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# The Effects of Autophagy on the Replication of Zika Virus

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# The Effects of Autophagy on the Replication of Zika Virus

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| | |
|---|---|
| ATG12 | Autophagy-Related Gene 12 |
| DMEM | Dulbecco's Modified Eagle Medium (Culture Medium) |
| FBS | Fetal Bovine Serum |
| ZIKV | Zika Virus |
| RPMI | Roswell Park Memorial Institute (Culture Medium) |
| RdRp | RNA-Dependent RNA Protein |
| RT-PCR | Reverse Transcription - Polymerase Chain Reaction |
| *IFNB1* | Interferon-Beta 1 Gene |
| qPCR | Quantitative Polymerase Chain Reaction |
| GBS | Guillain-Barre Syndrome |
| E | Envelope Protein |
| ED | Envelope Domain |
| NS | Non-Structural Proteins |

| | |
|---|---|
| eIF4E | Eukaryotic translation initiation factor 4E |
| LRP1 | Lipoprotein Receptor Port 1 |
| APL | Autophagolysosome |
| LAMP2A | Lysosomal Membrane Protein 2a |

# ABSTRACT

Zika virus is an enveloped virus with a single-stranded positive-sense RNA genome. The Zika virus is a member of the Flaviviridae family of viruses that was discovered to infect humans in 1952. The Flavivirus envelope is decorated by many copies of two membrane proteins that facilitate viral entry. The virus is primarily transmitted through mosquito bites. Another method by which Zika virus could be transmitted is through sexual contact with Zika infected partners or from an infected pregnant woman to fetus known as congenital transmission. Infected patients suffer from self-limiting febrile illness including headaches, skin rash myalgia, and conjunctivitis. Zika virus has a significant impact on pregnancies, causing developmental defects in fetal brain that leads to infants born with smaller head size, a disorder known as microcephaly. Zika virus has also been linked to the increase of the development of neurological disorder known as Guillain-Barre syndrome. Zika virus has been involved in multiple outbreaks around the world, beginning in 2007 where the Zika virus infected about 5,000 of the people of Yap Island in Micronesia. The following outbreak began between 2013-2014, affecting about 30,000 people in French Polynesia. In 2015-2016, Zika virus outbreak was reported to affect the Americas, where over 30,000 cases and about 4300 microcephaly cases. Upon entry into host cells, Zika virus begins to unpack and release the RNA genome to express the viral proteins as well as to replicate the RNA genome. Studies have revealed that autophagy, which is a cellular mechanism that protects host cells during starvation and pathogenic infections, is associated with Zika replication as it may facilitate the replication of the Zika virus. Here, we investigated the effects of autophagy in the replication of Zika virus using an autophagy inducer, rapamycin. For this study, we used a human trophoblast cell line which is physiologically relevant to congenital transmission of Zika virus. To better understand how autophagy is involved in Zika replication in

trophoblast cells, a knockout was generated on one of the autophagy gene known as ATG12. The ATG12 autophagy protein forms a complex with another autophagy protein known as ATG5. PCR analysis revealed that the relative Zika viral load was increased in cells treated with rapamycin when compared to non-rapamycin treated cells. Consistent with the rapamycin treatment results, Zika replication was lower in ATG12-/- than wild type cells. Interestingly, the effect of rapamycin on Zika replication was abolished in ATG12-/- cells. These findings suggest that autophagy facilitates Zika replication. Understanding the effects that autophagy imposes on the replication of the Zika virus could provide therapeutic opportunities through designing drugs that control the autophagy by either entirely blocking the autophagy mechanism or partially in patients who have been infected with Zika virus. Another avenue that would benefit from controlling autophagy is by strategizing delivery of autophagy blockers to pregnant patients to control the severity of developmental disorders in fetuses such as microcephaly.

# INTRODUCTION

## 1. Background

Zika virus (ZIKV) is a member of the Flaviviridae family of viruses that was discovered in 1947 (Agrelli, de Moura, Crovella, & Brandão, 2019; Weaver et al., 2016). The ZIKV was known to be a threat to public health globally due to the virus being vector-borne transmitted through mosquito bites (X. Zhang et al., 2017). Additionally, ZIKV transmission can also occur through sexual contact with partners infected with ZIKV (D'Ortenzio et al., 2016). Infected patients suffer from self-limiting febrile illness including headaches, skin rash myalgia, and conjunctivitis (Liu, Shi, & Qin, 2019). ZIKV has a significant impact on pregnancies, causing developmental defects in the head that leads to infants born with a smaller brain, a disorder known as microcephaly (Vianna et al., 2018; Yu et al., 2017). ZIKV has also been linked to a neurological disorder in adults known as Guillain-Barre syndrome (GBS), a neuromuscular disorder that results from the immunopathogenesis against the peripheral nervous system (Agrelli et al., 2019; Yuki & Hartung, 2012). ZIKV has been involved in multiple outbreaks around the world, beginning in 2007 where the virus infected about 5,000 of the people of Yap Island in Micronesia. The following outbreak began between 2013-2014, affecting about 30,000 people in French Polynesia (Agrelli et al., 2019). In 2015-2016, ZIKV outbreak was reported in the Americas, where up to 33 countries and territories were affected with around 30,000 ZIKV infection cases and over 4300 reported microcephaly cases. (Faria et al., 2016; Petersen, Jamieson, Powers, & Honein, 2016).

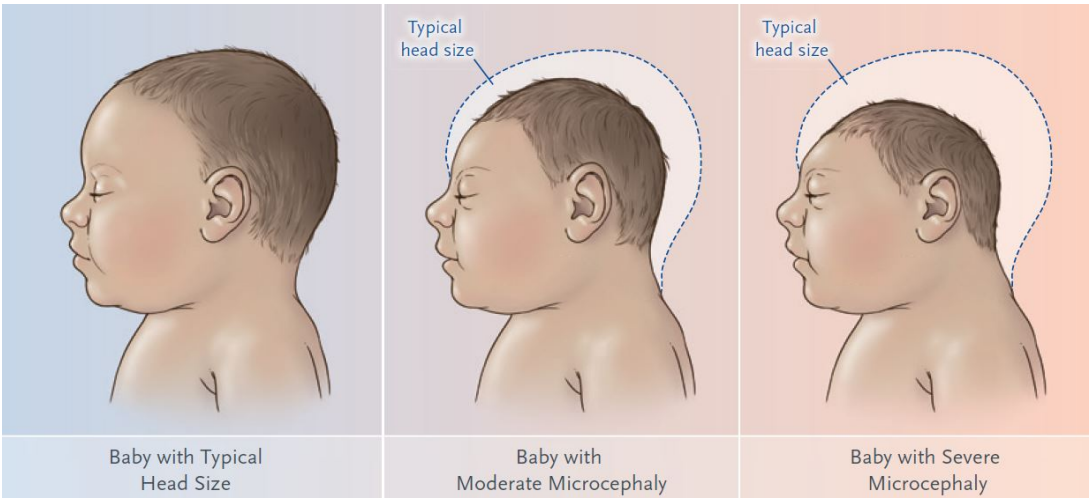


**Figure 1: Comparison between typical head size and moderate microcephaly and severe microcephaly**. Originally published in (Petersen et al., 2016)

## 2. Transmission

### 2.1 Transmission by mosquitoes

ZIKV is an arthropod-borne virus that relies on a mosquito vector to spread (D Musso et al., 2014). The transmission of ZIKV is carried mainly by *Aedes aegypti* mosquitos (Ioos et al., 2014). Mosquito transmission of ZIKV virus begins when a mosquito bites an individual infected with ZIKV for a blood meal (Abushouk, Negida, & Ahmed, 2016; Didier Musso & Gubler, 2016). The mosquito contracts the ZIKV from the infected individual and becomes a carrier of the ZIKV. The ZIKV-infected mosquito bites another individual that is not infected with ZIKV for a blood meal. The ZIKV-infected mosquito will transmit the ZIKV to the individual, infecting the individual with ZIKV. The transmission cycle continues when another mosquito that does not carry the ZIKV bites the ZIKV-infected individual (Abushouk et al., 2016).

## 2.2 Sexual Transmission

The transmission of the ZIKV is not only limited to mosquitos; other modes of transmission can also deliver the virus to healthy individuals. One of the modes of transmission of ZIKV is through sexual contact (Abushouk et al., 2016; Quicke et al., 2016). Sexual contact with partners infected with ZIKV has been proved to pass ZIKV to a naive individual. The ability of ZIKV to be transmitted sexually is due to the presence of the viral particles in the vaginal and seminal fluids (Abushouk et al., 2016; Didier Musso & Gubler, 2016).

## 2.3 Vertical Transmission

Another mode of transmission is through the passing of the ZIKV from infected mothers to their babies. The type of transmission that relies on the mother-to-baby transmission is known as vertical transmission or maternal-fetal transmission (Abushouk et al., 2016; Liu et al., 2019). The reason for the ZIKV to be transmitted to the fetus of the ZIKV -positive mother is that the virus can penetrate the placental barrier and replicate in the fetus (Quicke et al., 2016). The presence of the ZIKV particles in the amniotic fluids and in the fetus of the mothers infected with ZIKV is evidence of vertical transmission (Quicke et al., 2016; Silverstein, Aljouda, & Kumar, 2016).

