash Buffer. The plate was blocked by adding 200 µL of Block Buffer and placed on a plate shaker for 1 hour. The plate was then washed 4 times. 50 µL of assay buffer and 50 µL of either standard or samples were added to each well and incubated for 2 hours. The plate was then washed 4 times. 100 µL of detection antibody was added

and the plate was incubated for an hour. The plate was washed with 300 µL 4 times with Wash Buffer. Then 100 µL of diluted avidin-HRP solution was added to each well and incubated for 30 minutes. The plate was washed with 300 µL per 5 times with Wash Buffer, the wells were soaked in the wash buffer for 30 seconds to 1 minute for each wash.

Indirect ELISA

The wells of a microtiter plate were coated with 50 µl of the antigen by pipetting the antigen into the plate and then incubated on a plate shaker for 2 hours at 37°C. The plate was then washed three times with 200 µL of PBS before being blocked with 200 µL of 5% BSA for 2 hours at 37°C. The plate was then washed twice with PBS before being incubated with 100 µL per well of primary detection antibody for 2 hours at 37°C. The plate was then washed 4 times with PBS and then each well was incubated with 100 µL of secondary HRP conjugated antibody for 1-2 hours. The plate was then washed 4 times with PBS and then incubated with 100 µL of TMB substrate for 15-30 minutes. The reaction was then stopped by adding 100 µL of stop solution (2M H2SO4).

Neutralization Assay

Serial dilutions of D1-4G2-4-15 were made in 96 wells with DMEM supplemented with 10% FBS. Equal amounts of infectious particles were added to the wells and then incubated at 37°C for 1 hour to neutralize the virus. Afterwards the neutralized virus is added to a 96 well plate seeded with Huh7.5 cells and incubated at 37°C for 48 hours. The cells are then stained for DENV infected cells using the TCID50 staining protocol. The number of infected cells in the antibody neutralized condition are compared with the

number of infected cells in the non-neutralized condition to evaluate the level of neutralization.

Pharmaceutical Drugs

Etomoxir was bought from Cayman Chemical.

Chapter 2: Dengue Virus utilizes NBR1 to target the Lipid Droplet for Lipophagy

Abstract

Dengue virus (DENV) infection induces a proviral selective autophagy that targets lipid droplets to mobilize lipids and stimulates lipid metabolism, termed lipophagy. Selective autophagy is facilitated by an autophagy receptor binding to both poly-ubiquitinated target cargo and the phagophore component LC3, thus bridging the cargo to the phagophore. The mechanism of how the lipid droplet is specifically targeted in DENV infected cells is unknown. This study demonstrates that the selective autophagy receptor Neighbor of BRCA 1 (NBR1) is recruited to lipid droplets after DENV infection. Silencing NBR1 does not alter the induction of autophagy in DENV-infected cells, but specifically inhibits the DENV-induced depletion of lipid droplets.

Introduction

Autophagy is a catabolic cellular housekeeping process in which intracellular contents are captured in double membraned vesicles called autophagosomes. The contents are degraded when the autophagosome fuses with the degradative organelle called the lysosome. Autophagy represents a major contributor to the metabolism and homeostasis of the cell. In addition to generalized autophagy, selective autophagy is the specific targeting of cargo such as misfolded proteins, damaged organelles, and nutrient stores to autophagosomes [51,56]. Autophagy is also a contributor to host defense against invading pathogens such as viruses and bacteria [57,58]. As autophagy plays a major role in cellular metabolism, viruses commonly hijack various autophagy pathways to more efficiently replicate within infected cells.[77–80,78 79]

Dengue virus (DENV) is an ~11 kb positive-stranded RNA virus in the *Flaviviridae* family. It consists of four serotypes and is transmitted by the *Aedes aegypti* mosquito. As an emerging global pathogen, DENV infects almost 400 million people annually manifesting as disease in approximately one quarter of the time[8]. These infections result in approximately 500,000 hospitalizations every year due to diseases such as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome resulting in 22,000 annual global deaths [9]. There are currently no therapeutics for DENV infection, although there is a vaccine licensed in endemic countries that protects against ~2/3rds of disease [11,12]. Multiple studies have demonstrated that DENV induces a proviral autophagy in infected cells, as inhibition of autophagy significantly reduces the amount of infectious virus released by the cell [77,80,82–84] Heaton and Randall have shown that DENV induces a selective autophagy known as lipophagy, wherein lipid droplets (LD) are targeted to the autophagosomes [43]. Lipid droplets are dynamic organelles consisting of triglycerides and cholesterol esters. Lipophagy induces the release of free fatty acids which are trafficked to the mitochondria to undergo β-oxidation, resulting in increased ATP production by the infected cell [86,87]. DENV activates the AMPK-mTORC axis to trigger lipophagy in infected cells [82]. Viral proteins NS4A and NS4B are sufficient to translocate the lipid droplet-associated AUP1 to the autophagosome to induce lipophagy [83]. Lipid droplets are a common target for flaviviruses, contributing to a wide range of roles including metabolism, assembly, and viral spread [81,88–90]. Despite the lipid droplets' significance in flavivirus infection, the mechanism of how it is specifically targeted during infection remains to be elucidated.

Selective autophagy involves the ligation of poly-ubiquitin chains onto the cargo. An autophagy receptor can bind these poly-ubiquitin chains via a ubiquitin-associated domain and additionally bind to microtubule-associated protein 1 light chain 3 (LC3) on the surface of the developing autophagosome via a LC3-interacting region. These interactions target the cargo to the autophagosome and subsequently lysosomal degradation [51,87]. There are several different autophagy receptors, such as p62, optineurin, and NBR1 [92–94]. These receptors often target different cargo to the autophagosome for lysosomal degradation [95,96]. Evidence suggests specific autophagy receptors play a major role in infection with Herpes simplex virus 1, Sindbis virus and various bacterial pathogens [57,58,112,115,117,142]. Many investigations into how viruses interact with autophagy receptors have focused on viral inhibition of autophagy receptor mediated antiviral effects[118]. How viruses exploit autophagy receptors to replicate more efficiently is not as well studied.

Results

DENV induces ubiquitin accumulation at the lipid droplet

Tristan Jordan set out to investigate the specific targeting of the lipid droplet during DENV induced lipophagy. Ligation of poly-ubiquitin chains often targets cargo for autophagic degradation. K48-linked ubiquitin has been shown to accumulate at the lipid droplet during the ERAD-mediated degradation of apolipoprotein B, whereas the predominant ubiquitin chain linkage involved in selective autophagy is K63[85,143–146]. To characterize ubiquitin accumulation at the lipid droplet during DENV infection, he transfected cells with a ubiquitin construct deficient in generating K48 linkages (K48R). He fixed these cells

24hpi with DENV (or mock infected) and analyzed the colocalization of ubiquitin at the lipid droplet. Tristan observed increased colocalization of K48R-ubiquitin with the LD in DENV-infected cells compared to mock infected cells (Fig. 1A). Probing for endogenous K63-linked ubiquitin showed an increased co-localization with lipid droplets in DENV-infected cells, as compared to mock infected cells (Fig. 1B, C). Together these data suggest that K63-linked ubiquitination of lipid droplet proteins is increased during DENV infection.

DENV causes NBR1 to recruit to the lipid droplet surface

The accumulation of ubiquitin on LDs suggests a possible ubiquitin-dependent mechanism of selective autophagy. Tristan next examined which ubiquitin binding autophagy receptor is recruited to the lipid droplet during DENV infection. P62 and NBR1 have been previously reported to be associated with lipid droplets and as such were candidate proteins [54]. A previous study demonstrated that depletion of NDP52 or TAX1BP1 did not impact DENV replication so they were not tested [147]. HepG2 cells were mock- or DENV-infected for 48 hours and then treated with antibodies to Optineurin, p62, or NBR1, as well as antibodies raised against viral proteins NS3 or DENV E and then stained with ORO. After microscopy imaging he compared the amount of colocalizing lipid droplets and autophagy receptor antibodies between uninfected and infected cells. Optineurin and p62 exhibited basal levels of lipid droplet colocalization that did not increase following DENV infection (Fig. 2A-D). In contrast, NBR1 colocalization with lipid droplets significantly increased after 48 hours of DENV infection (Fig. 2E, F).

NBR1 is not necessary for DENV mediated induction of autophagosomes

To assess whether NBR1 is required for targeting the lipid droplet for autophagic degradation, Huh7.5 cells were transfected with either non-targeting control siRNA or one of two different siRNAs targeting NBR1 (Fig. 3). The Randall lab has previously shown that infecting cells with DENV for 24 hours significantly increases the number of LC3 puncta per cell as the virus induced autophagy causes an increase in the formation of autophagosomes within the cell [43]. To evaluate whether silencing NBR1 influenced this LC3 puncta formation, Huh7.5 cells were transfected with either non-targeting control siRNA or one of two different siRNAs targeting NBR1 and then infected the cells with DENV at an MOI of 5 for 24hrs. LC3 and DENV E were probed for using antibodies raised against either protein, imaged the cells on a confocal microscope, and the number of LC3 puncta per cell was quantified. Silencing NBR1 did not appear to inhibit the virus's ability to induce LC3 puncta formation (Fig. 4). In other replicates, the mock infected cells transfected with the second siRNA targeting NBR1 (NBR1-2) contained a higher than usual number of LC3 puncta per cell. Thus, the number of LC3 puncta per cell did not significantly increase between the mock and DENV infected, though there is an upwards trend. These results indicate that NBR1 is not necessary for the initial induction of autophagy and the formation of autophagosomes during DENV infection.

NBR1 is critical for DENV induced lipid droplet depletion

The cells were then either mock or DENV infected for 48 hours and then treated with antibodies against DENV NS3, and stained with oil red O (ORO), which stains neutral lipids such as those found within the lipid droplet. After microscopy imaging, the amount of cellular area covered by the lipid droplets in each cell was calculated and compared between mock and infected cells. In the control transfected cells, infection with DENV for

48 hours induced a mobilization of the lipid droplet's lipid content as demonstrated by a significant decrease in the lipid droplet abundance per cell in DENV infected cells compared to uninfected cells, as previously described[43]. Cells transfected with either of the distinct siRNA's targeting NBR1 did not display a decrease in lipid droplet abundance following DENV infection, indicating the autophagy receptor NBR1 plays a critical role in targeting the lipid droplet for autophagic degradation during dengue virus induced lipophagy (Fig. 5).

Impact of CRISPR mediated knockout of NBR1 on DENV induced lipophagy

To aid in characterizing NBR1's role in lipophagy a CRISPR Knockout cell line that had NBR1 knocked out was generated (Fig. 6). Quantifying the number of LC3 puncta in mock and DENV infected cells, verified the expected increase in LC3 puncta per cell in the control cells. The NBR1KO cells did not demonstrate a significant increase in the number of LC3 puncta per cell following DENV infection due to a higher number of puncta in the mock-infected cells. Despite this lack of statistical significance, there is still an upward trend that can be observed between the mock and DENV infected cells (Fig. 7). This phenotype is similar to what is observed in some of the replicates evaluating the effect siRNA targeting of NBR1 has on the amount of LC3 puncta as described earlier. This supports the hypothesis that NBR1 acts as the adaptor protein that bridges the lipid droplet to the nascent phagophore during DENV induced lipophagy. Afterwards control and NBR1KO cells were infected for 48 hours at an MOI of 5 and the lipid droplet abundance was measured; DENV induced mobilization of the lipid droplets were inhibited in NBR1KO cells (Fig. 8). These data indicate that NBR1 is the adaptor protein

responsible for targeting the lipid droplet to the autophagosome in DENV induced lipophagy.

NBR1 is required for induction of lipophagy by NS4A and NS4B

It was previously described that DENV proteins NS4A and NS4B are sufficient for induction of lipophagy [83,148]. To determine if NBR1 was necessary for DENV NS4A and NS4B induced lipophagy, Huh7.5 cells containing a Tet-on 3G gene were either transfected with non-targeting control siRNA or siRNA targeting NBR1 and then transduced with pseudovirus containing either an empty vector or a vector containing DENV NS4A and NS4B. Then they were either treated with 5 ng/mL of Doxycycline alone or infected with DENV for 48 hours. They were then stained with anti-NS4A antibody and ORO and imaged via microscopy. Non-targeting control siRNA transfected cells transduced with NS4A and NS4B or infected with DENV demonstrated a significant reduction in lipid droplet abundance compared to cells transduced with an empty vector. Cells that were transfected with siRNA targeting NBR1 and transduced with NS4A and NS4B or infected with DENV did not demonstrate a drop in lipid droplet abundance compared to the empty vector control (Fig. 9). These data indicate that NBR1 is necessary for lipophagy induced by DENV infection and NS4A and NS4B expression.

Discussion

Dengue virus induced lipophagy has been well described, but the mechanism by which the lipid droplet is specifically targeted has not been investigated [43,82,83]. This thesis examines this targeting mechanism and how lipophagy affects DENV infection. Tristan Jordan performed experiments that indicate upon infection with dengue virus, K63

polyubiquitin chains are ligated to the cytosolic surface of the lipid droplets (Fig. 1). These data suggest that the DENV induced lipophagy is ubiquitin mediated. When Jordan evaluated the colocalization of various ubiquitin binding autophagy receptors with lipid droplets, he observed that specifically the Neighbor of BRCA1 Gene 1 (NBR1) and not p62 nor optineurin increased accumulation on the surface of the lipid droplet during DENV infection (Fig. 2). These findings point to the autophagy receptor NBR1 as the adaptor protein responsible for bridging the lipid droplet to the autophagosome.

To confirm NBR1's role in DENV induced lipophagy, cells were treated with one of two different siRNAs targeting NBR1. siRNA mediated knockdown of NBR1 does not prevent DENV from inducing autophagy as shown by the increase in LC3 puncta in both control siRNA transfected cells and cells transfected with siRNA targeting NBR1. In one experiment, the cells transfected with one of the NBR1 targeting siRNA (NBR1-2) did not show increased LC3 puncta formation after DENV infection due to the mock infected cells showing a slight increase in LC3 puncta formation. When NBR1 is knocked out via CRISPR/Cas9 mediated inhibition, there is a similar increase in the number of LC3 puncta in the mock infected cells with a trending increase of LC3 puncta in the DENV infected cells. NBR1 knockdown may influence the amount of autophagosomes present in the cell at a basal level, which would explain this phenotype.

Transfecting cells with siRNA targeting NBR1 inhibits the virally induced autophagic flux of the lipid droplets (Fig. 5). Knocking NBR1 out via CRISPR also inhibits the DENV induced depletion (Fig. 8). This supports the hypothesis that NBR1 acts as the adaptor protein bridging the lipid droplet to the autophagosome during DENV infection.

Targeting NBR1 will allow investigators to specifically characterize the mechanism of lipid droplet depletion during DENV infection and possibly in non-infectious contexts.

Since DENV proteins NS4A and NS4B have been shown to be sufficient to cause lipophagy in cells, NBR1's necessity in the context of DENV NS4A and NS4B induced lipophagy was evaluated [83,148]. These experiments demonstrated that NBR1 is necessary for NS4A and NS4B induced lipophagy (Fig. 9). These results confirm the necessary role NBR1 plays in targeting the lipid droplet for autophagosomal degradation during DENV induced lipophagy. These data provide a novel and uncharacterized role for the autophagy receptor NBR1.