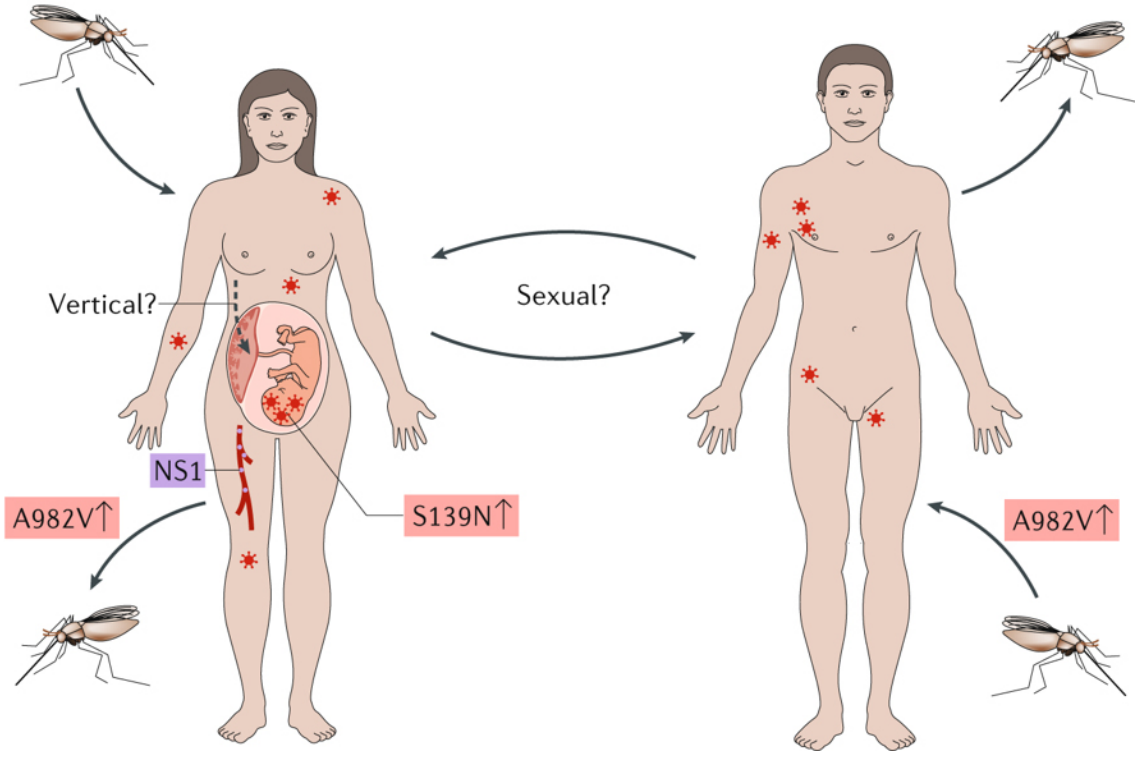


**Figure 2: Routes of entry for Zika virus**. Originally published in (Liu et al., 2019)

## 3. Genome

ZIKV is a single-stranded RNA virus. The RNA molecule of ZIKV is in a positive-sense conformation (Abushouk et al., 2016; X. Zhang et al., 2017). The positive-sense conformation of RNA molecules in viruses can be processed as a messenger RNA, which is subject for translational machinery (Paranjape & Harris, 2010). The process of hijacking the host translational machinery allows the ZIKV to express the genes that code for structural proteins as well as nonstructural proteins. The structural proteins that are encoded in the ZIKV genome are C, PrM, and E proteins. These proteins are responsible for making up the

viral particles of the ZIKV. The C protein in ZIKV forms the capsid that houses the genomic material of the ZIKV (Shan et al., 2016). The proteins that are encoded by the E gene form the glycoprotein envelope that surrounds the ZIKV capsid (Liu et al., 2019; Shan et al., 2016; X. Zhang et al., 2017). The PrM gene codes for a protein that works in concert with the E protein. Together, the PrM and the E protein form PrM-E heterodimers. The PrM-E heterodimers play a major role in the development of immature ZIKV visions (Zhu et al., 2016). Whereas the non-structural proteins that ZIKV codes for are NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5 (Liu et al., 2019; Shafique, 2017). The non-structural proteins are required for the replication process of the ZIKV genome inside the host cells (Shan et al., 2016).

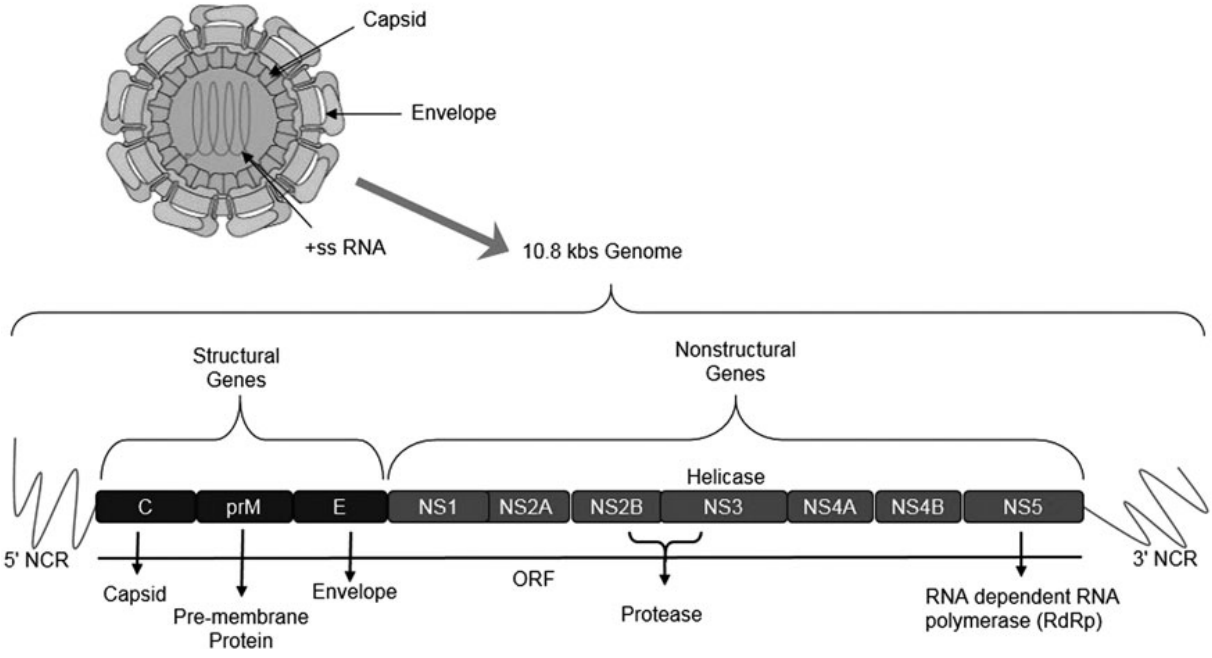


**Figure 3: Zika virus structure and genome**. Originally published in (Asif et al., 2017)

## 3.1 Structural proteins

### 3.1.1 Capsid

There are three structural proteins encoded by the ZIKV genome. From the 5' end of the genome, the C gene, which is the first gene of the structural genes, codes for the capsid protein (Fontaine et al., 2018; Liu et al., 2019; Shang, Song, Shi, Qi, & Gao, 2018). The capsid is a protein that surrounds the viral genome. One of the roles the capsid protein plays is to provide a level of protection to the viral genome from environmental factors (Nuanualsuwan & Cliver, 2003). The capsid is also responsible for the delivery of the virus into hosts cells and releasing genetic material in the cytoplasm (Bothner, Dong, Bibbs, Johnson, & Siuzdak, 1998; Nuanualsuwan & Cliver, 2003). The structure of viruses is broadly classified based on the 3-dimensional morphology of the capsid (Baron, 1996). The Flaviviridae family of viruses assumes an icosahedral structure (Agrelli et al., 2019; Plevka et al., 2011). The capsid protein forms a nucleocapsid when the RNA molecule of the ZIKV enters host cells. The nucleocapsid formation occurs as the virus is being assembled for budding out of the infected cell to infect neighboring cells (Shang et al., 2018). It has also been discovered that the capsid of the ZIKV localizes into the nucleus of host cells during the replication of the virus (Fontaine et al., 2018).

Additionally, the capsid protein of dengue virus, which is another member of the Flaviviridae family that is closely related to ZIKV, disrupts the assembly process of nucleosomes by binding to the histone protein in the nucleus. Blocking the nucleosome assembly of the infected host cells interferes with the host gene expression (Colpitts, Barthel, Wang, & Fikrig, 2011). It has also been discovered that the capsid protein of the ZIKV and

other members of the Flaviviridae can positively regulate the production of viral particles (Rawlinson & Moseley, 2015; Slomnicki et al., 2017).

### 3.1.2 Envelope

The third protein to be encoded by the ZIKV genome is the envelope protein, which surrounds the capsid of the ZIKV. The envelope is composed of glycoprotein protein formed as a result of expressing the envelope protein (E). The E protein is crucial for the viral infection of host cells. The structure of the E protein allows the virus to bind to host cellular receptors and to facilitate fusion with the host's membrane to release the virus inside the host cells (X. Zhang et al., 2017). To enable the virus to survive inside a human host, the structural properties of the E proteins can aid the virus in avoiding antibody neutralization (X. Zhang et al., 2017).

The E protein consists of four domains each serving multiple functions (X. Zhang et al., 2017). Three domains of the E protein are EDI, EDII, and EDIII (X. Zhang et al., 2017). The fourth domain of the E protein is at the base of the structure known as the stem-transmembrane domain that serves as an anchorage for the E protein domains (Shafique, 2017). The EDI domain is positioned between the EDII and EDIII domains and serves as a scaffold protein that is necessary for maintaining the structural orientations of the overall E protein. The EDI domain aids in the viral attachment to the cells and can facilitate neurovirulence through the position of conserved residues at Asn154, an N-linked glycosylation site. The E protein is also susceptible to conformational changes occurring by the EDI domain due to the nature of the pH environment to facilitate the fusion to host cells. These residues at the EDI domain pose an additional set of properties that favor the

production of the ZIKV particles, as well as an increase in neuroinvasiveness of the virus (X. Zhang et al., 2017).

EDII domain is positioned towards the peripheral part of the E protein, above the EDI domain (X. Zhang et al., 2017). This domain, with the aid of the EDI domain, facilitates the fusion with the membrane of host cells (Shafique, 2017; X. Zhang et al., 2017). The membrane fusion process is carried out through the 98–110 amino acid sequence, which is a highly conserved sequence that forms what is known as the fusion loop (X. Zhang et al., 2017). The fusion loops interact with the membrane of host cells, and the viral membrane as the E protein undergoes conformational changes with pH change (Gratton, Agrelli, Tricarico, Brandão, & Crovella, 2019; Shafique, 2017; X. Zhang et al., 2017). The consequence of fusion between the membrane of the ZIKV and the membrane of host cells is that host cells engulf the virus in an endosome. The inside of the endosome will have a very acidic environment that induces conformational changes to the ZIKV envelope, facilitating the fusion (Agrelli et al., 2019).

The third domain of the E protein is the EDIII, which is positioned towards the inner side of the E protein and is connected to the stem domain (Shafique, 2017). Sulfide-sulfide bridges contribute to the stability of the connection to the stem domain (X. Zhang et al., 2017). The EDIII domain provides sites complementary to cellular receptors to facilitates binding. The structure of the EDIII domain is known to play a role in triggering antibodies to neutralize the E protein of the ZIKV. This domain is subject to being used for designing monoclonal antibodies to target the ZIKV and other members of the Flaviviridae (Shafique, 2017)

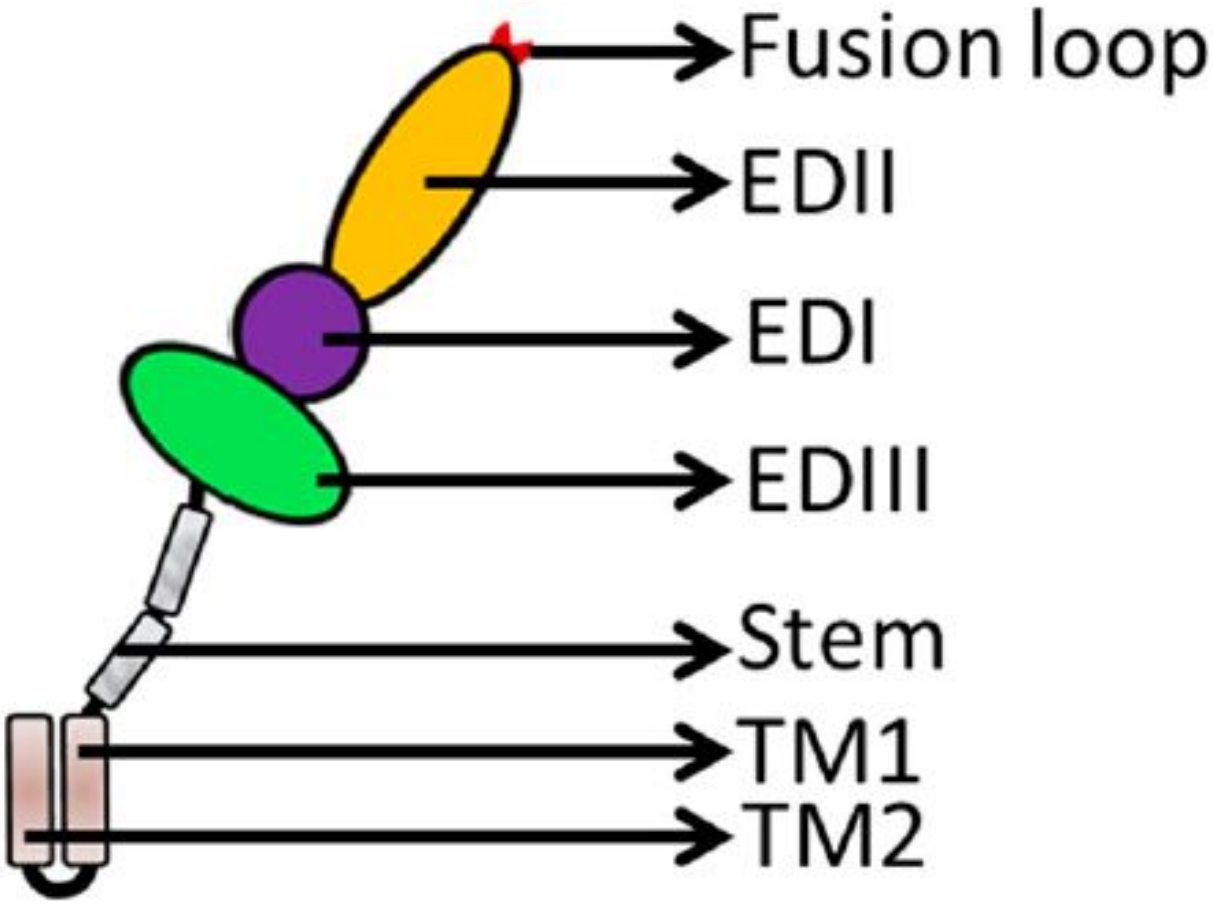


**Figure 4: Structural domains of envelope protein**. (X. Zhang et al., 2017)

### 3.2 Non-structural protein

After the viral genome is being released in the cytoplasm of the infected host cell and translated into a polyprotein, the host proteases begin cleaving the polyprotein with the help of viral proteases into 10 structural and non-structural proteins (Abushouk et al., 2016; X. Zhang et al., 2017). The non-structural (NS) proteins play a crucial role in viral replication as well as the participation in virulence and immune evasion. The NS1 protein is one of the seven non-structural proteins. The main function of the intracellular NS1 protein is to

facilitate the replication process of RNA of the ZIKV (Nogales et al., 2017; Sager, Gabaglio, Sztul, & Belov, 2018; Zou et al., 2015). When the NS1 is localized into the lumen of the ER it undergoes processing, allowing the NS1 to be associated with the membrane (mNS1) (Asif et al., 2017). When the NS1 proteins are either membrane-bound or secreted, the complement system activation will be prevented and the Toll-like receptors become activated (Zhu et al., 2016).

The NS3, along with the NS2B acting as a cofactor, is responsible for cleavage the polyprotein of the ZIKV (Li, Phoo, & Luo, 2014; Zou et al., 2015). The NS3 also participates in the replication of the viral RNA by acting as a helicase (Li et al., 2014). The helicase activity prevents the formation of dsRNA ahead of the replication complex (Jankowsky & Fairman, 2007). Additionally, NS3 also carries a nucleotide phosphatase and RNA triphosphatase activities (Li et al., 2014).

NS4A and NS4B are membrane-integrated proteins that mediated the replication process of the Flaviviridae family of viruses (Zou et al., 2015). These two proteins are cleaved by the NS3 protein in complex with the NS2A proteins by their protease activity (Li et al., 2014). The replication complex formation is facilitated by the NS4A protein when it interacts with the vimentin (Zou et al., 2015). Additionally, both NS4A and NS4B proteins are also responsible for interfering with the antiviral response in infected cells. Both NS4A and NS4B proteins interfere with the JAK/STAT pathway. According to Ma et al, the interactions of RLR-MAVS are being blocked by the NS4A, suggesting that the NS4A has a suppressive activity towards the RLR pathway. In stem cells, both NS4A and NS4B proteins interfere with the signaling of AKT-mTOR. As a result, cellular autophagy mechanism is

induced. Links between the interference of AKT-mTOR and microcephaly development have been reported (Ma et al., 2018).

The NS5 protein is also responsible for the replication of the RNA of ZIKV. The NS5 protein carries an enzymatic activity that is known as RNA-dependent RNA polymerase (RdRp) (Lin, Chang, Yu, Liao, & Lin, 2006). The RdRp is a type of polymerase that is specialized in replicating RNA molecules (Li et al., 2014; Zou et al., 2015). The NS5 is also responsible for the RNA modifications by acting as a methyltransferase and guanyltransferase (Grant et al., 2016; Li et al., 2014; Lin et al., 2006). The capping of the RNA also allows the RNA to be recognized for translation by the eukaryotic initiation factor 4 E (eIF4E). The recognition of the viral cap by the eIF4E triggers the host translational machinery of the viral RNA (Coutard et al., 2017). NS5 proteins also have negative effects on the immunological pathways. The presence of the NS5 proteins in infected cells inhibit the signaling for the JAK-STAT pathways that are induced by type I IFN (Ashour et al., 2010; Best et al., 2005; Grant et al., 2016; Lin et al., 2006).

**4. Replication Cycle:**

Upon the entry of a host cell, ZIKV begins its replication cycle to produce mature virion that leaves the infected cells to infect other cells, repeating the cycle (Heinz & Stiasny, 2017). The replication cycle consists of several events that are crucial for the success of the replication cycle. The first step of the replication cycle is the receptor recognition and attachment of the host cells by the ZIKV. One of the major components of the envelope in the ZIKV is the E protein, which consists of multiple domains known as EDI, EDII, and EDIII. The EDIII facilitates the entry of ZIKV by interacting with surface receptors of the host cells (Heinz & Stiasny, 2017; X. Zhang et al., 2017). These cell surface receptors are

heparan sulfates, carbohydrate receptors, ribosomal protein SA, and low-density lipoprotein receptor-related protein 1 (LRP1) (X. Zhang et al., 2017). The interaction with the cell surface receptors induces the cells to uptake the ZIKV through endocytosis, keeping the virus in an endosomal vacuole. This process is known as receptor-induced endocytosis (Heinz & Stiasny, 2017; X. Zhang et al., 2017).