The adaptor protein NBR1 has many characterized roles especially as an autophagy receptor, but this dissertation has demonstrated a novel role for it as the receptor responsible for targeting the lipid droplet to the autophagosome during DENV infection[96]. Results indicate that NBR1 is likely also responsible for targeting the lipid droplet for lysosomal degradation in DENV-independent lipophagy. More work needs to be done investigating general lipid mobilization and metabolization utilizing a specific lipophagy stimulant. By investigating NBR1's role in DENV infection lipophagy's role in DENV infection can also be elucidated. Previous work has shown that inhibition of bulk autophagy negatively impacts virus replication but the discovery of NBR1 as the lipophagy adaptor allows for the specific inhibition of lipophagy [43]. The next chapter will be focused on determining how the virus is impacted by specifically inhibiting lipophagy.

Chapter 3: NBR1 is necessary for a non-canonical Dengue Virus secretion method

Abstract

Infection with dengue virus or transfection with dengue viral proteins NS4A and NS4B induces an autophagic response that targets the lipid droplet to mobilize lipids thus stimulating lipid metabolism. These experiments have demonstrated that the autophagy adaptor NBR1 is necessary for this lipophagic response. Silencing NBR1 allows the characterization of the effects that specifically inhibiting lipophagy has on the DENV life cycle. Inhibition of NBR1 protein expression does not impact DENV RNA replication or assembly of infectious virions. However silencing NBR1 negatively impacts DENV egress from the cells. DENV utilizes NBR1 to leave the cell in a non-canonical secretion method. Exogenous addition of free fatty acids restores DENV egress in cells that have had NBR1 silenced. Similar to the related flavivirus ZIKV, this uncharacterized secretion method possibly allows DENV to escape the cell in an extracellular vesicle protecting it from antibody neutralization. The data from this study suggests that NBR1 and DENV induced lipophagy play a critical role in the autophagic secretion of DENV.

Introduction

Many viruses utilize autophagy to efficiently infect cells, this autophagy can contribute to the viral infection via a number of different mechanisms [77]. One such mechanism involves the autophagy-assisted secretion of the virus or secretory autophagy [119]. The flavivirus Zika virus (ZIKV) has been shown to utilize Lyn kinase to leave the cell in autophagosome derived vesicles [111]. Another group demonstrated that DENV leaves the cell in autophagosomes that are also associated with lipid droplets [78].

DENV induced lipophagy may be involved in the secretory autophagy assisted release of DENV.

DENV infection causes the accumulation of k63 linked poly-ubiquitin chains on the cytosolic surface of the lipid droplets (Fig. 1). These polyubiquitin chains specifically recruit NBR1 to the lipid droplet to allow for the autophagic degradation of the lipid droplet to mobilize the lipids. Heaton and Randall have previously shown that inhibition of autophagy via treatment with autophagy inhibitor 3-Methyladenine (3MA) or siRNA mediated knockdown of important autophagy proteins such as Beclin-1 or ATG5 negatively affects DENV RNA replication [43]. With the identification of NBR1 as the specific autophagy receptor for lipophagy, future investigations can specifically target lipophagy to observe its impact on DENV.

The DENV-life cycle has multiple stages. It must bind to a cell surface receptor and enter the cells. Then it uncoats and translates its RNA genome into the polyprotein which is subsequently cleaved by viral and host proteases. Afterwards the virus replicates its RNA genome and packages it into the capsid before leaving the cell to infect other cells. By silencing NBR1 and evaluating these life cycle steps, which step in the viral life cycle is impacted by inhibiting lipophagy can be determined. Earlier it was confirmed that DENV expresses viral proteins in cells with reduced NBR1 expression (Figs. 4,5,8, and 9). These demonstrate that the virus can enter the cell and translating its RNA genome despite the lack of NBR1. The next step is to evaluate whether DENV requires NBR1 to replicate its RNA genome, virion assembly, and egress. This chapter will demonstrate that NBR1 is required for an alternative autophagy associated egress pathway similar to those previously described in flaviviruses and various other viruses.

Poliovirus uses autophagy to egress from cells in a non-lytic manner. Investigators observed that the normally lytic poliovirus was able to non-lytically secrete from Hela cells without inducing lysis after the cells had been treated with the autophagy stimulator Tamoxifen [149]. Inhibition of the autophagosome protein LC3 severely reduced non-lytic release of poliovirus, these data taken together suggest that poliovirus utilizes autophagy to exit the cell via a non-canonical egress method [136]. Hepatitis A virus (HAV) is typically considered to be a non-enveloped virus, but investigators identified that viral particles that have been secreted into the serum of infected patients are contained in a quasi-envelope [150]. Analysis of the proteins in the quasi-envelope via mass spectrometry reveal that the quasi-envelope contains the proteins Charged multivesicular body protein (CHMP) 1A and 1B; proteins associated with the multivesicular body (MVB) a critical component of the secretory autophagy pathway [151,152]. These results support the possibility that HAV utilizes autophagy to secrete from cells in a largely uncharacterized alternative egress pathway. Li et al demonstrated that the flavivirus ZIKV relies on Lyn kinase to secrete in autophagosome derived extracellular vesicles positive for LC3 and Rab11 [111]. Electron microscope analysis of ZIKV infected cells reveal that large vesicles containing multiple virions and smaller vesicles containing single virions can be visualized within the cells further strengthening the hypothesis that ZIKV is capable of leaving the cell in two distinct pathways [153,154]. Wu et al demonstrated that inhibition of autophagy impairs DENV's ability to leave the cell via a significant secretion pathway as illustrated by the severe reduction in extracellular DENV. They also indicate that this autophagy-dependent pathway releases DENV contained in extracellular vesicles that contribute to viral

resistance to antibody neutralization. These vesicles also contain the autophagosome protein LC3 and the lipid droplet providing evidence for a link between the targeting of the lipid droplet to the autophagosome and the non-canonical secretion of DENV [78].The upcoming results will show that inhibition of NBR1 expression does not significantly impact DENV replication nor assembly, but it does severely reduce DENV secretion via a non-canonical egress method.

Results

Silencing NBR1 inhibits DENV egress but not replication or assembly

Since lipophagy is a proviral response the next step was to identify the stage in the viral life cycle reliant on NBR1 [43,82]. The previous experiments demonstrated that DENV entry and translation are unaffected in cells that have had NBR1 expression inhibited via either siRNA transfection or CRISPR knockout. To evaluate whether DENV RNA replication is affected by inhibiting lipophagy, a RT-qPCR experiment was performed to measure the amount DENV RNA across various timepoints (6 hpi, 24hpi, 48hpi, and 72 hpi) in infected Huh7.5 cells that had been transfected with control non-targeting siRNA or siRNA targeting NBR1. There was little to no difference in the amount of RNA replication between the control transfected cells and cells transfected with siRNA targeting NBR1 (Fig. 10).

To investigate the effect lipophagy had on viral assembly and egress, a TCID50 titer assay was used to quantify both cell-associated and extracellular infectious DENV from infected cells that had been transfected with non-targeting siRNA or one of two different siRNAs targeting NBR1. To evaluate the effect that inhibiting NBR1 expression has on DENV assembly, the amount of cell-associated infectious DENV after 48 hours of

DENV infection was evaluated. The supernatant was collected, and the cells were washed, refreshed with fresh media, and freeze/thawed repeatedly to lyse them and release any cell-associated virus into the media. There was not a significant difference in the amount of cell-associated infectious DENV between control transfected cells and cells transfected with siRNA targeting NBR1 (Fig. 11A). Thus, virion assembly is independent of NBR1. The amount of extracellular infectious DENV in the first collected supernatant was measured and unlike the cell-associated infectious DENV, there was a significant reduction in the amount of extracellular infectious DENV in the supernatants from cells transfected with siRNA targeting NBR1 (Fig. 11B). Measuring the amount of extracellular infectious DENV in cells in which NBR1 has been knocked out via CRISPR revealed a similar reduction (Fig. 12). These data provide evidence that DENV RNA replication and assembly are not impacted by NBR1, but viral egress is reliant on NBR1.

With this investigation into the mechanism of how the lipid droplet is targeted for autophagic degradation during DENV, a novel role for the autophagy adaptor protein NBR1 has been identified. As summarized in the model, infection with DENV induces the ligation of K63 poly-ubiquitin chains to the surface of the lipid droplet. These poly-ubiquitin chains then bind to the UBA domain of the autophagy adaptor protein NBR1 which also binds to the LC3 on the nascent phagophore via its LIR domain, bridging the lipid droplet to the autophagosome. The autophagosome then engulfs either part or all of the lipid droplet. The autophagosome fuses with a lysosome forming the autolysosome degrading the lipid droplet into free fatty acids. This leads to an overall increase in the amount of infectious DENV virions and DENV RNA released from the cell (Fig. 13).

The supernatant of infected cells that have been transfected with NBR1 targeting siRNA also had a significant reduction in the amount of extracellular DENV RNA in addition to the decrease in infectious DENV particles (Fig 14). These results demonstrate that silencing NBR1 is not impacting the infectivity of released DENV virion, it is instead affecting the release of infectious virions from the cell. The remainder of this chapter will be focused on characterizing the mechanism of this autophagy assisted DENV egress and attempting to determine the link between DENV induced lipophagy and DENV egress.

DENV uses NBR1-dependent lipophagy to egress via a non-canonical secretory route

To understand the link between lipophagy and egress, the nature of the NBR1-assisted egress must be characterized. Previous studies have shown that the flaviviruses DENV and Zika virus (ZIKV) have two forms of secreted virions that differ in buoyant density, with a lower density fraction containing virions in extracellular vesicles and a higher density fraction containing free floating virions not contained in vesicles [78,111,155]. To further interrogate this NBR1/autophagy-dependent DENV secretion, an experiment performed by Li et al where they demonstrated that ZIKV egresses from infected cells in the previously described buoyant densities was adapted to measure the buoyant densities of secreted DENV in cells that have been treated with siRNA targeting NBR1 [111]. Secretion of the more infectious lighter fraction was dependent on Lyn expression; this fraction was determined to be infectious ZIKV enclosed in autophagosome derived extracellular vesicles.

To perform this experiment in the context of DENV infected cells with or without NBR1 expression, DENV infected supernatants from control transfected cells and from cells transfected with siRNA targeting NBR1 were fractionated on a discontinuous sucrose gradient, ten 1 mL fractions were collected and the amount of DENV RNA and infectious DENV in each fraction were then quantified. There were two infectious peaks containing both DENV RNA and infectious particles in the control transfected cell supernatant. A heavy peak in one of the 40% sucrose fractions and a lighter peak in one of the 10% sucrose fractions (Fig. 15 A, C). This sedimentation pattern is very similar to what Li et al described for ZIKV using similar conditions[111]. When the amount of infectious virus and viral RNA in the supernatant of the siRNA targeting NBR1 transfected cells was measured, the heavier density peak in the 40% sucrose fraction could be observed, but not the lighter density peak (Fig. 15 B, D). Comparing the specific infectivity, the ratio of infectious particles to the RNA copy number as calculated by the RNA amount, of the two infectious peaks reveals there is no significant difference in specific infectivity (Fig. 16). Knocking out NBR1 via CRISPR and analyzing the sedimentation patterns of released DENV particles demonstrates a similar phenotype (Fig. 17). These results led to the formation of a hypothesis that the DENV induced breakdown of lipid droplets contributes to an alternate egress route for DENV.

Since the autophagic breakdown of lipid droplets results in the release of free fatty acids (FFA), and Heaton and Randall demonstrated that the addition of FFA rescues infectious DENV production in autophagy-deficient cells, whether exogenous addition of FFA would have an impact on the infectious peaks was evaluated [43,86,87]. After the cells were infected for four hours, the infected media was replaced with uninfected media

supplemented with 1:1000 BSA alone or Oleic Acid-BSA and left to incubate for 48 hours. The amount of infectious DENV in each fraction of each condition was then measured. The control transfected cell supernatant maintained in media supplemented with BSA alone contained two infectious peaks in the 40% fraction and 10% fraction and the supernatant from the cells transfected with siRNA targeting NBR1 maintained in media supplemented with BSA alone only contained both the heavy infectious peak. These results were expected due to the previous experiments (Fig. 15, 17). When the amount of infectious DENV in the fractions of infected supernatants of either control non-targeting siRNA or siRNA targeting NBR1 transfected cells that were maintained in media supplemented with 1:1000 Oleic acid was measured, both transfection conditions contained the heavy and light infectious peaks (Fig. 18). This indicates that the free fatty acids released by lipophagy are significantly contributing to the alternative egress route of the virus. These data suggest that DENV induced lipophagy is critical for DENV to utilize an autophagy dependent secretion method. More work needs to be done to identify how the release of free fatty acids enhances DENV release in this non-canonical autophagy pathway.

Determining the link between DENV induced lipophagy and secretory autophagy

DENV induced lipophagy causes the release of free fatty acids which can then be utilized by the mitochondria to produce ATP via a process called β-oxidation allowing the cell to make use of the stored energy in the lipid droplet. To test the hypothesis that DENV induced lipophagy enhances ATP production to restructure the cell's secretory network allowing the virus to utilize an alternate way to escape from the infected cell, the supernatants from DENV infected cells that had either been treated with DMSO or 20 μM

of the β-oxidation inhibitor, etomoxir, were collected and fractionated on a discontinuous sucrose gradient, and the amount of infectious DENV in each fraction was measured. In the DMSO control condition, there were 2 infectious peaks as expected, but in the etomoxir condition, the overall amount of virus is sharply reduced in both peaks. Despite this severe reduction in the amount of infectious virus, etomoxir treatment more strongly impacted the heavier peak than the lighter peak (Fig. 19). These data point to a possibility that both the autophagy-independent and autophagy-dependent viral egress pathways rely on the process of β-oxidation. β-oxidation is a major metabolic process and inhibiting it has broad implications which make these data difficult to interpret. More work needs to be done to determine the role of lipid metabolism and energy production in this NBR1-dependent pathway.

Since an obvious correlation between β-oxidation and the autophagy-assisted secretion method was not observed, this led to the hypothesis that DENV induced lipophagy causes an increase in the number of free fatty acids in the cell and this increase in the cell's fatty acid content triggers a secretion pathway that DENV is somehow able to hijack. To assess this hypothesis, an ELISA to evaluate the amount of IL-1β in the supernatant of cells in varying contexts was performed. IL-1β is a leaderless cytosolic protein that requires secretory autophagy to exit the cell, so IL-1β is being used as a general marker for secretory autophagy [121]. The amount of IL-1β released was measured in four different conditions: Mock infected supplemented with BSA, mock infected supplemented with oleic acid, DENV infected supplemented with BSA, and DENV infected supplemented with oleic acid. Infecting with DENV and/or supplementing cells with oleic acid caused an increase to the amount of IL-1β in the supernatant (Fig. 20).

These results indicate that it may be possible that DENV induced increase in the cell's free fatty acid content may be stimulating a secretion pathway. More work can be done to determine how the mobilization of lipids affects secretory autophagy. However, the remainder of this chapter will describe attempts to characterize the NBR1-dependent infectious peak.