Following the ZIKV entry through the induced endocytosis, the virus begins the process of membrane fusion (Cann, 2001; Heinz & Stiasny, 2017; Savidis et al., 2016; X. Zhang et al., 2017). The membrane fusion allows the envelope of the ZIKV to fuse with the endosomal membrane, leading to the uncoating of nucleocapsid and the release of the viral genome into the host cytoplasm (Agrelli et al., 2019; Cann, 2001; Savidis et al., 2016; X. Zhang et al., 2017). The membrane fusion is triggered by the acidic environment inside the endosomal vacuole. This acidic environment induces a conformational change of the E protein (Cann, 2001; Savidis et al., 2016; X. Zhang et al., 2017). The fusion process is facilitated by the EDII domain of the E protein, which contains a region known as the fusion loop (X. Zhang et al., 2017).

Once the RNA molecule of ZIKV is released in the cytoplasm, the RNA molecule begins the translation process (Agrelli et al., 2019). The translation process of ZIKV produces the essential structural and non-structural proteins that are essential for the viral assembly (Agrelli et al., 2019; X. Zhang et al., 2017). The translational event also produces an enzyme crucial to the replication process of ZIKV. This enzyme is RdRp. The RdRp utilizes the viral RNA to synthesize new RNA molecules (Cann, 2001; Saiz et al., 2016). The RdRp activity is carried out by one of the non-structural proteins NS5 (Lin et al., 2006; Zhao et al., 2017). The replication process occurs in the endoplasmic reticulum, where the newly

synthesized viral RNA undergoes modifications that prevent their degradation from the cytoplasmic environment, as well as allows as the RNA molecules to be recognizable by the eIF4E for translational events (Coutard et al., 2017; Grant et al., 2016; Li et al., 2014; Lin et al., 2006).

Once both translational and replicative events are completed, the assembly of ZIKV begins (Atif, Azeem, Sarwar, & Bashir, 2016; Saiz et al., 2016). The assembly process of ZIKV where the newly RNA molecule assembles with the capsid, PrM, and envelope proteins (Heinz & Stiasny, 2017; Saiz et al., 2016; Sikka et al., 2016). The product of the assembly is an immature virion. These virions channel through the Golgi apparatus where they use the trans-Golgi network (TGN) to utilize the exocytotic pathway (Atif et al., 2016; Heinz & Stiasny, 2017; Saiz et al., 2016). The virions then complete their maturation by cleaving the PrM by furin enzyme (Heinz & Stiasny, 2017; Saiz et al., 2016). Once the Zika virions are packaged and have become fully maturated, they are released by the process of exocytosis and begin infecting neighboring cells (Atif et al., 2016; Heinz & Stiasny, 2017).

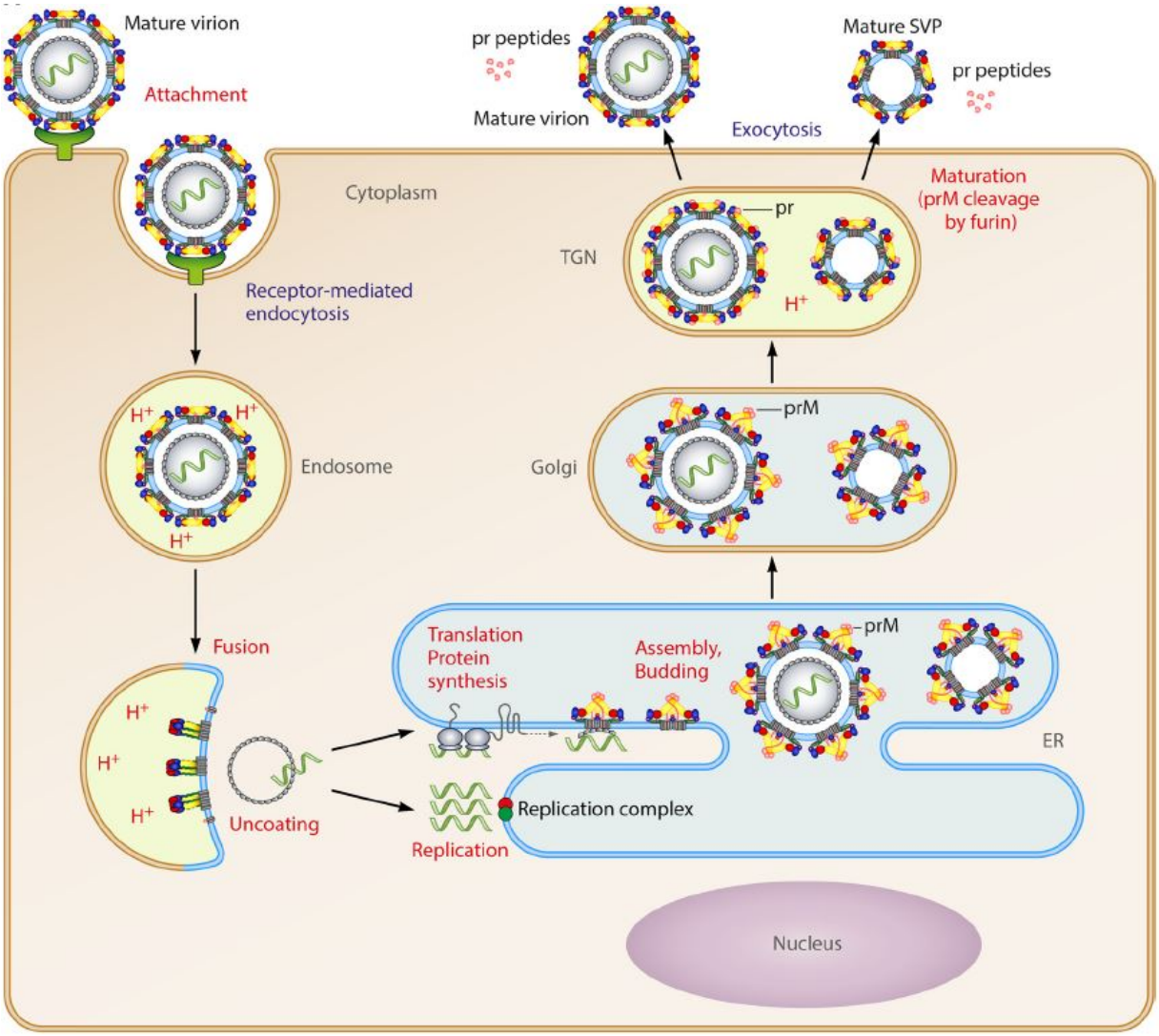


**Figure 5**: **Zika virus replication cycle.** Originally published in (Heinz & Stiasny, 2017)

## 5. Pathogenesis

ZIKV pathogenesis in humans causes a range of diseases from self-limiting illness to severe disorders (Liu et al., 2019). ZIKV infections through mosquito bites allow entry via the skin cells; fibroblast, keratinocytes and immature dendritic cell (Abushouk et al., 2016;

Didier Musso & Gubler, 2016; Plourde & Bloch, 2016). The entry of ZIKV is facilitated by entry and adhesion factors that assist the viral infection and autophagy induction, which has been associated with enhanced viral replication. ZIKV disseminates into other tissues such as spleen, brain, spinal cord, kidney, eye and placenta in pregnant women. ZIKV dissemination is facilitated using migration into the blood circulation through the lymphatic system (Plourde & Bloch, 2016).

One of the mechanisms ZIKV that contribute to the pathogenesis is the induction of apoptotic events in neural progenitor cells. This mechanism may significantly impact brain development and leading to microcephaly development (Miner, Diamond, & microbe, 2017). GBS development can also occur as ZIKV targets the neural cells. Reports indicate that neural illness begins six days before the development of the neurological symptoms, suggesting the presence of ZIKV infection in neural cells (Cao-Lormeau et al., 2016).

## 6. Autophagy

As the ZIKV infects cells and undergoes the replicative cycle, cellular pathways that induce autophagy formation are triggered (Saiz et al., 2016). Autophagy is a cellular mechanism that is initiated due to cellular maintenance or in response to environmental stresses (Glick, Barth, & Macleod, 2010; Gratton et al., 2019; Jung, Ro, Cao, Otto, & Kim, 2010; Rabinowitz & White, 2010). Autophagy mechanisms are classified into three different types; macroautophagy, microautophagy, and chaperone-mediated autophagy (Gratton et al., 2019; Jung et al., 2010; Mizushima & development, 2007; Rabinowitz & White, 2010). Macroautophagy, which represents the term autophagy, is a mechanism that forms a double-membrane vesicle know as an autophagosome (Gratton et al., 2019; Klionsky, Eskelinen, & Deretic, 2014; Shoji-Kawata & Levine, 2009; Silvestrini et al., 2018). This vesicle is formed

around cellular waste components that are destined for degradation (cargo). The autophagosomal vesicle undergoes membrane fusion with a lysosome, forming autophagolysosome (APL) (Ballou & Lin, 2008; Gratton et al., 2019; Klionsky et al., 2014; Silvestrini et al., 2018). The fusion with the lysosome causes degradation of the APL's cargo due to high acidity (Glick et al., 2010). Microautophagy is the process of direct lysosomal uptake of the cargo where it gets degraded. The chaperone-mediated autophagy pathway utilizes the chaperone HS70 and a lysosomal membrane protein 2A (LAMP2A) (Glick et al., 2010; Mejlvang et al., 2018; Mizushima & development, 2007).

The process of autophagy is regulated by multiple factors. One of these factors is mTOR (Ballou & Lin, 2008; Jung et al., 2010; Ma et al., 2018). mTOR is a member of the PIKK, phosphatidylinositol kinase-related kinase (Jung et al., 2010). This kinase phosphorylates serine/threonine residues (Ballou & Lin, 2008). Regulation of mTOR activity affects the signaling pathways that induce autophagy. The activation of mTOR has a negative regulatory effect on the autophagy formation. Activated mTOR inhibits the induction of the autophagy. Environmental stress condition alters the activity states of mTOR, blocking their functions (Ballou & Lin, 2008; Jung et al., 2010). One of these stressors is starvation. Under starvation conditions, cells utilize autophagy to recycle nutrients and misfolded proteins to produce precursors necessary for the generation of essential nutrients for survival (Ballou & Lin, 2008; Shoji-Kawata & Levine, 2009).

The formation of the autophagosome requires the assembly of autophagy-related genes (ATG) (Wesselborg & Stork, 2015). To date, about 31 ATG proteins have been discovered that are associated with autophagosome formation (Glick et al., 2010; Mizushima & development, 2007). These ATG proteins work in concert throughout the process of

autophagy. For example, ATG17, ATG29, ATG31, and ATG11 proteins are responsible for the formation of pre-autophagosome structures (PAS) (Glick et al., 2010; Jung et al., 2010; Mizushima & development, 2007). Other ATG proteins, such as ATG8, are responsible for the membrane fusion with the lysosome (Jung et al., 2010; Mejlvang et al., 2018; Mizushima & development, 2007). Some of the ATG proteins form complexes with other ATG proteins in order for them to become functional. An example of ATG complexes is the ATG12-ATG5 complex, which functions in facilitating the conjugation of ATG8 with phosphatidylethanolamine (PE) (Jung et al., 2010). The ATG12-ATG5 conjugate forms a complex with ATG16 that aids in the formation of the phagophore, in addition to recruiting LC3B-II (Glick et al., 2010).

Interestingly, autophagy pathways can also be induced in response to intracellular pathogens (Rabinowitz & White, 2010; Shoji-Kawata & Levine, 2009). Viral infections stress the cell and cause the inhibition of mTOR pathways induces autophagy in the infected cells. Viral infections cause the formation of virus-containing autophagic vesicles in an attempt to degrade the virus. The antiviral mechanism of autophagy can limit viral replications of some viruses, including Sindbis virus, resulting in reduced viral titers in the CNS (Gratton et al., 2019).

The placental immune system utilizes autophagy formation as one of the defensive mechanisms used against pathogenic stresses. One of the pathogenic stressors that induce autophagy formation is ZIKV. The induction of autophagy in placental cells infected with ZIKV could be triggered due to the expression of the non-structural genes NS4A and NS4B (Chiramel & Best, 2018; Ma et al., 2018; S. Miller, Kastner, Krijnse-Locker, Buhler, & Bartenschlager, 2007; Sven Miller, Sparacio, & Bartenschlager, 2006; Paul &

Bartenschlager, 2015). Reports have indicated that NS4A and NS4B proteins promote autophagy formation using the inhibitory Akt-mTOR pathway (Chiramel & Best, 2018). Autophagy formation in cells infected with ZIKV seems to act as a pro-viral mechanism (Yuan, Zhang, Li, & microbiology, 2017). Recent studies have suggested that autophagy formation may facilitate ZIKV replication and transmission (Cao, Parnell, Diamond, & Mysorekar, 2017). The autophagic vacuole is thought to provide a protective barrier from the immune system (Zhang, Li, Yuan, & microbiology, 2017). ZIKV also is also involved in blocking the lysosomal fusion with the autophagosome to prevent its degradation and to provide a safer environment for replication (Yuan et al., 2017).

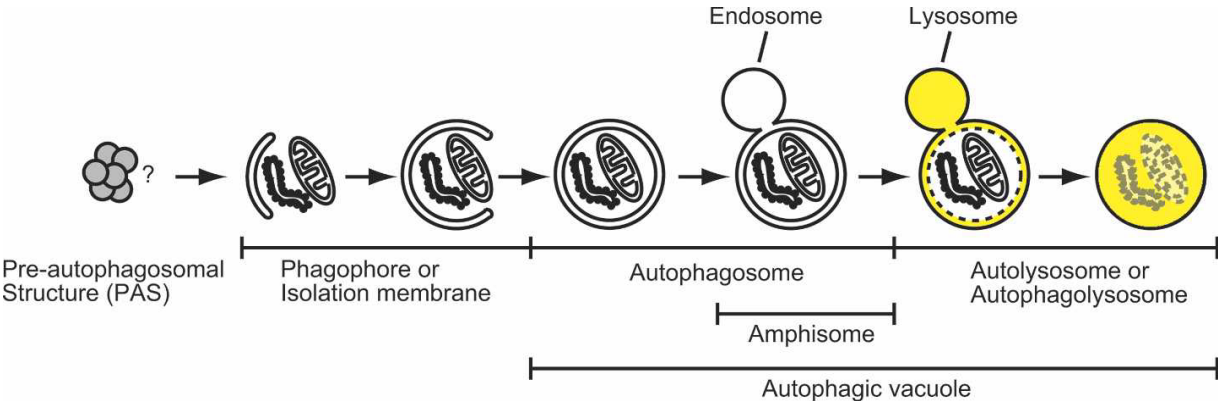


**Figure 6:** Autophagy formation steps. Originally published in (Mizushima & development, 2007)

## SPECIFIC AIMS AND HYPOTHESIS

In our study, we wanted to evaluate the effect of autophagy on the replication of ZIKV in human trophoblasts, which are physiologically relevant to ZIKV vertical transmission and fetal infection. To investigate whether the replication of ZIKV is hindered by autophagy deficiency, we created an autophagy gene knockout known as ATG12 in a human trophoblast cell line. Then, we wanted to see if induction of autophagy by rapamycin, an mTOR inhibitor and an autophagy inducer would result in higher viral loads. We hypothesized that the use of rapamycin would enhance ZIKV replication.

**Aim: Determining whether the autophagy formation in trophoblast would facilitate the replication of ZIKV**

We generated ATG12 gene knockout in trophoblast cells to determine whether autophagy deficiency would hinder the replication of ZIKV. We have also treated cells with rapamycin to determine if autophagy induction would increase the viral replication of ZIKV in trophoblast cells.

## MATERIALS AND METHODS

### 1. Cell culture and virus

The primary cell line that was used for this study is human placental trophoblast. For this experiment, the trophoblast cells were cultured at 37C in a 5% $CO_2$ incubator. The cells were cultured in RPMI medium with 10% of FBS and 1% pen-strep antibiotic. Trophoblast ATG12 gene knockout (*ATG12*$^{-/-}$) was generated and was kindly provided by my lab colleague. The ATG12$^{-/-}$ trophoblast cells were cultured in RPMI medium with 10% FBS and 1% pen-strep antibiotic. The trophoblast cells were incubated at 37ºC in a 5% $CO_2$ incubator.

Vero cell line was also used in the study for ZIKV propagation. The cells were cultured in DMEM medium with 10% FBS and 1% pen-strep antibiotic. Vero cells were incubated at 37ºC in the $CO_2$ incubator.

ZIKV was kindly supplied by one of my lab colleagues. The virus was propagated in Vero cells for 72hrs. The ZIKV was stored at -80ºC.

### 2. Reagents and antibodies

The antibodies used for the western blot analysis were mouse anti-ATG12 (Cat# MAB6807) that was purchased from R&D systems; rabbit anti-LC3B (Cat# 2775), and Actin (Cat# 8456) were purchased from Cell Signaling Technology (Danvers, MA, United States). Rapamycin (Cat# 553210) was purchased from EMD Millipore Corp (Billerica, MA, United States). PrimeScript™ RT Reagent Kit (Perfect Real Time) (Cat# RR07A) was purchased from Takara Bio Inc (Mountain View, CA, United States).

### 3. Western blotting

The Western blot technique was used to confirm that the ATG12 knockout of the trophoblast had been generated. The wild type (WT) and the ATG12$^{-/-}$ were incubated until the cells were about 90% confluent. The cells were extracted by removing the old medium and treating the cells for 1 minute with cold PBS, which then was removed. The cells were then dislodged by pipetting fresh medium. Once the cells were suspended in fresh medium, the cells were pelleted by centrifugation at 1000 rpm. After removal of the supernatant, the isolated cells were lysed with cold lysis buffer and placed on ice. The constituents of the lysis buffer included 0.5% NP-40, 50 nM Tris-HCL, pH 7.4, 2 mM EDTA, protease inhibitors and 150 mM NaCl. The cells were then subjected to centrifugation at 10,000 rpm, and the supernatant was collected for western blot analysis. The supernatant was treated with loading buffer and subjected to SDS gel electrophoresis. The protein bands generated on the SDS gel were transferred to the membrane. The membrane was blocked with 5% milk for 1 hour, then probed with anti-ATG12 and anti-LC3 primary antibodies that were purchased from Cell Signaling. The membranes were incubated with the primary antibodies overnight at 4°C. The antibodies were diluted in 5% milk and the concentration were following manufactures specifications. Anti-actin antibodies were used as a control for this experiment. Then, the membrane was incubated with secondary antibodies for 1hr at 4ºC. The membrane was then treated with a chemiluminescent substrate and was exposed to a film. The film was then developed using a film developer.