Characterization of the lower buoyant density infectious peak

Both Li et al and Wu et al described ZIKV and DENV as leaving the cell in autophagy-associated extracellular vesicles; so a hypothesis stating that the lighter infectious peak, hereafter referred to as "Peak 2" from now on, consisted of DENV contained in extracellular membranous vesicles and the heavier infectious peak, hereafter referred to as "Peak 1", is made up of free floating DENV virions as shown in the model (Fig. 21) was formed [78,111]. To assess this hypothesis, an attempt was made to determine if Peak 2 could be detected via an indirect ELISA. The hypothesis was that Peak 1 would be detected via Indirect ELISA, but Peak 2 would only be detected after treatment with 1% Igepal detergent disrupts the membranous vesicles releasing the virions contained within. Fractions corresponding to Peak 1 and Peak 2 were collected from the supernatants of Huh7.5 cells that had either been infected for 48 hours or 72 hours and then either treated with 1% Igepal or PBS solution for 30 minutes. If peak 2 consists of DENV contained in extracellular vesicles and peak 1 is made up of free-floating virions, then there would be equal signal in Peak 1 in both the PBS treated and 1% Igepal treated conditions. Peak 2 should have little to no signal in the PBS treated condition, but increased signal after 1% Igepal treatment. There was little to no signal in the negative controls, mock infected supernatants and media containing 10% FBS only, regardless of

whether the samples were treated with 1% Igepal (Fig. 22C). In the 48 hours post-infection condition, Peak 2 was detected along with Peak 1 and the input, but there is no increase in the signal after 1% Igepal treatment (Fig. 22B). In the 72 hours post infection condition there is increased signal across the board compared to the 48 hour post infection samples. Treatment with 1% Igepal does not increase the amount of signal in the input sample but does increase the signal in the Peak 1 and 2 samples (Fig. 22A). These results are inconclusive as they do not confirm whether Peak 2 is membrane bound or not, so other experiments must be performed to determine the nature of the more buoyant Peak 2. It is possible that the detergent treatment was also impacting the virion structure and this the epitope availability for the ELISA

Next a protease protection assay was performed to identify the morphology of the infectious particles in Peak 2. If the infectious particles that make up Peak 2 are contained in extracellular vesicles then the DENV E protein within the vesicle will be protected from Protease K degradation. Treating the sample with 1% Igepal will render the DENV E protein susceptible to protease degradation. The protease protection assay was performed on both the overall unfractionated infected supernatant and the fraction correlating to infectious Peak 2. Western blotting for DENV E on both the untreated whole supernatant and Peak 2 stained positive confirming the presence of DENV in these samples. When the samples were treated with Proteinase K, both samples no longer tested positive for DENV E (Fig. 23). These results indicate that the DENV virions in Peak 2 are not protected from protease degradation. These results taken along with the results of the earlier ELISA suggest that the virions in Peak 2 may not be enclosed in an

extracellular vesicle. One caveat to this interpretation is that the extracellular vesicle stability may be compromised during the experimental procedure.

Wu et al described the autophagic-associated extracellular vesicle that DENV egresses from as conferring resistance to DENV from antibody mediated neutralization [78]. To determine if silencing NBR1 caused DENV transmission to be more susceptible to neutralization by Anti-DENV E antibodies the supernatants from infected cells that were either transfected with non-targeting control siRNA or siRNA targeting NBR1 were collected and equivalent amounts of infectious particles from these supernatants were incubated with different concentrations of anti-DENV E antibody and then used to infect Huh7.5 cells. After 48 hours of infection, the level of infectivity across the different concentrations of neutralizing antibodies was evaluated by comparing the number of infected cells in wells with a given concentration of antibody to the wells with no neutralizing antibody. There was an expected decrease in infectivity correlating to an increase in antibody concentration. DENV from cells that had been transfected with siRNA targeting NBR1 demonstrated a lower infectivity when compared to virus from the control transfected cells (Fig 24). These data suggest that NBR1 is conferring some resistance to neutralization of the released virus.

To further characterize the identity of the infectious Peak 2, Huh7.5 cells were treated with either DMSO or the drug, GW4869. GW4869 inhibits the membrane enzyme nSMase, which releases ceramide important for exosome shedding. Therefore nSMase inhibition results in a decrease in exosome biogenesis and release [156]. Huh7.5 cells were infected with DENV for 4 hours and then the infected media was replaced with uninfected media containing either DMSO or GW4869 and the cells were left to incubate for 48 hours.

Treating cells with GW4869 did not significantly impact the amount of infectious DENV released from cells compared to cells that had been treated with DMSO (Fig 25). There are several potential reasons for this result, but it seems to suggest that DENV does not leave the cell in exosomes.

Discussion

Targeting NBR1 via siRNA degradation and evaluating the effect this knockdown had on the different steps of DENV infection, such as entry, translation, replication, assembly, and egress, shed light on the role of NBR1 and possibly lipophagy in DENV infection. The microscopy experiments demonstrated that DENV can infect cells and translate its genome in Huh7.5 cells transfected with siRNA targeting NBR1. Knockdown of NBR1 had no significant impact on the amount of viral RNA replication as measured by RT-qPCR indicating that DENV RNA replication does not rely on NBR1. siRNA mediated silencing of NBR1 did not significantly alter the amount of cell-associated infectious particles as measured by TCID50 compared to control non-targeting siRNA transfected cells. These results demonstrate that infectious viral particle assembly is not affected by the defect in DENV induced lipophagy. However, the supernatant of cells that had been transfected with siRNA targeting NBR1 had significantly fewer infectious particles as measured by TCID50 (Fig. 11). This led to the hypothesis that lipophagy is necessary for an autophagy-dependent secretion method such as those found in both DENV and other viruses [78,79,151]

To investigate this NBR1-dependent viral egress phenotype, an experiment done by Li et al was adapted to evaluate how DENV egresses in NBR1-silenced cells compared to control cells[111]. Fractionating DENV infected supernatant on a discontinuous sucrose gradient and measuring the amount of DENV RNA and infectious particles in each fraction, resulted in the observation of 2 infectious peaks. Silencing NBR1 via either siRNA transfection or CRISPR transduction caused a severe reduction of both the RNA amount and number of infectious particles in the lighter “Peak 2", but not in the heavier “Peak 1”. Comparing the specific infectivity of both peaks reveal that they both represent similarly significant avenues of subsequent infection (Fig 16). NBR1 is responsible for infectious virus egressing in some altered method that possibly involves the capture of DENV virions in vesicles which are released from the cell via secretory autophagy. Exogenously added oleic acid, a monounsaturated fatty acid, restores the abolished Peak 2 in the fractionated supernatant of cells transfected with NBR1 targeting siRNA supporting the hypothesis that this NBR1 dependent peak is due to DENV induced lipophagy instead of another function of NBR1. Future work should be done to confirm that NBR1’s role in lipophagy is mediating this low density DENV secretion.

To investigate how lipophagy can result in the non-canonical secretion of DENV, the effect that etomoxir mediated inhibition of β-oxidation has on this non-canonical NBR1-dependent secretion pathway was evaluated. β-oxidation appears to be required for robust release of the virus via either pathway as both peaks were drastically reduced in the supernatants of cells treated with etomoxir (Fig 19). Since Peak 1 was more affected by inhibition of β-oxidation than Peak 2, it is possible that β-oxidation primarily supports the canonical secretion of DENV while some other process is the major

contributor to the non-canonical autophagy-dependent secretion. It is also likely that etomoxir treatment impacts an earlier step in the DENV life cycle and work should be done to determine the role of β-oxidation in DENV translation, replication, and assembly. It is possible that the link between DENV induced lipophagy and secretory autophagy is not due to energy production and instead is related to another cellular process or pathway.

Another hypothesis was formed stating that DENV utilizes NBR1 to target lipid droplets to the autophagy pathway to mobilize the lipid droplet's fatty acids, and this mobilization of free fatty acids stimulates a secretion pathway that DENV is able to hijack and use to escape the cell. To test this hypothesis, IL-1β was used as a marker for secretory autophagy and the amount of IL-1β in the supernatant was evaluated via ELISA. Infection with DENV caused an increase in IL-1β release which may be an indicator of the hypothesis being accurate or that DENV infection caused the activation of caspase-1 inducing an increase in IL-1β [157]. Mock infected cells that were maintained in media supplemented with exogenous free fatty acids demonstrated an increase in IL-1β release supporting the hypothesis that an increase in mobilized lipids could stimulate secretory autophagy, perhaps to relieve lipotoxicity when the concentration of cellular lipids is too high for autophagic degradation to maintain homeostasis. DENV may be able to then somehow enter these vesicles and egress. Another possibility involves DENV activation of caspase-1 as a method of viral secretion, though the involvement of the free fatty acids and lipid droplets is unclear in this hypothesis. DENV and lipid induced secretory autophagy represents a heretofore undescribed interaction between DENV and the cellular secretion pathway that warrants more investigation.

To identify what structural factor or factors cause Peak 2 to sediment at a lower density fraction than Peak 1 a hypothesis was formed stating that Peak 2 was made up of extracellular vesicles that contain DENV virions. To evaluate this hypothesis, an indirect ELISA, a protease protection assay, and a neutralization assay were all performed. The hypothesis stated that if DENV was contained in membranous vesicles then, it would not be bound and detected by the antibodies in the ELISA unless the vesicles are first digested by 1% Igepal. While detection of peak 2 increased with Igepal treatment, it still showed significant detection without Igepal treatment. Peak 1 was detected without Igepal treatment, but the Igepal treated peak 1 also demonstrated increased signal. Peak 1's Igepal induced increase in signal may be due to a conformational change in the DENV E protein leading to enhanced antibody binding. Peak 2's signal increase may be due to the same process or the degrading of a membranous vesicle. Since the negative controls did not show any signal regardless of Igepal treatment, it is unlikely that there is a significant amount of non-specific binding. These results are inconclusive and do not reveal whether the infectious virions in Peak 2 are contained in extracellular vesicles. Since antibody detection did not provide solid evidence as to the nature of peak 2, the susceptibility of Peak 2 to Proteinase K was evaluated.

A protease protection assay was performed to determine whether the DENV virions in peak 2 were protected from protease mediated degradation, but neither the whole supernatant nor peak 2 were protected from proteinase K degradation. There are 3 possibilities that explain this result: 1) the virions in peak 2 may not be contained in extracellular vesicles, 2) they are contained in vesicles, but the vesicles are permeable to

Proteinase K, or 3) they leave in vesicles that do not maintain their structure for long in the storage conditions they were kept in (4°C in sucrose solution). There is more work to be done to determine the structure and morphology of the virions of peak 2. To further investigate this, whether DENV released from cells transfected with siRNA targeting NBR1 have a functional disadvantage compared to virus released from cells transfected with non-targeting control siRNA was evaluated.

To investigate whether there was a functional difference between DENV released from control transfected cells compared to DENV released from cells transfected with siRNA targeting NBR1, a neutralization assay was performed. DENV from NBR1 targeting siRNA transfected cells are more susceptible to antibody neutralization than DENV from the control cells. This supports the hypothesis that the virions in Peak 2 are contained within extracellular vesicles that protect them from neutralization. These data corroborate the findings of Wu et al who described DENV as being secreted in autophagy-associated vesicles which protect the virus from antibody neutralization [78]. Being able to escape the cell in a form that allows the virus to resist antibody neutralization is an advantageous adaptation. This would make NBR1 and DENV induced lipophagy attractive targets for potential antiviral therapies. There is plenty of work to be done to properly establish whether DENV stimulated lipid metabolism is responsible for contributing to DENV's resistance to neutralization or some other role of NBR1.

Next, to further assess this hypothesis, whether DENV is released in a subset of extracellular vesicles known as exosomes was evaluated. The effect that GW4869 had on DENV secretion was evaluated. There was no significant difference between the amount of virus secreted from the DMSO control cells and the amount of virus secreted

from the GW4869 treated cells. These findings indicate that DENV may not be secreting in exosomes. However, it is still possible that DENV is secreting in another form of extracellular vesicle such as microvesicles or apoptotic bodies. Another possibility is that DENV is somehow able to bypass the need for nSMase and induce biogenesis in the presence of an nSMase inhibitor. To assess this hypothesis, it may be useful to evaluate the effect that another exosome inhibitor has on DENV secretion. And finally it is possible that DENV does not secrete in an extracellular vesicle at all, but can instead leave the cell in a lipidated form similar to HCV [158].

The experiments indicate that DENV is utilizing NBR1 and likely lipophagy to escape the cell via a non-canonical secretion pathway. Lipid metabolism has not been described as contributing to secretion pathways before, making this an exciting and novel contribution to the field of virology and cell biology. The virus that leaves this pathway is likely in a vesicle capable of protecting it from antibody neutralization. Future work must be done to better comprehend and characterize this pathway as it represents a major avenue for DENV secondary infection and may provide insight into how other viruses utilize autophagy to egress. This work may also aid in understanding the uncharacterized cellular processes involved in secretory autophagy.

Chapter 4: Conclusion

Findings and Model

Dengue virus induced lipophagy has been well described, but the mechanism by which the lipid droplet is specifically targeted had not yet been investigated [43,82,83]. This project examines this targeting mechanism and how lipophagy impacts the DENV life cycle. Tristan Jordan observed that after infection with dengue virus, K63-linked, and not K48 linked, polyubiquitin chains accumulate at the cytosolic surface of the lipid droplets. This supported the possibility that the DENV induced lipophagy is ubiquitin mediated (Fig. 1). Interestingly, K63-linked polyubiquitin is often associated with non-degradative roles whereas K48-linked polyubiquitin tends to target cargo for proteasome dependent degradation [159]. When Tristan evaluated the colocalization of various ubiquitin binding autophagy receptors with lipid droplets, he observed that only Neighbor of BRCA1 Gene 1 (NBR1) increased accumulation on the surface of the lipid droplet in DENV infected cells (Fig. 2). These results indicate that the autophagy adaptor protein NBR1 is recruited to the cytosolic surface of the lipid droplet during DENV infection.

To characterize NBR1's role in DENV induced lipophagy, cells were transfected with one of two different siRNAs targeting NBR1 or control non-targeting siRNA. siRNA mediated knockdown of NBR1 does not prevent DENV from inducing autophagy as shown by an increase in LC3 puncta in DENV infected cells in both the control siRNA transfected condition and in cells transfected with siRNA targeting NBR1 (Fig. 4). However transfecting cells with siRNA targeting NBR1 inhibits the virally induced autophagic flux of the lipid droplets as demonstrated by the lack of reduction in lipid droplet abundance in DENV infected cells compared to mock infected cells (Fig. 5). These

data demonstrate that NBR1 function after the induction of the autophagy during DENV infection, but before the depletion of lipid droplets. This phenotype is consistent with the hypothesis that NBR1 acts as the specific autophagy receptor bridging the lipid droplet to the autophagosome during DENV infection.

Viral proteins NS4A and NS4B have been shown to be sufficient to induce lipophagy in cells [83,148]. We observed that NBR1 is necessary for NS4A and NS4B induced lipophagy (Fig. 9). These results confirm the necessary role NBR1 plays in targeting the lipid droplet for autophagosomal degradation during DENV induced lipophagy as well as corroborate Zhang et al's findings that NS4A and B are sufficient to induce lipophagy.