### 4. ZIKV infection and rapamycin treatment

The WT and ATG12$^{-/-}$ trophoblast cells were cultured to about 90% confluence. The medium was removed, cells were washed with cold PBS for 1 minute, and dislodged by

applying fresh RPMI medium. WT and ATG12$^{-/-}$ cells were each was seeded in a 24-well plate and incubated until reaching confluence. The medium was then removed, and the cells were treated with ZIKV in all wells for 3 hours. The medium containing the virus was then removed and replaced with a fresh RPMI medium. Half of the wells in the 24-well plate were treated with 100 nM rapamycin, while the other half were treated with 50 nM rapamycin for 3 hours. The medium was replaced with fresh medium thatalso contained rapamycin. The cells in the 24-well plate were incubated at 37°C in a CO2 incubator for 24hrs or 48hrs . At these time points, the WT and the ATG12$^{-/-}$ cell samples were lysed using TRK lysis buffer.

**5. RNA extraction and qPCR analysis**

Total RNA was extracted from the lysed WT or the ATG12$^{-/-}$ trophoblast cells using the E.Z.N.A. Total RNA Kit I by OMEGA. The isolated RNA samples were subjected to RT-PCR using the PrimeScript™ RT Reagent Kit (Perfect Real Time) to generate cDNA. The cDNA samples were then subjected to qPCR analysis. The targets for the cDNA samples consisted of ZIKV, *IFNB1*, and Actin. The ZIKV primers used were 5' CCGCTGCCCAACACAAG-3' and 5'CCACTAACGTTCTTTTGCAGACAT-3'. The *IFNB1* primer (Hs01077958_s1) used in the qPCR analysis was purchased from ThermoFisher Scientific. The viral load was calculated using the -ΔΔCT method and was compared to the values of the beta-actin genes.

## RESULTS

### 1. Confirmation of ATG12 Gene Knockout in Trophoblast Cells

The trophoblast gene knockout of *ATG12* was generated previously by a lab colleague. To determine whether the gene has been deleted successfully, Western blot analysis was performed on WT and *ATG12*$^{-/-}$. At about 55 kDa, we observed a band in the WT lane. The anti-ATG12 antibody was detected at in WT cells. The anti-ATG12 antibody was not detected at the same molecular weight, 55 kDa in ATG12$^{-/-}$ cells. This suggests observation suggests that the ATG12 protein was absent in ATG12$^{-/-}$ cells, confirming a successful gene knockout of the ATG12.

To further confirm that the *ATG12* gene was successfully knocked out, another autophagy-related protein that is associated with the ATG12-ATG5 conjugates was investigated. This protein is LC3B-II. Anti-LC3 antibody was used to detect the presence of LC3B-I and LC3B-II proteins. The Anti-LC3B antibody detected the presence of LC3B-I at 16 kDa in the WT cells. At 14 kDa molecular weight, the anti-LC3B antibody did not detected the presense of LC3B-II in the ATG12$^{-/-}$ cells. The absence of LC3B-II protein is an indication of defects in ATG12-ATG5 conjugate as they are required for the lipidation of the LC3B-II. These observations further confirmed that *ATG12* gene was successfully knocked out in trophoblast cells. (Fig. 7)

### 2. ZIKV Infection of ATG12 Deficient Trophoblast Cells

To investigate the effects of autophagy formation on the replication of ZIKV, we transfected both WT and ATG12$^{-/-}$ trophoblast cells with ZIKV. The total RNA was extracted from both WT and ATG12$^{-/-}$ trophoblast cells at three time points; 24, 48, and

72hrs. RT-PCR was performed to synthesize cDNA. The cDNA samples were then subjected to qPCR analysis. We have used primers targeting ZIKV, and *IFNB1* to investigate the viral load in response to autophagy deficiency, and to correlate the antiviral response to ZIKV load. The qPCR analysis revealed that there is a significant decrease in the relative viral load in response to autophagy deficiency at both 24 and 48hrs post-ZIKV infection when compared to the WT with a $P < 0.01$. Past 48hrs, the relative viral load began to decline gradually. These findings suggest that autophagy-deficiency hindered the ability of ZIKV to replicate efficiently (Fig. 8).

The relative *IFNB1* levels were significantly lower in ATG12$^{-/-}$ cells in the first 48hrs post the ZIKV infection with $P < 0.05$. At 72hrs post-infection, the *IFNB1* levels began to decline gradually. The *IFNB1* patterns are correlated with the relative viral load of ZIKV, likely as a secondary result of reduced viral replication (Fig. 9).

### 3. ZIKV Infection of Trophoblast Cells Treated with Rapamycin

Now that we have confirmed that autophagy deficiency in trophoblast cells significantly reduced the relative viral load, we wanted to determine whether autophagy induction by rapamycin in trophoblast cells would increase the relative viral load in trophoblast cells. Rapamycin is an mTOR inhibitor that induces autophagy formation. To investigate the of rapamycin on ZIKV viral load, we treated both WT and ATG12$^{-/-}$ with two different concentrations of rapamycin; 50 nM or 100 nM. The total RNA was extracted for PCR analysis at 24 and 48hrs. The qPCR analysis revealed that rapamycin treatment in trophoblast cells that at 24hrs post-infection, there was an increase in the relative viral load of ZIKV in WT cells treated with 100 nM rapamycin. The relative viral load of ZIKV in WT trophoblast cells treated with 50 nM rapamycin did not increase as much compared to WT

cells treated with 100 nM rapamycin. Interestingly, both 50 nM and 100 nM rapamycin treatment did not increase the viral load in ATG12$^{-/-}$ trophoblast cells (Fig. 10). However, at 48hrs, similar ZIKV loads were observed in both WT and ATG12$^{-/-}$ trophoblast cells that had been treated with either 50 nM or 100 nM rapamycin. These findings suggest that 100 nM of rapamycin treatment was sufficient enough to induce autophagy formation in WT trophoblast cells, thus increasing the viral load of ZIKV. The low relative viral load in 50 nM rapamycin-treated WT trophoblast cells may be an indication of an insufficient concentration of rapamycin to induce enough autophagy formation. The findings at 48hrs indicate that the ZIKV replication does not rely solely on the process of autophagy (Fig. 11).

We also trophoblast cells investigated the *IFNB1* levels in ZIKV infections in response to rapamycin treatment. At 24hrs post infection, *IFNB1* levels were elevated in WT at both 50 nM and 100 nM rapamycin concentrations when compared to the ATG12$^{-/-}$ trophoblast cells (Fig. 12). At 48hrs, WT cells treated with 100 nM rapamycin displayed an increase in *IFNB1* levels. These findings were consistent with the *IFNB1* patterns in Figure 9, suggesting that the *IFNB1* levels are correlated to the relative viral load of ZIKV (Fig. 13).

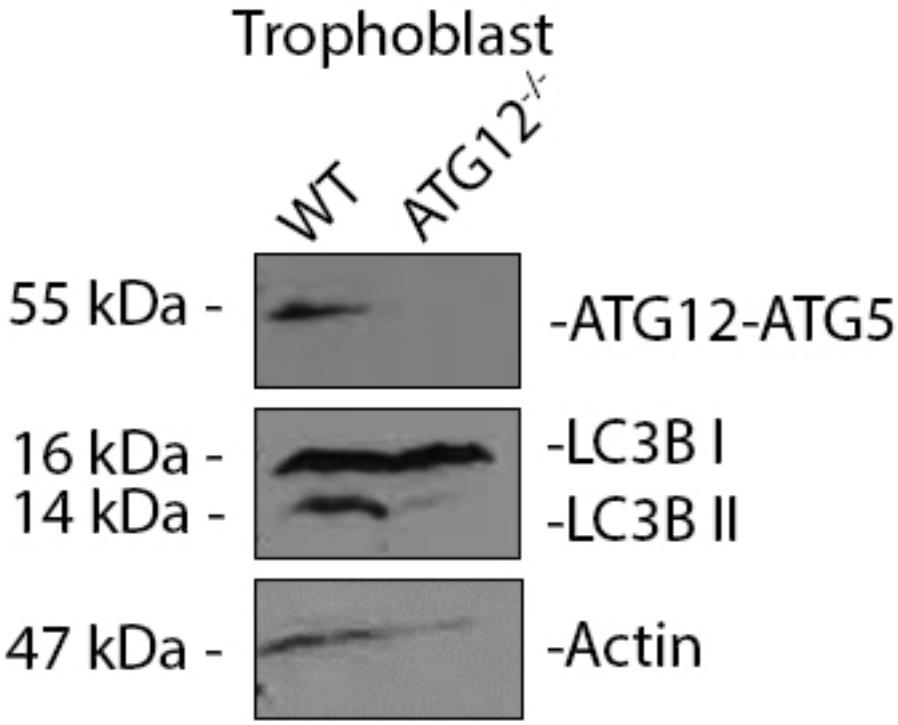


**Figure 7**: **ATG12 gene was successfully deleted in trophoblast cells**. Cellular proteins of trophoblast were separated on SDS-PAGE. Membranes were probed for the presence of ATG12, LC3BI/II, and Actin proteins. The first lane represents WT trophoblast cell lysate. The second lane contains ATG12$^{-/-}$ trophoblast cell lysate. Anti-ATG12 did not detect a product in ATG12$^{-/-}$ at 55 kDa, which would correspond to the ATG12-ATG5 conjugate. Similarly, anti-LC3B antibody did not detect LC3B-II in ATG12$^{-/-}$ cells. In summary, this Western blot analysis confirms the successful gene deletion of *ATG12* in trophoblast cells.

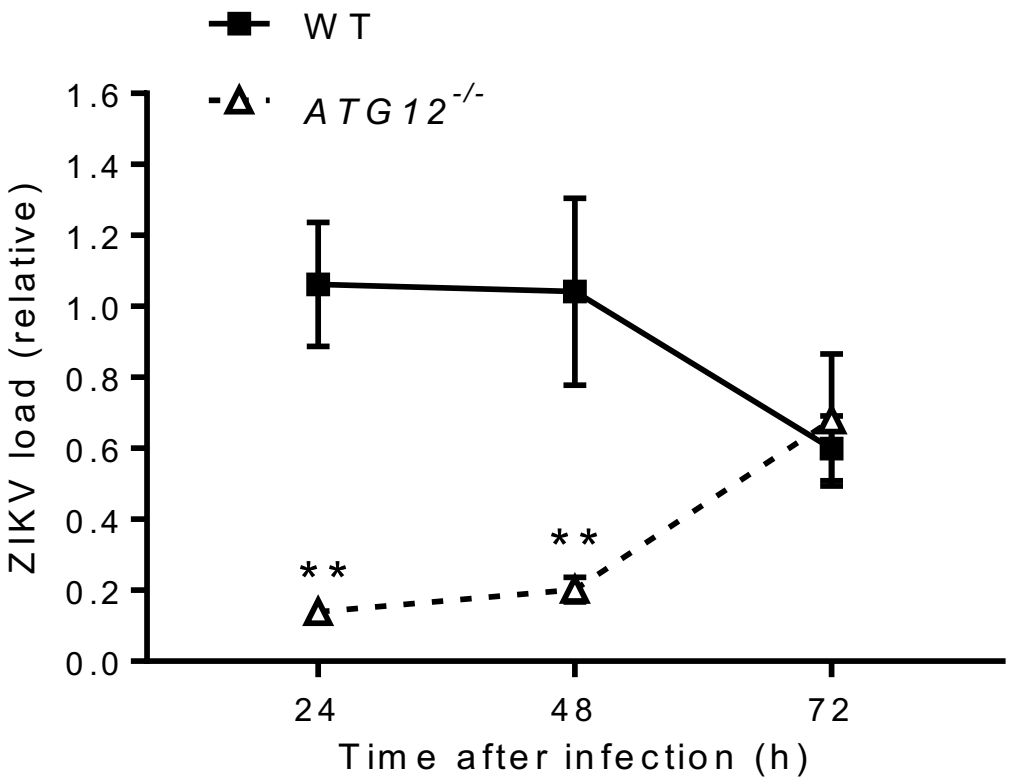


**Figure 8: ZIKV viral load was decreased in ATG12$^{-/-}$ cells.** The relative viral load in WT trophoblast cells is significantly higher in WT than in ATG12$^{-/-}$ trophoblast cells during the first 48hrs. The viral load then begins to drop after 48hrs of the ZIKV infection. The plots indicate the mean of three replicates and error bars indicate the standard deviation. $P < 0.01$, two-tailed student T test. This experiment was repeated twice.

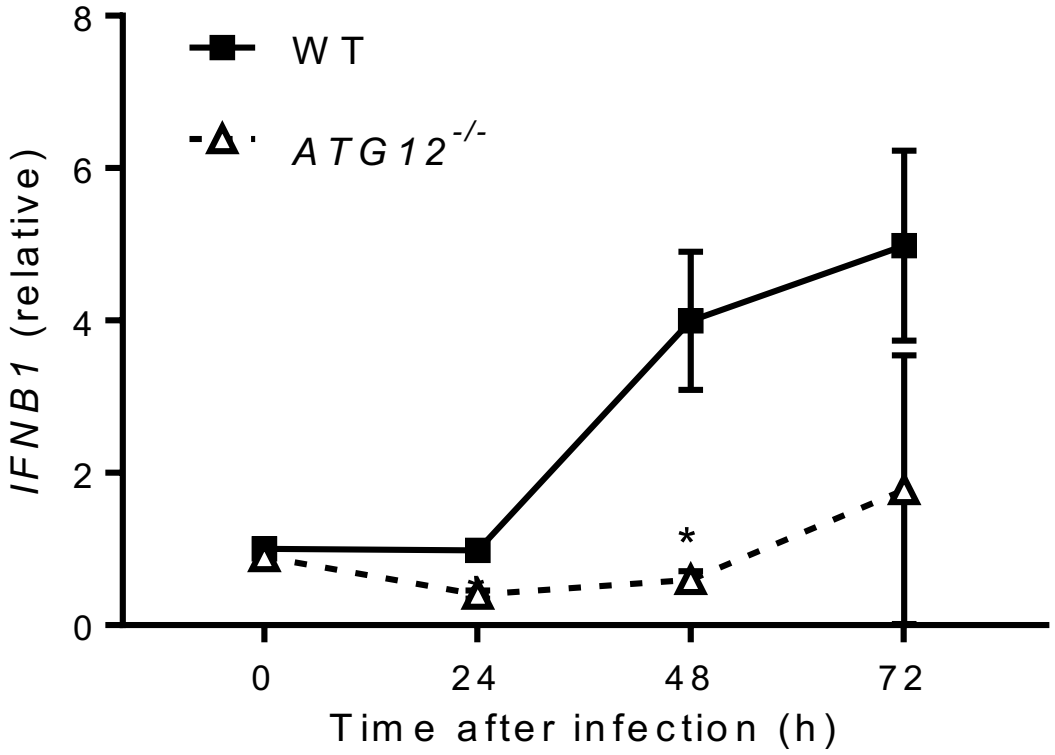


**Figure 9: *IFNB1* levels decreased in ATG12$^{-/-}$ cells in response to ZIKV.** The relative levels of the IFNB1 in response to the viral infection is at basal levels 24hs post infection. The IFNB1 levels begin to rise significantly 48 and 72hrs post infection. The plots indicate the mean of three replicates and error bars indicate the standard deviation. $P < 0.05$, two-tailed student T test. This experiment was repeated twice.

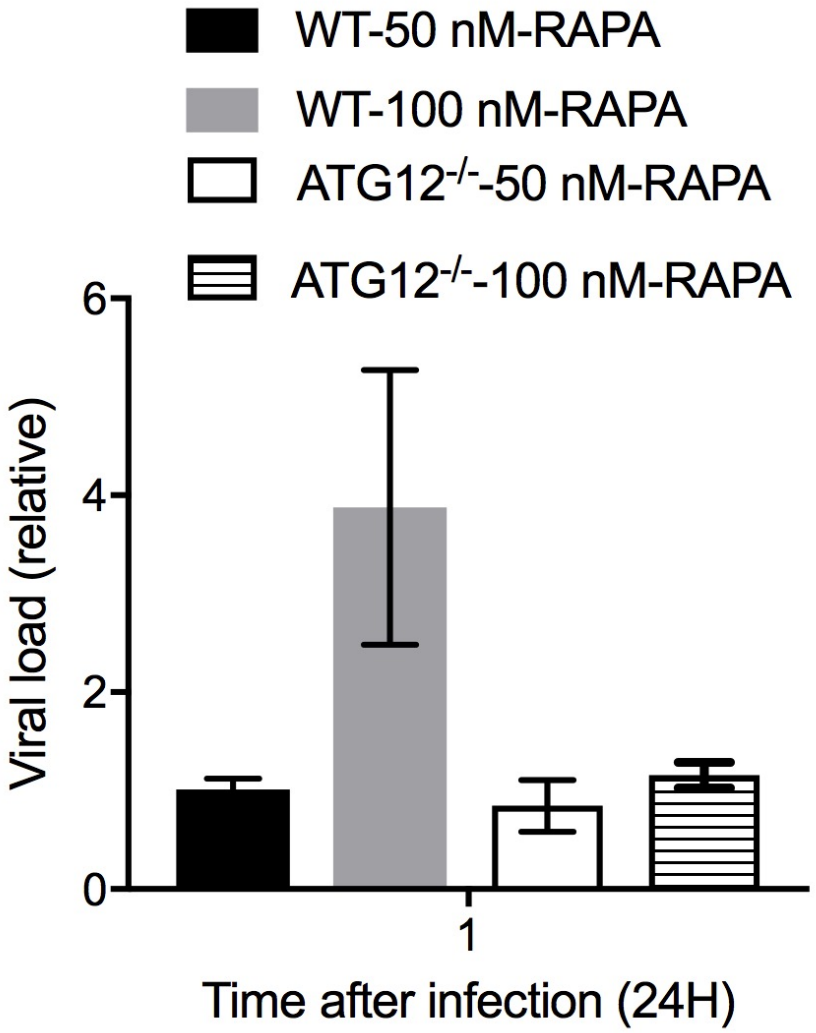


**Figure 10: ZIKV viral load increased in WT trophoblast cells in response to 100 nM rapamycin 24hrs post infection.** Both WT and ATG12-/- were treated with either 50 nM or 100 nM of rapamycin drug. WT cells treated with 100 nM of rapamycin display elevated levels of relative ZIKV viral load when compared to the ATG12-/- at 24hrs post infection. There is a trend towards significance in the readings that was not achieved due to the small sample size. Columns indicate the mean of three replicates and error bars indicate the standard deviation. This experiment was conducted once.