Nicholas Heaton's previous study demonstrated that inhibition of general autophagy impaired DENV's ability to produce infectious virus, but not how lipophagy specifically aids in DENV infection can be determined. Targeting NBR1 via siRNA degradation allows for the evaluation of the impact of DENV infection without affecting bulk autophagy. Knockdown of NBR1 had no impact on the amount of viral RNA replication as measured by qPCR nor the assembly of infectious particles as shown by the amount of cell-associated infectious particles as measured by TCID50 (Fig. 10, 11A). The defect in DENV induced lipophagy did not have a significant impact on either of these viral life cycle steps. However, the supernatant of cells that had been transfected with siRNA targeting NBR1 had significantly fewer infectious particles as measured by TCID50 (Fig. 11B). This led to the hypothesis that lipophagy is necessary for an autophagy-dependent secretion method such as those found in both DENV and other viruses [78,79,151]. These results as summed up by the model provided evidence for a novel role of lipophagy as a critical regulator of viral secretion (Fig. 13).

These results represent an important and exciting possibility for the study of virally induced autophagy and uncharacterized modes of viral egress. Fractionating the infected supernatant from control transfected cells on a discontinuous sucrose gradient produced two infectious peaks as previously described in ZIKV (Fig. 15 A, C)[111].The heavier peak, Peak 1, may be made up of free floating virus and the lighter peak, Peak 2, may consist of DENV inside of autophagy associated vesicles. Silencing NBR1 abolishes the lighter infectious peak supporting the hypothesis that lipophagy is necessary for an uncharacterized autophagy mediated secretion of DENV (Fig. 15 B, D). Since lipophagy causes a release of free fatty acids (FFA), we evaluated the ability of exogenous FFA to complement the defective lipophagy in NBR1 silenced cells (Fig. 13). Supernatant from siNBR1 transfected cells that have been supplemented with oleic acid restored the low density infectious peak after sucrose fractionation similar to the supernatant from control transfected cells (Fig. 18). These data further support the hypothesis that DENV induced lipophagy leads to secretory autophagy of DENV. This strengthens the earlier hypothesis that NBR1's role in targeting the lipid droplet for autophagic degradation is responsible for the egress phenotype.

The next set of experiments were aimed at identifying the link between lipophagy and secretory autophagy. The level of IL-1β release in DENV infected cells compared to mock infected cells as well in cells supplemented with oleic acid compared to cells supplemented with BSA alone were evaluated (Fig. 20). A hypothesis stating that the increase of free fatty acids in the cell via either DENV induced mobilization of the lipid stores or exogenous addition triggered a sort of secretion state within the cell that DENV was then able to utilize to escape the cell. To measure the levels of secretory autophagy

an ELISA was performed to measure the levels of the proinflammatory cytokine IL-1β since it is only able to be secreted from the cell via secretory autophagy [121]. Both DENV infection and addition of oleic acid induced an increase in the amount of IL-1β in the supernatant. While it is possible that DENV infection is causing caspase-1 activation which will lead to IL-1β release, the fact that either DENV infection or oleic acid induces IL-1β release supports the hypothesis representing a potential link between lipophagy and secretory autophagy.

Another hypothesis that may help explain the link between lipophagy and secretory autophagy is that DENV induced lipophagy results in enhanced ATP production which is necessary for restructuring the host secretion network to allow robust egress of the infectious particles. There is plenty of evidence that (+) single strand RNA viruses such as DENV restructure the endoplasmic reticulum to facilitate efficient genome replication; lipophagy may be a crucial part of a similar process to aid in virion secretion [29,160]. Free fatty acids mobilized from the lipid droplet are trafficked to the mitochondria to undergo β-oxidation to produce ATP [86,87]. Etomoxir inhibits fatty acid oxidation via inhibition of CPT1a, so cells were infected with DENV and then treated with etomoxir to inhibit β-oxidation. Evaluating the amount of infectious particles in each of the infected supernatant fractions, demonstrated that both Peak 1 and Peak 2 had significantly reduced infectivity compared to the fractionated infected supernatants from DMSO treated cells. Peak 1 was more strongly affected by etomoxir treatment than the proposed secretory autophagy infectious peak, Peak 2 (Fig. 19). This suggests that the hypothesis that Peak 2 is reliant on oxidation of the DENV mobilized fatty acids is likely incorrect. It appears fatty acid

oxidation is necessary for complete viral release in both the canonical NBR1-independent pathway and the non-canonical NBR1-dependent pathway.

To better understand the nature of this NBR1-dependent egress, characterization of the two infectious peaks is important. To test the hypothesis, Peak 1 consists of free floating DENV virions and Peak 2 is made up of extracellular vesicles containing DENV virions, multiple assays aimed at determining if the infectious DENV particles that made up Peak 2 were contained in a protective membranous vesicle of some sort were performed. The ELISA and protease protection assays suggested that these DENV particles were not in enclosed in a membranous vesicle as the virion was capable of being bound by detection antibody and digested by Proteinase K without treatment with the membrane disrupting detergent Igepal (Fig. 22, 23).

To further characterize the role of NBR1 in DENV egress the susceptibility of DENV from either control transfected cells or cells transfected with siRNA targeting NBR1 to antibody neutralization was evaluated. The hypothesis was that if the Peak 2 DENV infectious peak is enclosed in extracellular vesicles, then DENV released from control transfected cells would be more resistant to neutralization than DENV from cells with silenced NBR1. The data supports the hypothesis suggesting that NBR1 dependent release of DENV is protected against antibody neutralization potentially due to being in a vesicle (Fig. 24).

To determine whether DENV is secreted from the cell within exosomes, infected cells were treated with 20 μM of the exosome biogenesis inhibitor, GW4869 and the amount of released infectious DENV was measured. There was no significant difference

in the amount of released infectious DENV between the control DMSO treated cells and cells treated with GW4869 (Fig. 25).

Potential Weaknesses and Unanswered Questions

Though Tristan demonstrates that both ubiquitin and NBR1 accumulate on the lipid droplet surface, future experiments should be done to confirm that the UBA domain of NBR1 is necessary for its association with the lipid droplet. It is possible that NBR1 may be able to bind to p62, which appears to associate with the lipid droplet in both uninfected and DENV infected cells, via its PB1 domain. This would mean that NBR1 is not the sole adaptor protein responsible for targeting the lipid droplet for degradation during DENV induced lipophagy. This is unlikely though as Tristan has unpublished data demonstrating that siRNA mediated inhibition of p62 does not significantly impact DENV induced lipid droplet mobilization indicating that p62 is neither necessary nor sufficient for DENV induced lipophagy. More work needs to be done to determine if NBR1 cooperates with another autophagy adaptor protein to target lipid droplets for autophagic degradation.

There is plenty of work to be done to further characterize NBR1's role in lipid droplet mobilization. NBR1 plays many important roles in the cell, some of which are not autophagy related [96]. Future investigations into NBR1-dependent lipid droplet mobilization need to be done to confirm whether NBR1 is acting as a specific autophagy receptor in this context. Assessing which domains are necessary for NBR1 to contribute to DENV induced lipophagy would be invaluable in answering this question.

There are still open questions about the process of fatty acids enhancing non-canonical viral egress, for example it has been shown that exogenous addition oleic acid

is capable of restoring Peak 2 in cells that have been silenced for NBR1, but it is unknown whether other types of fatty acids can support DENV egress in a similar manner.

Fatty acid oxidation represents a major source of energy for the cell so it is to be expected that its inhibition would have a broad impact on DENV infection. To investigate the impact of β-oxidation on the DENV life cycle a future study could be done evaluating the various steps of the viral life cycle in cells that have been treated with etomoxir. This would include evaluating the DENV translation via either western blot or microscopy, RNA replication via qPCR, and viral assembly and egress via quantification of both cell - associated and supernatant infectious particles. Such a study would provide valuable insight into determining the virally enhanced production of ATP impacts DENV infection. Etomoxir represents a broad inhibition of fatty acid oxidation which is a significant aspect of the cell's metabolism outside of the context of infection. It would be ideal if one could inhibit the oxidation of the fatty acids that were specifically mobilized by DENV and NBR1's involvement. Until such a method is developed, it seems unlikely that the link between the energy production due to DENV induced lipophagy and the autophagy assisted egress of the virus will be identified.

The results of the ELISA and the protease protection assay looking to characterize the infectious peaks may be explained by an instability of the extracellular vesicle (Fig. 22, 23). If the vesicle containing DENV were short-lived then it may have degraded shortly after being sedimented in the sucrose gradient, but before the respective assays. The fractions were stored in sucrose solution at 4°C for as brief a time as possible, but it is unknown whether they were stored in ideal conditions. Future work should be done to

optimize storage of these proposed vesicles to ensure they are able to be accurately characterized.

Next Steps

To establish whether NBR1's role as an autophagy adaptor protein is contributing to virally induced lipid droplet depletion or one of its non-autophagy related roles in secretion an investigator could utilize NBR1 mutants [96,161]. This would aid in determining if NBR1 is directly interacting with the LC3 to bridge autophagosomes to the lipid droplet. NBR1's ability to oligomerize via its CC domains or bind to other autophagy receptors via the PB1 domain should be investigated to determine their necessity in DENV induced lipophagy. It is also important to determine whether NBR1 is the specific autophagy receptor in general lipophagy or only in lipophagy induced by DENV infection and if there are any differences in the mechanisms of either. Investigations into NBR1's role in lipophagy in an uninfected cell necessitate the use of a specific lipophagy stimulant to answer. Stably knocking out NBR1 via CRISPR and then transducing in a NBR1 construct with a ΔLIR, ΔUBA or ΔPB1 mutation and evaluating its colocalization with the lipid droplet and lipid droplet depletion in DENV infected cells would provide valuable insight into how NBR1 is contributing to DENV's hijacking of lipid metabolism.

Silencing the lipid droplet protein(s) that are ubiquitinated and evaluating DENV egress is one method to confirm the hypothesis that NBR1's role as a specific autophagy receptor is responsible for the depletion of lipid droplets in DENV infected hepatocytes. The ubiquitinated lipid droplet protein would need to be identified before these experiments could be done. Another avenue of investigation would be to determine the signaling pathways necessary for this NBR1 dependent egress and how these pathways

differ from those responsible for mobilizing the lipid droplet. NBR1's role as the autophagy adaptor protein in lipid metabolism presents a novel and exciting avenue for many future studies in both DENV virology and cell metabolism.

It has been demonstrated that DENV infection enhances fatty acid biosynthesis [35]. The ability to specifically inhibit the autophagic degradation of lipid droplets represents a novel method to investigate the nature of DENV induced lipid droplet biosynthesis. Inhibiting NBR1 expression via either siRNA transfection or CRISPR transduction and then evaluating the lipid droplet flux in DENV infected cells would allow future researchers to begin to investigate the nature of DENV induced lipid biogenesis and perhaps lipid biogenesis in uninfected cells.

To evaluate whether types of fatty acids other than oleic acid can support DENV egress, one could utilize the sucrose gradient experiment to measure released DENV particles and supplement with diverse types of FFA. Another monounsaturated fatty acid such as palmitoleic acid or a saturated acid like stearic acid or arachidic acid could be used to test a diverse range of free fatty acids. The results of such an experiment could provide valuable insight on how determining the mechanism of how lipid metabolism contributes to DENV egress. Using NBR1 mutants or silencing other specific autophagy receptors such as p62 or Optineurin and evaluating the effect on DENV or other viruses' ability to utilize this non-canonical autophagy assisted pathway could help determine how viruses hijack autophagy receptors to leave the cell. There are many options to investigate the relationship of these autophagy adaptor proteins and secretory autophagy.

The data indicating that the addition of FFA or infection with DENV enhances IL-1β release points to another potential hypothesis. IL-1β secretion is commonly the result

of caspase-1 activation. It has also been shown that activation of the caspase-1 complex induces the export of the autophagic vacuole into the extracellular space in human endothelial cells [127]. DENV induced lipophagy may lead to activation caspase-1 which could cause the cell to secrete the virus. To assess this hypothesis, an investigator could utilize western blotting to observe whether caspase-1 is cleaved after DENV infection or after the addition of oleic acid. To confirm these results, one should also evaluate caspase-1 cleavage in DENV infected cells and cells that have been supplemented with additional free fatty acids that have been transfected with siRNA targeting NBR1. If the hypothesis is correct, then cell lysates from DENV infected cells or cells supplemented with oleic acid will have an increased amount of caspase-1 compared to either mock infected or BSA treated cells. Furthermore, silencing NBR1 will prevent caspase cleavage in cells that have been infected with DENV and supplemented with BSA alone. NBR1 silenced cells that have been treated with oleic acid whether they be DENV infected or mock infected will still demonstrate caspase-1 cleavage. This is because the exogenous addition of free fatty acids will bypass the necessity for DENV induced NBR1-dependent targeting of the lipid droplet for autophagic degradation. To help ensure that DENV induced lipophagy is specifically responsible, investigators should transduce cells with DENV NS4A and NS4B which, as previously discussed, are sufficient for inducing lipophagy and then evaluate caspase-1 cleavage. Other experiments that take a broader look at caspase-1 involvement in DENV induced secretory autophagy include evaluating viral assembly and egress in cells that have been silenced for caspase-1 or treated with the pan-caspase inhibitor ZVAD-FMK. This hypothesis is supported by data published by Wei et al who demonstrated that treating WS1 cells, a fibroblast cell line, with the

caspase-1 inhibitor, ac-YVAD-cmk inhibited both caspase-1 cleavage and IL-1β release as well as significantly reduce infectious DENV virion production [162]. They did not evaluate assembly of the viral particle compared to release of infectious particles, but these results support the hypothesis that DENV induced lipophagy causes caspase-1 activation which induces autophagy dependent secretion of IL-1β and DENV.

Determining whether there is a link between lipophagy, caspase-1 cleavage, the inflammasome and viral egress would provide insight into the mechanism of DENV induced secretory autophagy, identify a potential link between lipophagy and secretory autophagy, as well as provide the basis for several other studies looking to investigate the interplay between DENV, the inflammasome response, and autophagy. Another hypothesis is that the rapid increase in the intracellular free fatty acid level threatens to put the cell in a lipotoxic state and to aid in the elimination of the lipids, the cell shifts into a secretory state instead of a degradative one. To assess this hypothesis, an investigator should measure different secretory cargo instead of just one to reduce the likelihood of unintentionally only measuring the effect that DENV infection or exogenous addition of FFA has on an irrelevant pathway instead of secretory autophagy. Some examples of cargo that are released from the cell via secretory autophagy include the previously mentioned IL-1β, the proinflammatory cytokine IL-18, and the damage associated molecular pattern molecule High Mobility Group Box 1 (HMGB1) among other proteins that lack a N-terminal signal sequence [119,163]. To investigate this free fatty acid induced secretory state being proposed, an investigator could perform a series of experiments aimed at determining whether the DENV or FFA induced autophagy is more degradative or secretory. One method to accomplish this is to evaluate the ratio of LC3 II to LC3 I in

cells that have either been infected with DENV or been supplemented with oleic acid. LC3 II is the mature form of LC3 found on autophagosomes and autolysosomes while LC3 I is the precursor form. An increase in cytosolic LC3 II/I ratio indicates an increase in macroautophagy suggesting a more degradative state [164]. Another method to determine degradative autophagy compared to secretory autophagy is to transduce into cells a LC3 construct that is tagged with both GFP and RFP. This RFP-LC3-GFP probe will fluoresce a yellow signal when in autophagosomes as both the GFP and RFP give off a green and red signal respectively, but fusion with the lysosome will cause the pH sensitive GFP tag to degrade leaving only the red signal. These fluorescent signals can then be observed and measured via either confocal microscopy or flow cytometry. If the overall cell signal is yellow, then the cell is in a secretory autophagy favored state and if the cell signal shifts red, then the cell has entered a degradative autophagy favored state. To observe the effect that inhibiting autophagic degradation has on the amount of DENV in the supernatant and the sucrose fractionated infectious peaks, an investigator could treat cells with Bafilomycin A1. This drug is a vacuolar-type H^+-ATPase inhibitor which inhibits the formation of lysosomes. The expected result is that treatment with this drug will impair DENV release since the data suggests that lysosomal degradation of the lipid droplet is supporting DENV egress. Perhaps treating the cells at later time points will provide insight into how non-degradative autophagy enhances viral egress. If an earlier degradative lipophagy supports a later non-degradative autophagy mediated secretion of DENV, then treatment with bafilomycin A1 at a later time point would enhance DENV secretion. This would indicate a novel relationship between lipophagy and viral secretion.

A future experiment could evaluate the susceptibility of Peak 1 and Peak 2 to antibody neutralization which would better confirm whether NBR1 is necessary for the secretion of DENV in vesicles that prevent antibody neutralization. If Peak 2 represents DENV in vesicles, the prediction is that it is resistant to neutralization while Peak 1 is sensitive It is also possible that DENV induced lipophagy causes a population of DENV particles that are lipidated similar to what has been seen with HCV [158]. To determine whether DENV particles are lipidated or one of the three described types of extracellular vesicles (exosomes, microvesicles or apoptotic bodies), an investigator could utilize electron microscopy to visualize the infectious particles in the two infectious peaks. More research needs to be done to identify the link between virally induced lipophagy and the secretion of infectious particles as well as better characterizing the virions that make up Peak 2.

Significance

NBR1 has been described as playing an antiviral role in plant immunity against turnip mosaic virus and cauliflower mosaic virus, but this is the first study to demonstrate a potential proviral role in mammalian cells and a role in lipophagic degradation [107,108]. In addition to its roles in regulating lipid metabolism, this study has identified that NBR1 and lipophagy are critical in an uncharacterized viral secretion method Identifying the mechanism of how DENV induced lipophagy supports release of the virus would be of great benefit to the field of virology and potentially provide insights into currently uncharacterized roles of metabolism and secretory autophagy.

Metabolism and energy production is a critical cellular process that operates at the core of many of the cell functions. It also represents a significant target for viral hijacking

to efficiently complete the viral life cycle. The DNA virus adenovirus uses viral E4ORF1 to enhance host glycolysis to provide energy for increased nucleotide synthesis [165,166]. The flavivirus ZIKV increases glucose utilization during the Krebs cycle [167]. Lipid metabolism especially represents a major cellular metabolic pathway and viral target. Vaccinia virus and dengue virus cause an upregulation of fatty acid biosynthesis [35,168]. This study provides valuable understanding into how viruses can utilize host metabolic pathways to support their life cycle and open future avenues of investigation into both these viruses and the cellular metabolic pathways.

These findings also demonstrate a heretofore undescribed link between a major metabolic process in lipophagy and a significant yet uncharacterized pathway being hijacked to be utilized as a viral egress pathway in secretory autophagy. Many viruses hijack autophagy to enhance infection via a number of different mechanisms including facilitating egress from the cell and this process of autophagy-associated secretion is still largely undescribed, it is crucial that more work be done to study these non-canonical viral egress pathways [78,81,169,170]. This study provides further insight into DENV induced lipophagy and secretory autophagy and how it supports DENV infection. These and any future investigations into secretory autophagy of DENV provide a potential valuable target for the much needed development of therapeutics against a significant emerging global pathogen.

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Figures

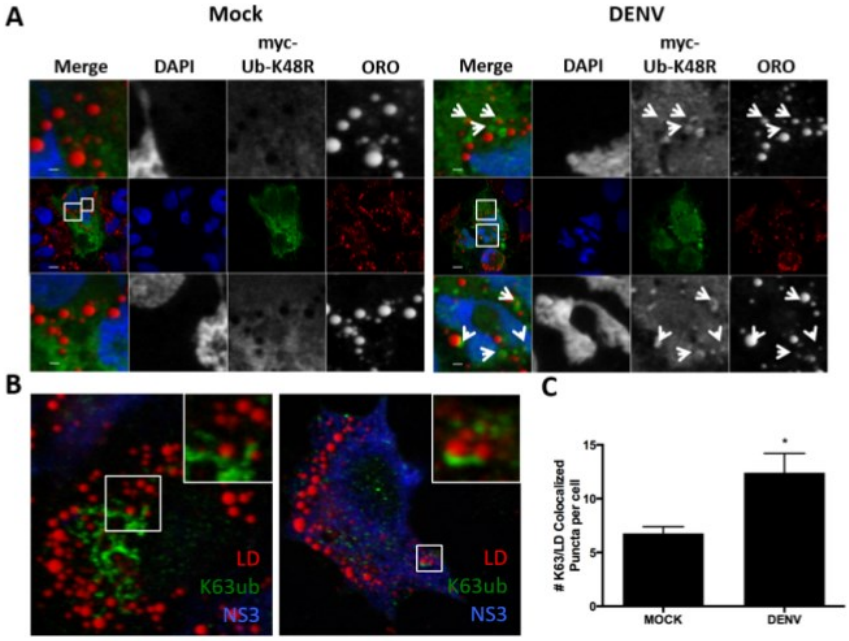

Figure 1. K63 linked ubiquitin accumulates at the lipid droplet during DENV infection. (A) HepG2 cells were mock- or DENV-infected at an MOI=5 for 6 hours, then transfected with a myc-tagged K48R ubiquitin. Cells were fixed in 4% PFA 30hpi and stained for myc-K48R ubiquitin and lipid droplets with oil red O (ORO). (B) HepG2 cells were mock- or DENV-infected at an MOI of 0.5 for 24 hours, fixed in 4% PFA and stained for endogenous K63 ubiquitin, DENV NS3, and ORO. (C) Quantitation of colocalized K63 ubiquitin and ORO puncta per cell from (B).

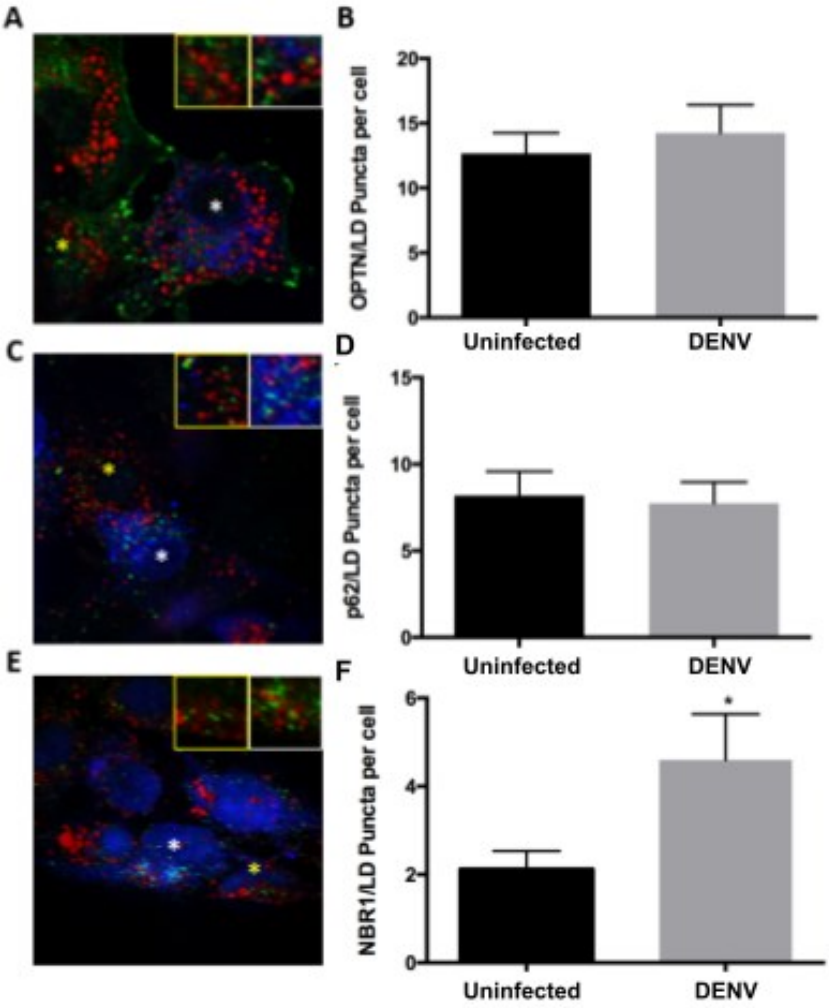

Figure 2. NBR1, but not p62 or OPTN, is recruited to lipid droplets during DENV infection. HepG2 cells were infected MOI=0.5 for 24h, fixed in 4%PFA, and probed for (A) p62 and DENV NS3, (b) OPTN and NS3, or (C) NBR1 and DENV E and ORO. Colocalization of (B) p62, (D) OPTN, or (F) NBR1 with ORO was quantified. Yellow asterisk and box = uninfected cells, white asterisk and box = DENV-infected cells

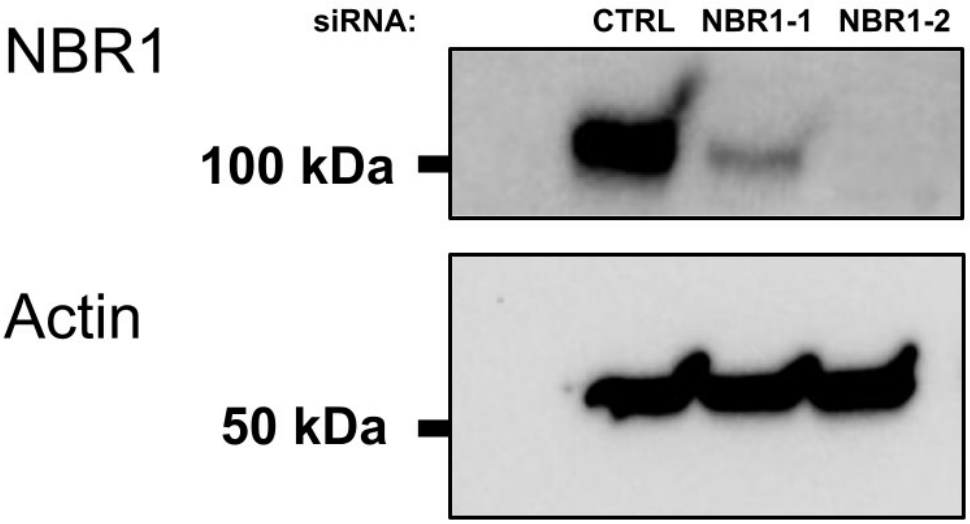

Figure 3. siRNA mediated knockdown of NBR1. Western blot of Huh7.5 cells transfected with non-targeting control siRNA or one of two different siRNAs targeting NBR1. The blot was then stained with either anti-NBR1 antibody (Top Row) or anti-Actin antibody (Bottom Row).

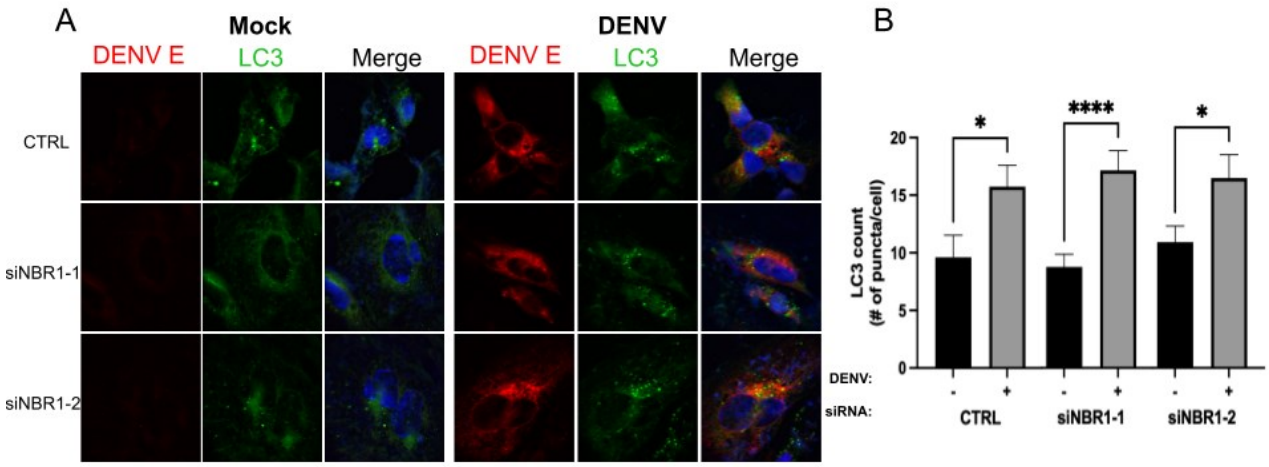

Figure 4. Silencing NBR1 does not inhibit dengue virus's inducement of autophagy. (A) Huh 7.5 cells were transfected with either non-targeting control siRNA or one of two different siRNAs targeting NBR1 and then infected with DENV (MOI 5) for 24 hours. The cells were then fixed and probed with antibodies raised against DENV E protein (red) and LC3 (green) and treated with DAPI (blue) before being imaged on a confocal microscope. (B) Quantification of the number of LC3 puncta per cells.

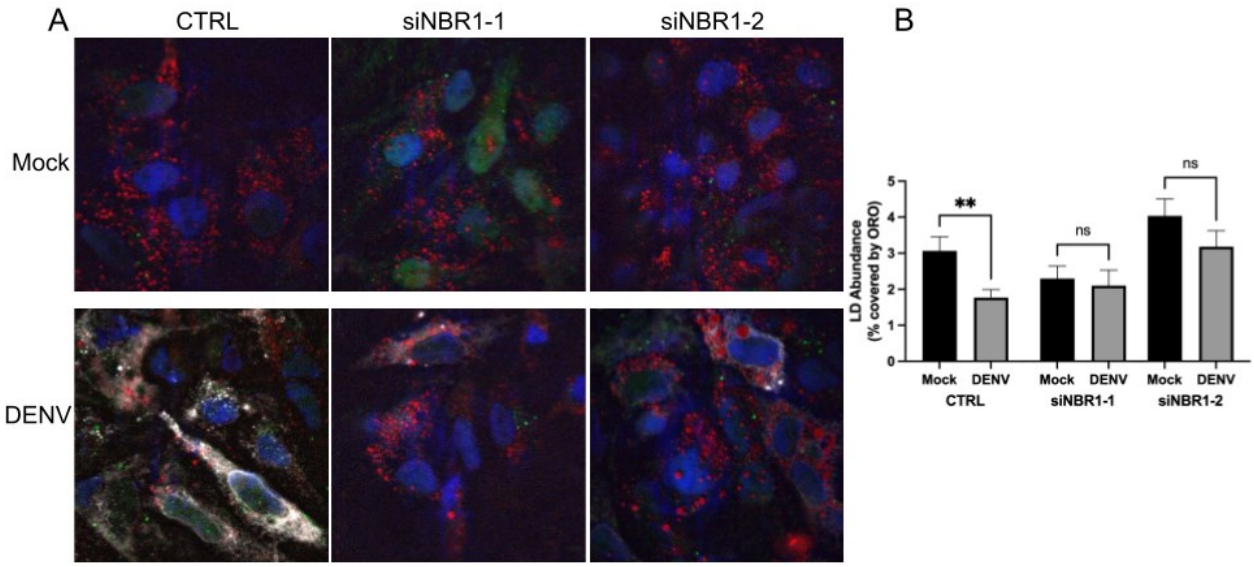

Figure 5. NBR1 is necessary for Lipid droplet depletion during DENV infection: (A) Huh7.5 cells were transfected with non-targeting control siRNA (Left Column) or one of two different siRNAs targeting NBR1 (Center and Right Columns) and then either mock (Top) or DENV (Bottom) infected for 48 hrs and then fixed with 4% PFA and stained with anti-DENV E antibody (Gray), Oil Red O (Red), and DAPI (Blue). (B) Quantification of the percentage of the area of each cell covered by lipid droplets from (A).

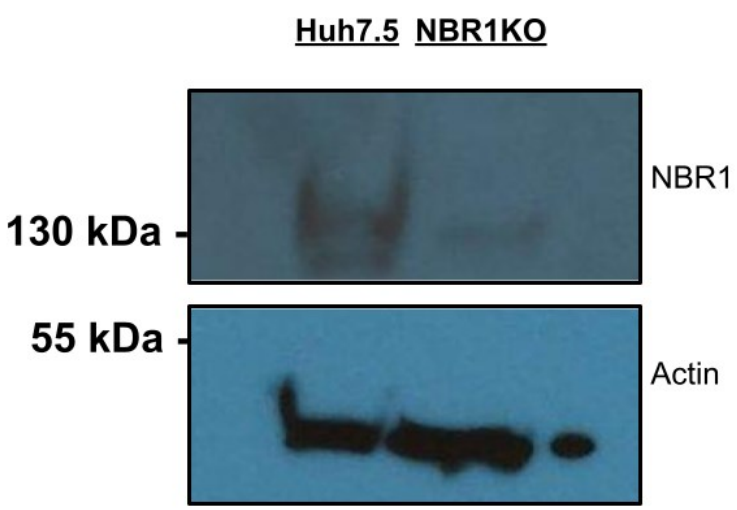

Figure 6: Western blot of CRISPR mediated Knockout of NBR1. Western blot of control Huh7.5 cells and NBR1KO cells. The blot was then stained with either anti-NBR1 antibody (Top Row) or anti-Actin antibody (Bottom Row).

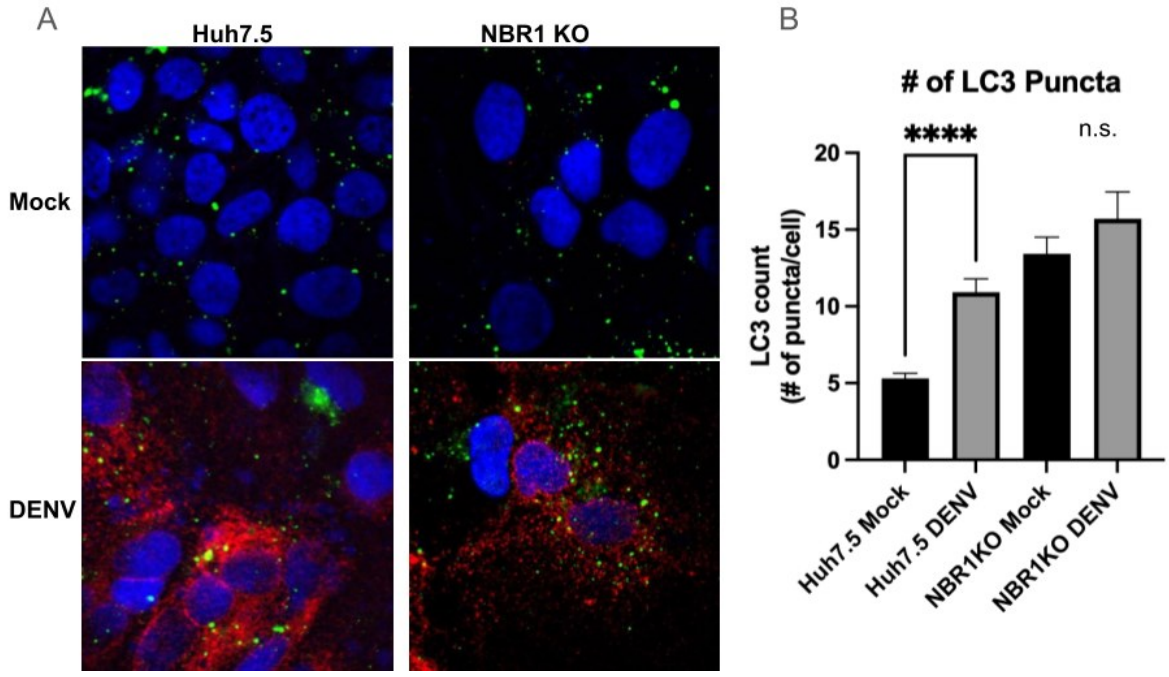

Figure 7: Impact of CRISPR mediated knockout of NBR1 on LC3 puncta formation: (A) Control Huh7.5 cells (Left column) and NBR1KO (Right column) were either mock (Top) or DENV (Bottom) infected for 24 hrs and then fixed with 4% PFA and stained with anti-LC3 antibody (Green), Anti-DENV E antibody (Red), and DAPI (Blue). (B) Quantification of the number of LC3 puncta per cell in (A).

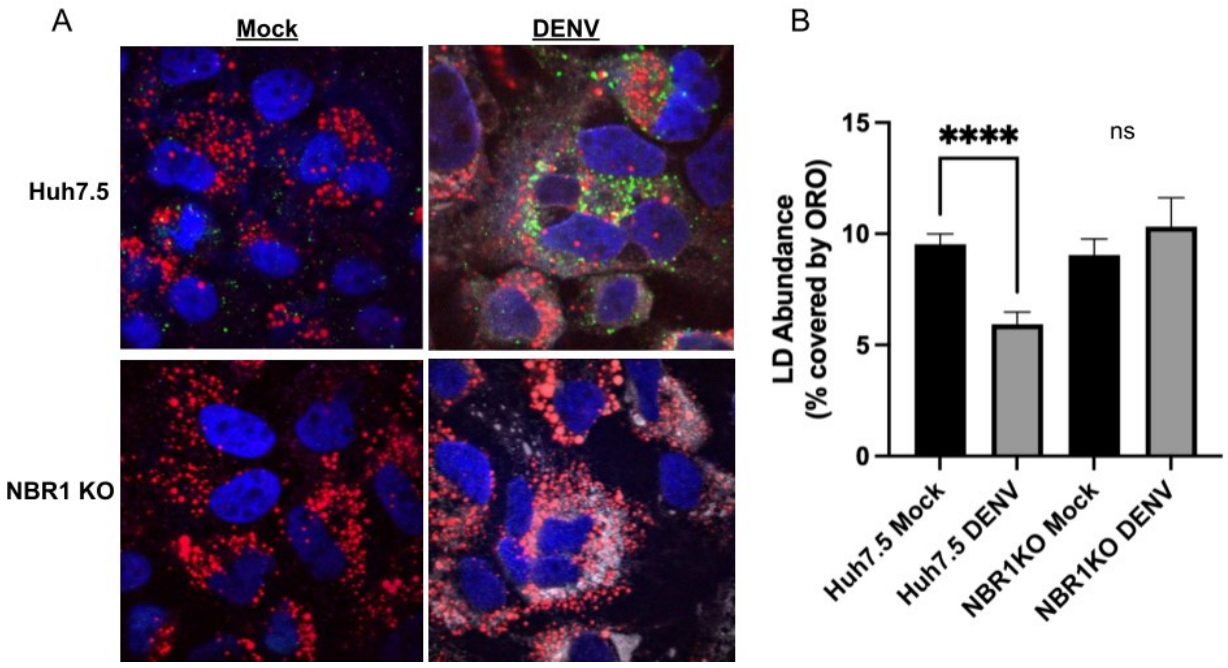

Figure 8: Impact of CRISPR mediated knockout of NBR1 on LD Abundance: (A) Control Huh7.5 cells (Left column) and NBR1KO (Right column) were either mock (Top) or DENV (Bottom) infected for 48 hrs and then fixed with 4% PFA and stained with anti-DENV NS4A antibody (Gray), Oil Red O (Red), anti-NBR1 antibody (Green), and DAPI (Blue). (B) Quantification of the percentage of the area of each cell covered by lipid droplets from (A).

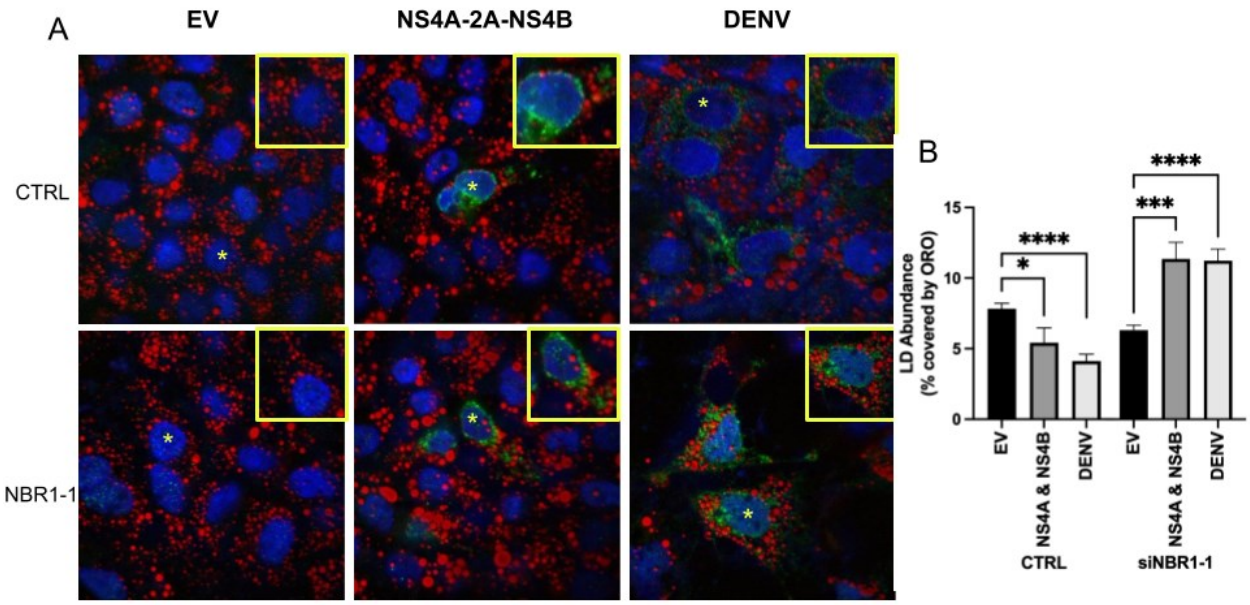

Figure 9: Silencing NBR1 prevents DENV NS4A and NS4B induced autophagic flux of lipid droplets (A) Huh7.5 cells with a Tet-on 3G gene were either transfected with non-targeting control siRNA (Top Row) or siRNA targeting NBR1 (Bottom Row) and then transduced with pseudovirus containing either an empty vector (Left), a vector containing DENV NS4A and NS4B (Center) or were left non-transduced (Right) . Then they were either treated with 5 ug/mL of Doxycycline (Left and Center) or infected with DENV (Right) for 48 hours. Cells were probed with anti-DENV NS4A antibodies, Oil Red O, and DAPI and imaged via fluorescence microscopy. Inset contains enlarged images of cells noted by asterisks. (B) Quantification of the percentage of the area of each cell covered by lipid droplets from (A).

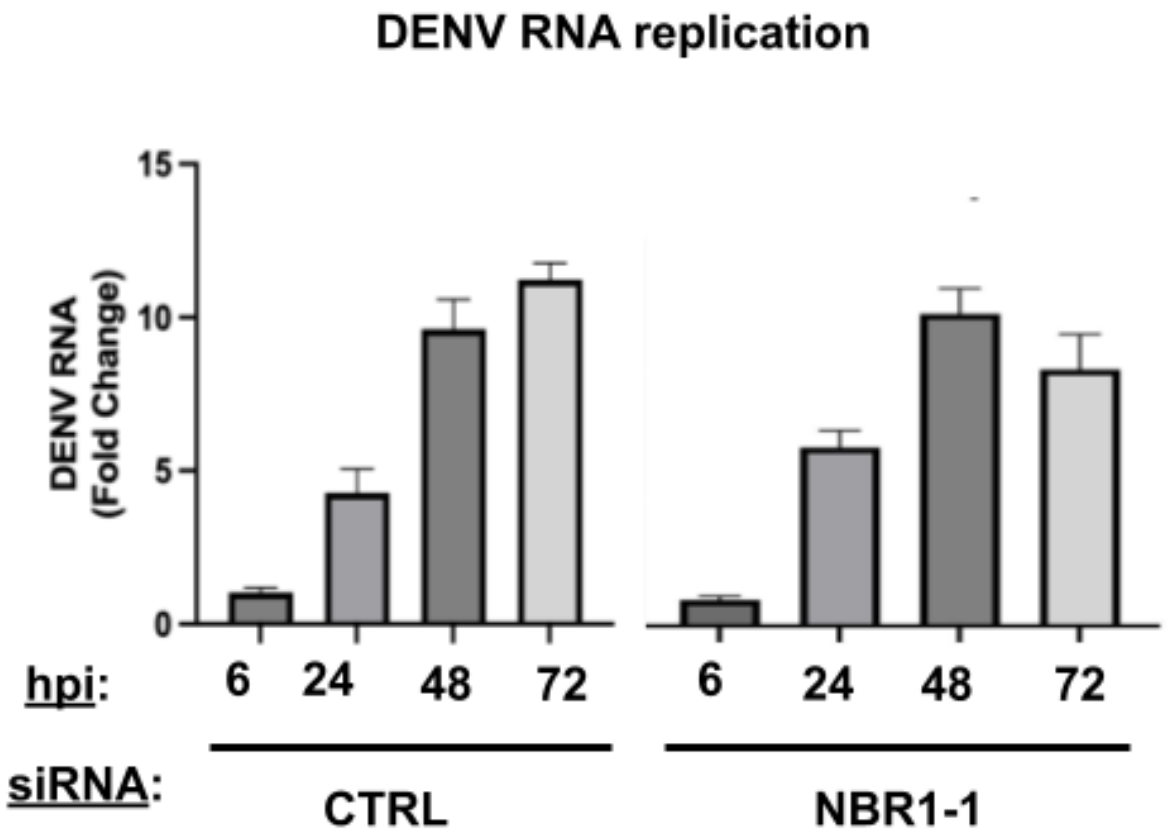

Figure 10: Silencing NBR1 has minimal effect on DENV RNA replication. Huh7.5 cells were transfected with either non-targeting control siRNA or siRNA targeting NBR1 before being infected with DENV (MOI = 5) for either 6, 24, 48, or 72 hours. The cells were then lysed, and the RNA was isolated and measured via RT-qPCR

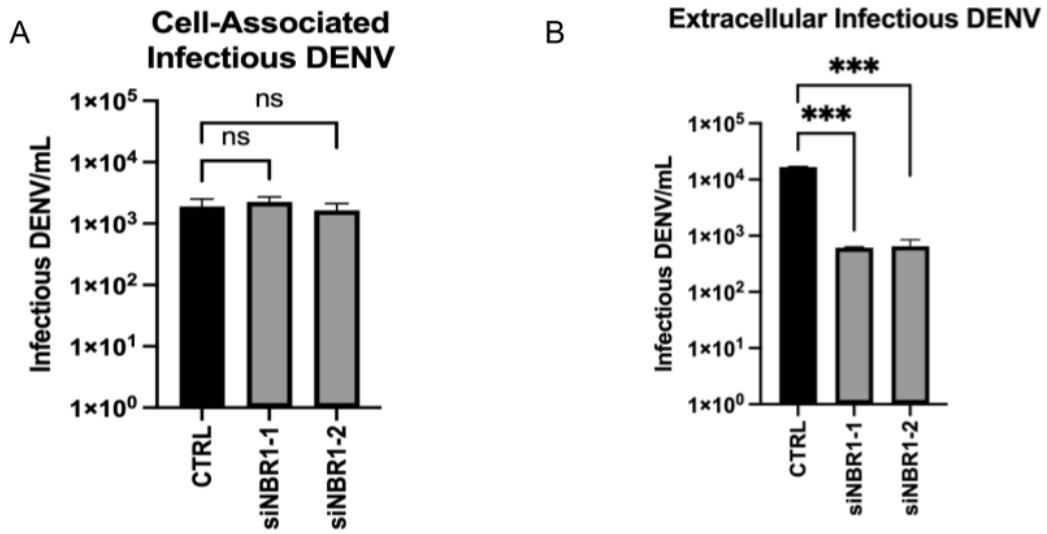

Figure 11: NBR1 is required for DENV egress, but not assembly. Cells transfected with either non-targeting control siRNA or one of two different siRNAs targeting NBR1 were infected with DENV (MOI = 5) for 4 hours. (A) The DENV containing media was then replaced with fresh media and left to incubate for 48 hours at 37°C. The supernatant was collected and the amount of extracellular infectious DENV was then quantified via TCID50. (B) The supernatant was replaced before freeze/thawing the cells to lyse them. The amount of cell-associated infectious DENV was then quantified supernatants via TCID50.

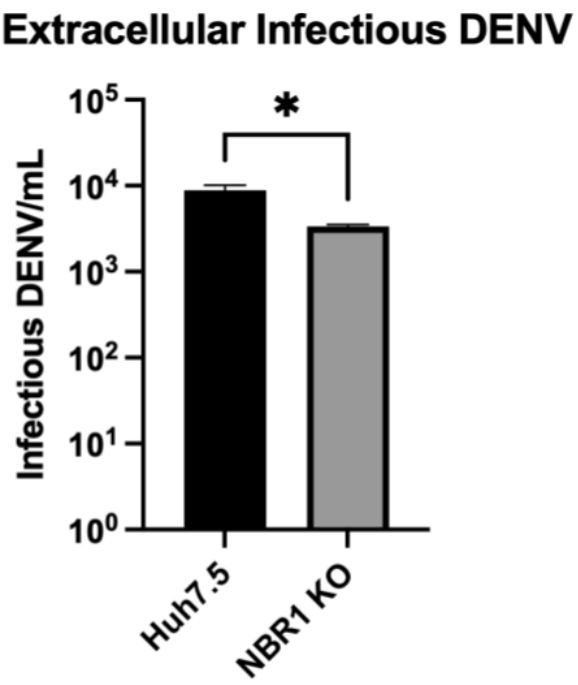

Figure 12: CRISPR mediated knockout of NBR1 reduces DENV release. Cells transduced with a lentiviral pseudovirus containing NBR1 targeting guide RNA and Cas9 were infected with DENV (MOI = 5) for 4 hours. The DENV containing media was then replaced with fresh media and left to incubate for 48 hours at 37°C. The supernatant was collected and the amount of extracellular infectious DENV was then quantified via TCID50.

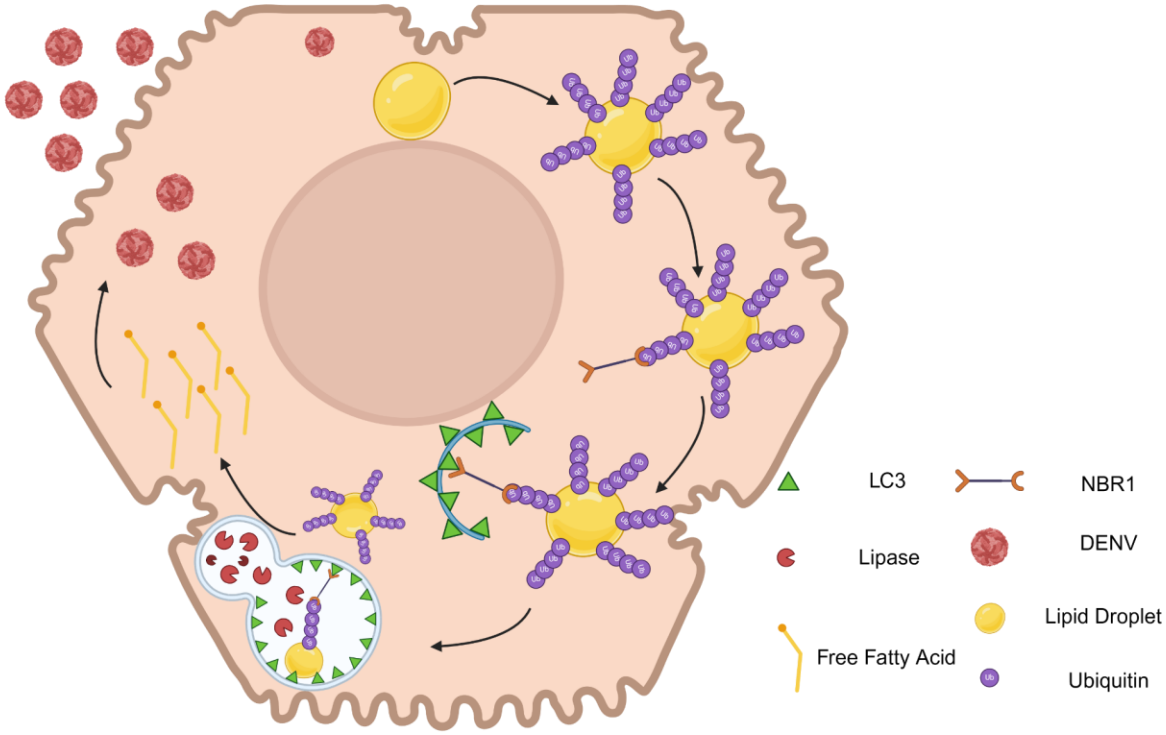

Figure 13: Model of DENV induced lipophagy. DENV induces the ligation of K63 poly-ubiquitin chains to the cytosolic surface of the lipid droplet. NBR1 is then recruited to the lipid droplet and binds to the nascent phagophore. Autophagic degradation of lipid droplets mobilizes the fatty acids and causes an increase in DENV egress.

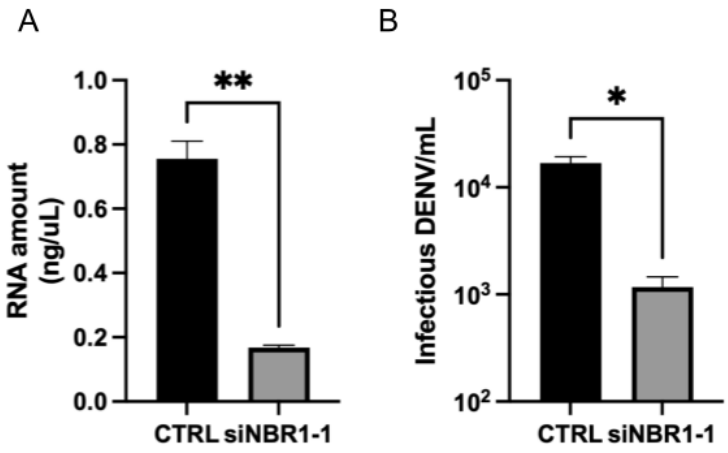

Figure 14: Silencing NBR1 severely reduces the amount of Extracellular DENV RNA and Infectious particles. Huh7.5 cells were infected at a MOI 5 for 4 hours before refreshing the media (A) Viral RNA was isolated via spin columns and the amount of DENV RNA in the supernatant was measured via RT-qPCR. (B) The amount of infectious DENV was measured via TCID50

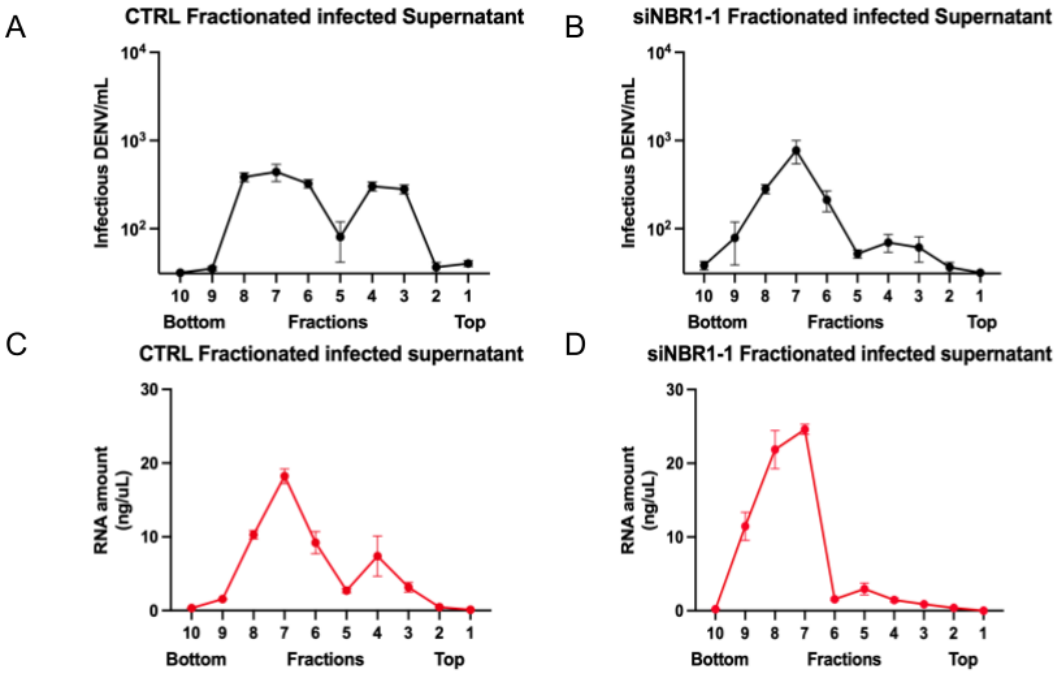

Figure 15: NBR1 is necessary for release of low-density infectious DENV. Huh7.5 cells were transfected with either non-targeting control siRNA or siRNA targeting NBR1 and then infected with DENV (MOI = 5) for 4 hours. The supernatant was then replaced with uninfected media and left to incubate at 37°C for 48 hours. The supernatant was then run on a discontinuous sucrose gradient. Ten fractions were collected and the amount of infectious DENV particles (A & B) or the amount of DENV RNA (C & D) were quantified.

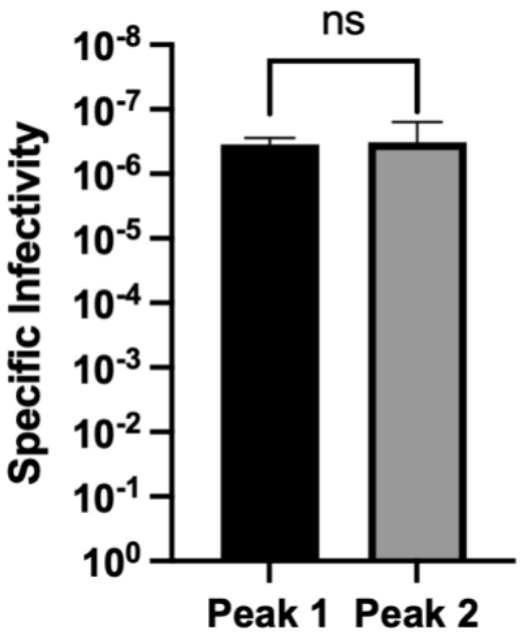

Figure 16: Low density and high density DENV peaks have similar specific infectivity. The specific infectivity of the two peaks were compared by calculating the ratio of infectious DENV as calculated by TCID50 and the DENV copy number as calculated by the amount of DENV RNA.

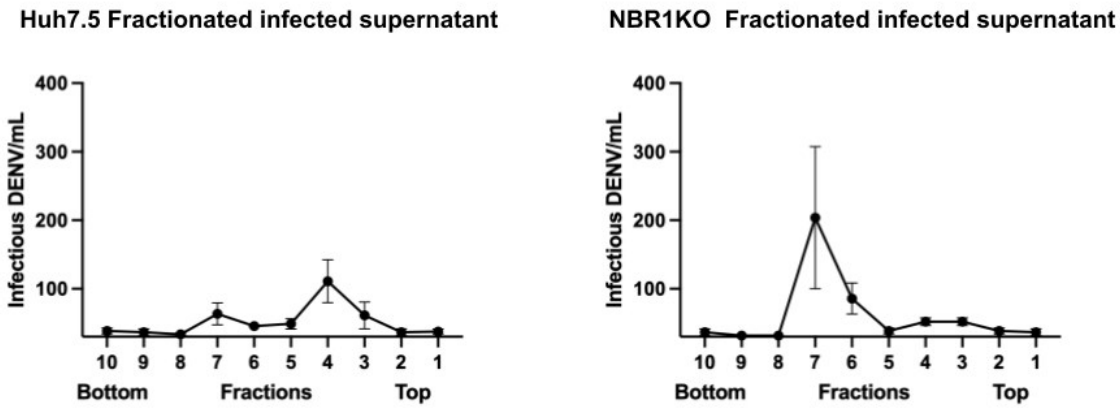

Figure 17: Impact of CRISPR Mediated knockout of NBR1 on secretion of low density DENV. Huh7.5 cells or NBR1KO cells were infected with DENV (MOI = 5) for 4 hours. The supernatant was then replaced with uninfected media and left to incubate at 37°C for 48 hours. The supernatant was then run on a discontinuous sucrose gradient. Ten fractions were collected and the amount of infectious DENV particles.

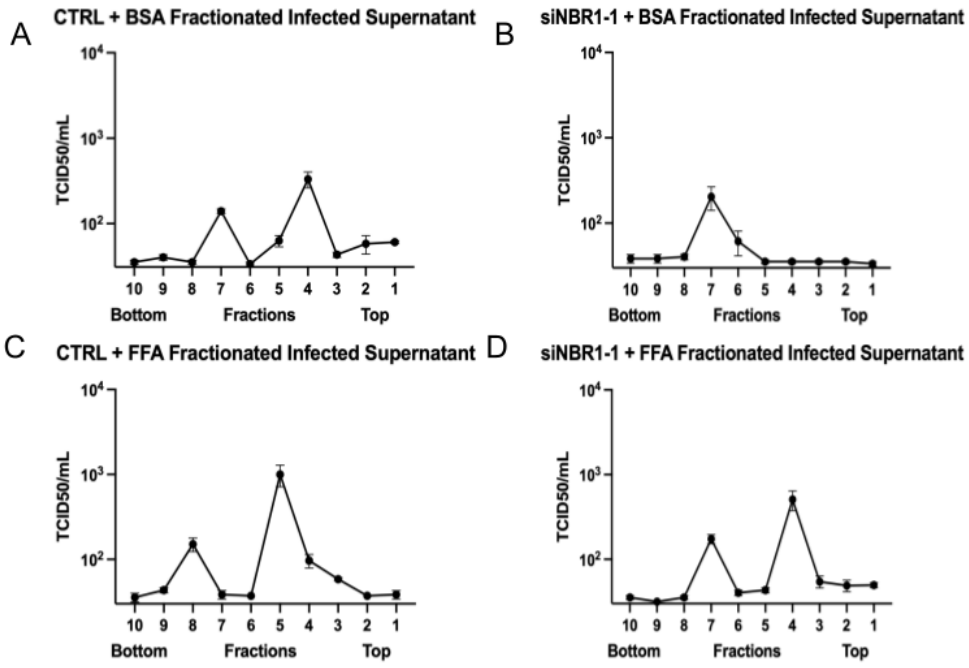

Figure 18: The exogenous addition of FFA complements secretion of low-density of infections DENV from NBR1 silenced cells. Huh7.5 cells were transfected with either non-targeting control siRNA or siRNA targeting NBR1 and then infected with DENV (MOI = 5) for 6 hours. The supernatant was then replaced with uninfected media containing either 1:1000 free fatty acids (FFA) conjugated to BSA (C & D) or BSA alone (A & B) and left to incubate at 37°C for 48 hours. The supernatant was then run on a discontinuous sucrose gradient and ten 1 mL fractions were collected. The amount of infectious DENV virions in each fraction were then quantified via TCID50

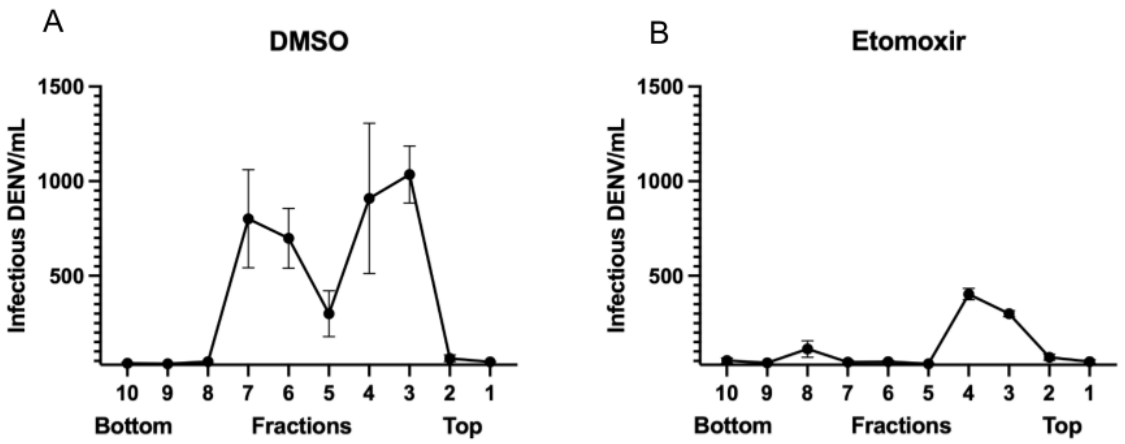

Figure 19: Impact of Etomoxir on DENV secretion. Huh7.5 cells were infected with DENV at a MOI 5 for 4 hours before replacing the supernatant with media containing either (A) DMSO or (B) 20 μM of Etomoxir and then each fraction was collected. The amount of infectious DENV in each fraction was measured via TCID50.

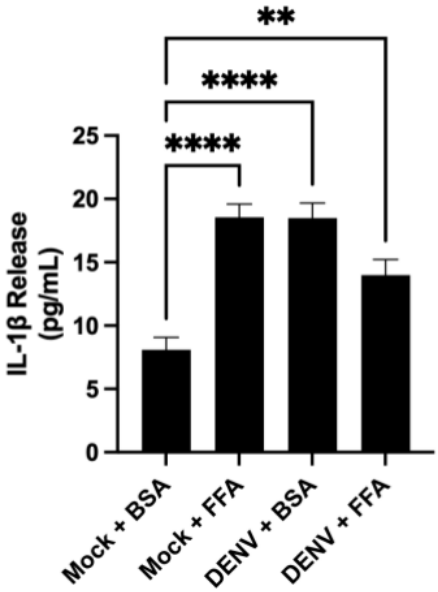

Fig 20: DENV infection and exogenous FFA addition enhances IL-1β. Huh7.5 cells were either mock infected or infected with DENV at an MOI of 5 for 4 hours and then refreshed with media supplemented with either BSA or Oleic Acid (FFA). The amount of IL-1β was then measured via Sandwich ELISA.

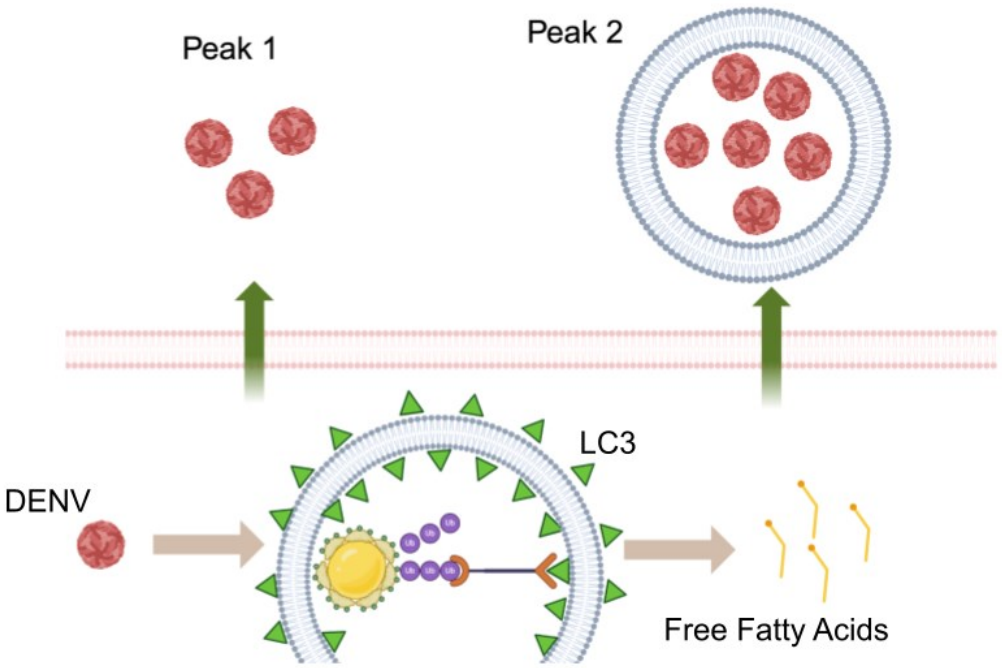

Figure 21: Model of Autophagy-assisted secretion of DENV. DENV induced lipophagy causes a release of free fatty acids that results in two different infectious peaks.

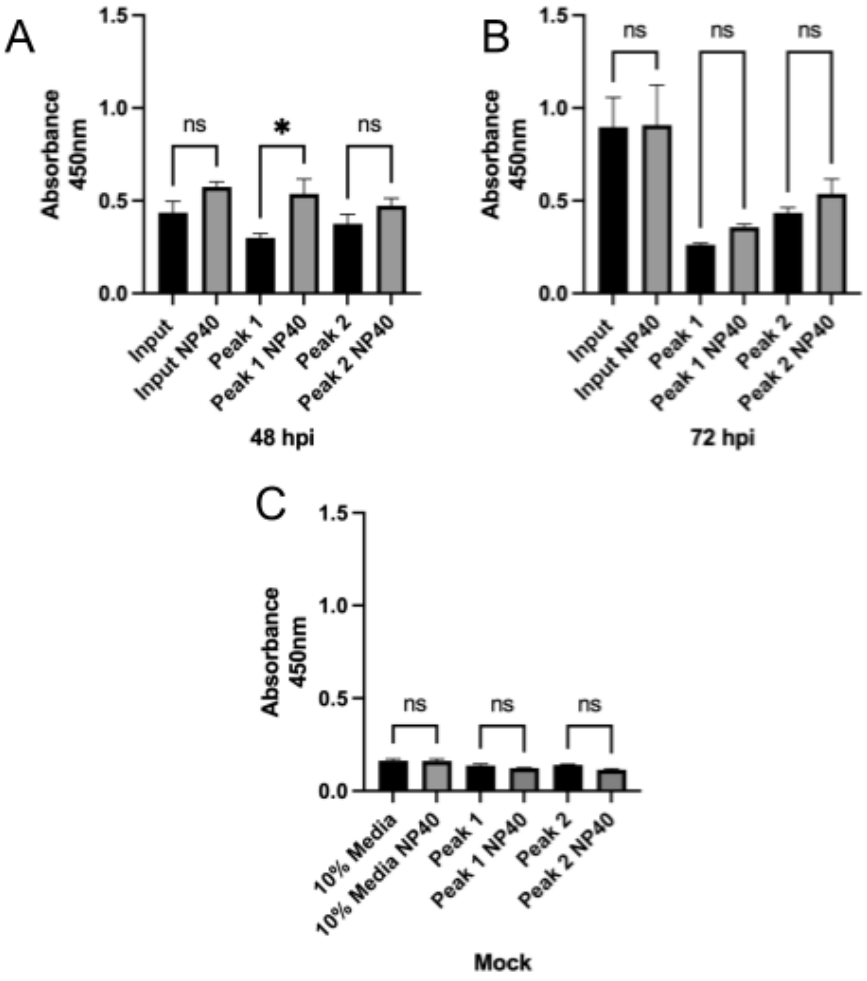

Figure 22. Detection of secretory autophagy DENV fractions via indirect ELISA. Huh7.5 cells were infected with DENV at an MOI of 5 for 4 hours and then refreshed with uninfected media. An indirect ELISA measuring the antibody mediated detection of DENV with and without 1% Igepal treatment (NP-40) was performed on fractionated 48hpi supernatant (A), 72hpi supernatant (B), or mock fractions and 10% media (C).

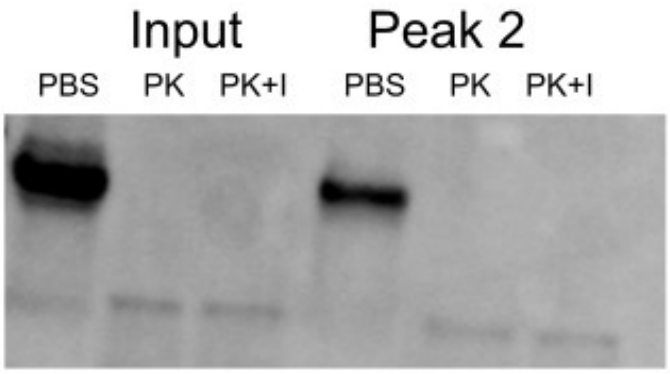

Figure 23. Protease Protection Assay of secretory autophagy DENV fractions. Whole infected supernatant and the fraction of fractionated infected supernatant that correlates to Peak 2 were treated with PBS, 0.2 mg/mL Proteinase K (PK), or 0.2 mg/mL Proteinase K with 1% Igepal (PK + I) and DENV E was probed via Western Blot.

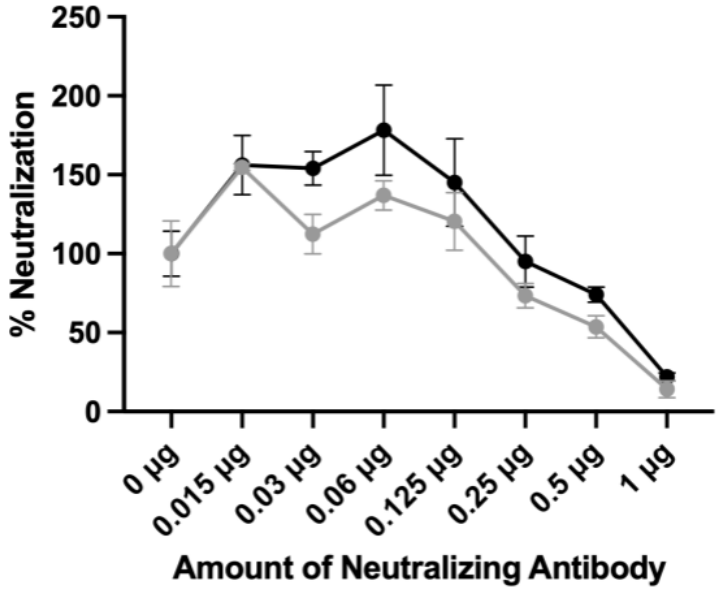

Figure 24. NBR1 contributes to DENV resistance to antibody neutralization. Equivalent amounts of DENV from either control transfected cells (black) or cells transfected with NBR1 targeting siRNA (gray) were neutralized for an hour with different amounts of antibody before infecting cells in a 96 well plate. The percent neutralization per antibody amount was then calculated and graphed.

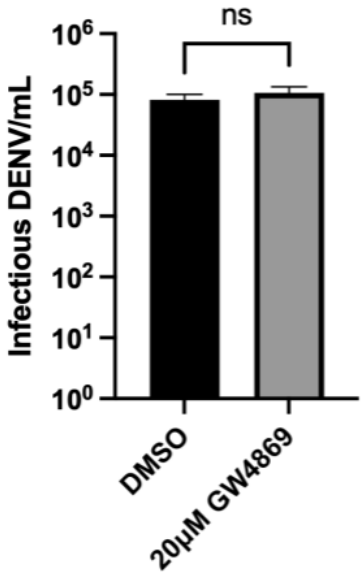

Figure 25. GW4869 does not significantly impact the amount of DENV release. Huh7.5 cells were infected with DENV at an MOI of 5 for 4 hours and then refreshed with uninfected media supplemented with either DMSO or 20 μM GW4869. The amount extracellular DENV was then measured via TCID50