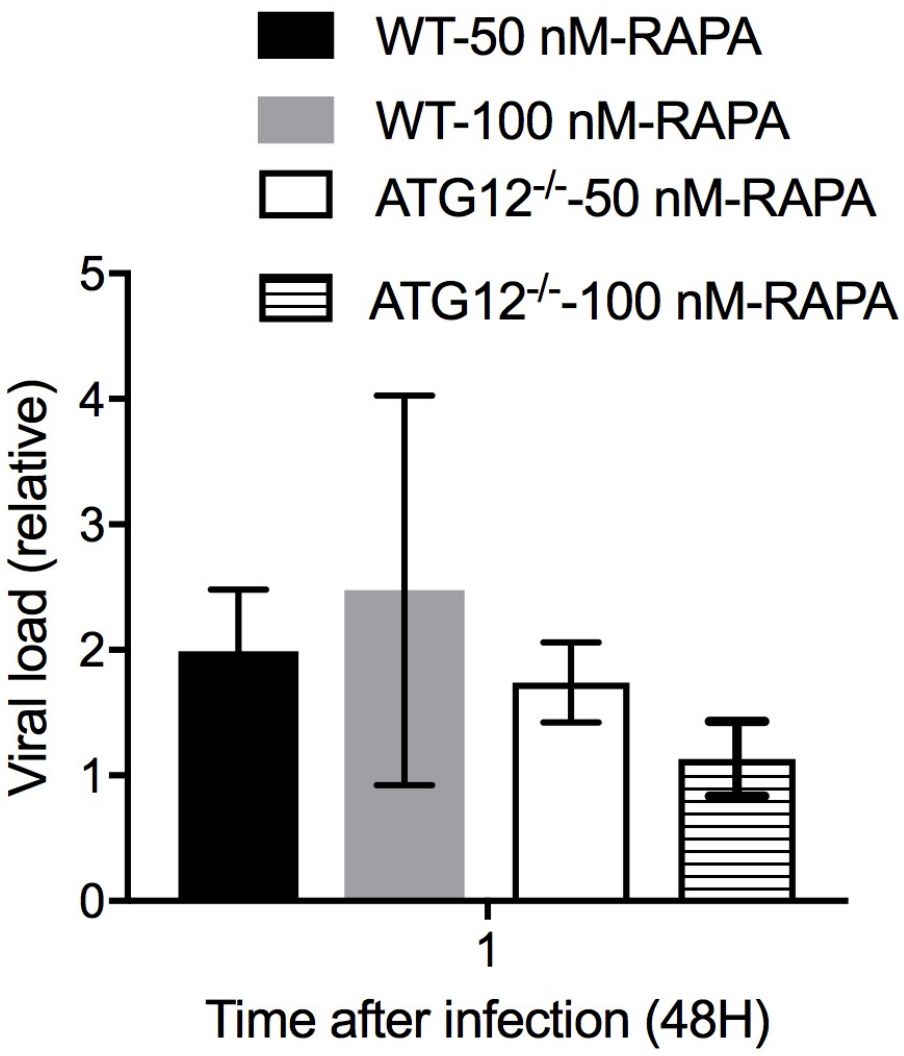


**Figure 11: Similar ZIKV viral load in both cells treated with 50 nM or 100 nM rapamycin 48hrs post infection.** At 48hrs, the relative viral load of ZIKV in WT and ATG12$^{-/-}$ treated with either 50 nM or 100 nM of Rapamycin begins to reach equilibrium. There is a trend towards significance in the readings that was not achieved due to the small sample size. Columns indicate the mean of three replicates and error bars indicate the standard deviation. This experiment was conducted once.

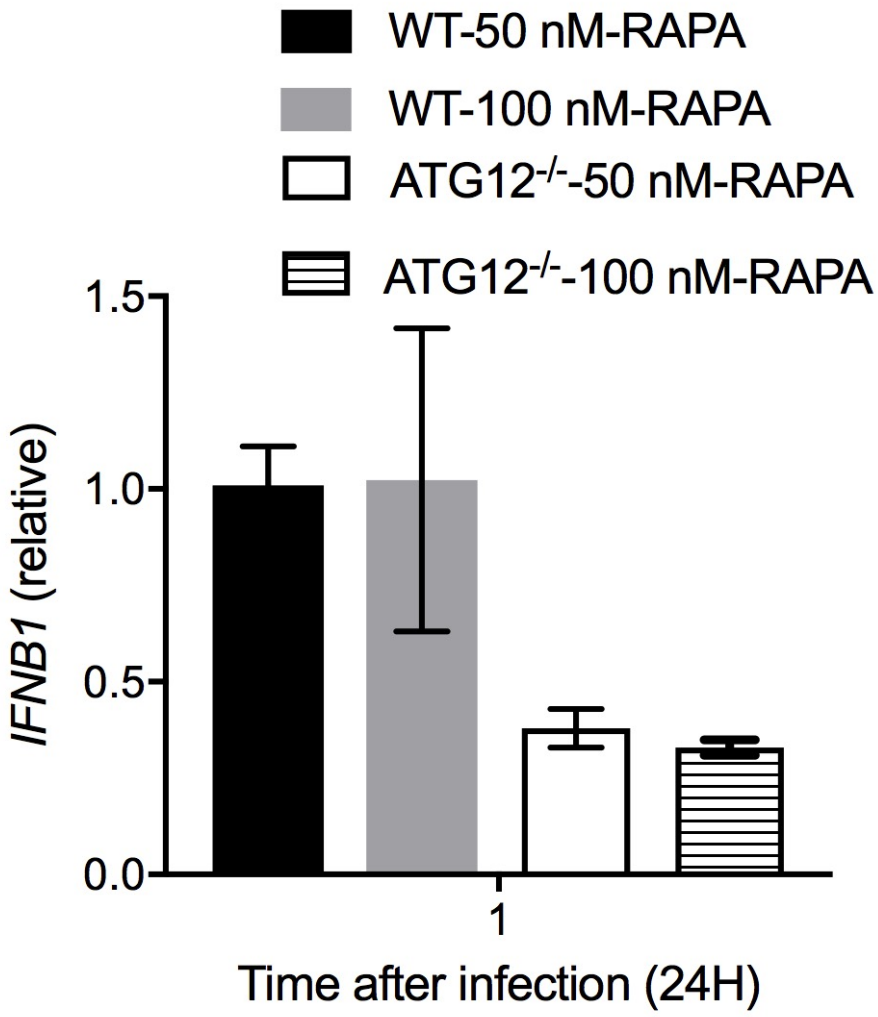


**Figure 12: *IFNB1* levels increased in cells infected with ZIKV in response to 50 nM and 100 nM rapamycin 24hrs post infection.** Both WT and ATG12$^{-/-}$ were treated with wither 50 nM or 100 nM of Rapamycin drug. At both dosages of Rapamycin, *IFNB1* levels in WT were higher than ATG12$^{-/-}$ at 24hrs post infection.. There is a trend towards significance in the readings that was not achieved due to the small sample size. Columns indicate the mean of three replicates and error bars indicate the standard deviation. This experiment was conducted once.

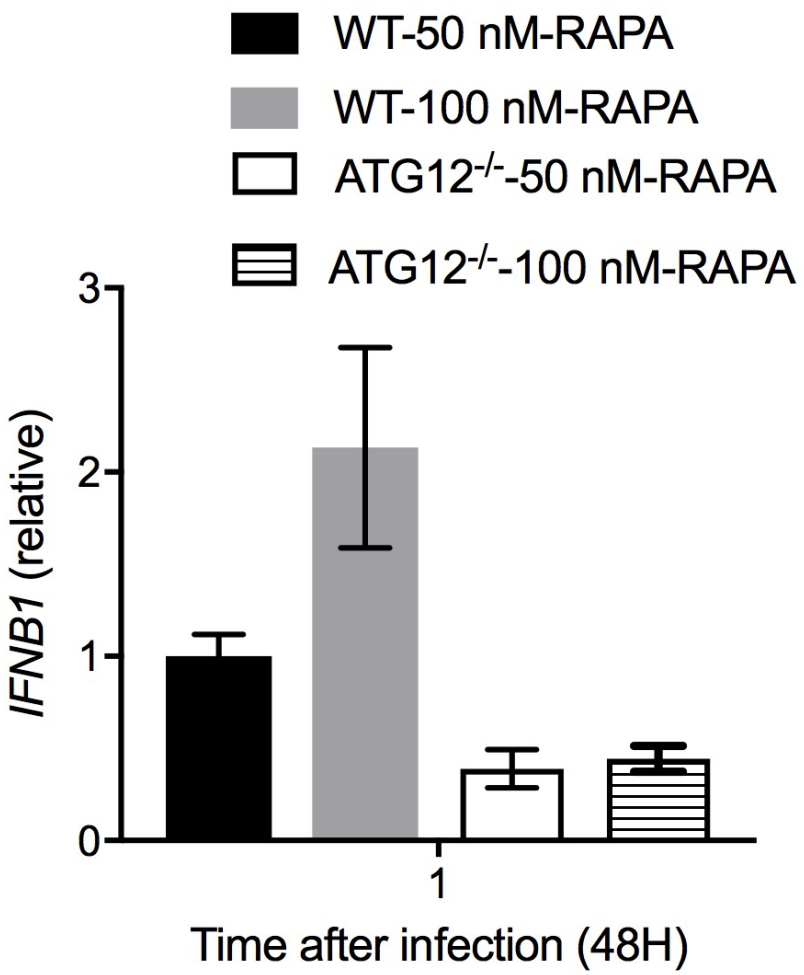


**Figure 13: Further increase in *IFNB1* levels in cells infected with ZIKV in response to 100 nM rapamycin 48hrs post infection.** At 48hrs, the *IFNB1* levels in WT cells treated with 100 nM rapamycin increased by one-fold. There is a trend towards significance in the readings that was not achieved due to the small sample size. Columns indicate the mean of three replicates and error bars indicate the standard deviation. This experiment was conducted once.

## DISCUSSION

Our primary goal in this study was to investigate the role of autophagy formation in trophoblast cells in the replication of the ZIKV. We have created a scenario for the ZIKV replication where the target cells were incapable of establishing autophagy. Then we investigated how autophagy formation would impact the viral replication of ZIKV.

The fetus is protected from pathogenic infections through the placental barrier. When a pathogen invades the placental barrier and infect placental cells, an immune response becomes triggered, and other protective mechanisms such as autophagy are activated. During pathogenic infections, autophagy formation captures the pathogen and fuses with the lysosomal compartment to degrade the pathogen. In our study, we have confirmed that autophagy formation provides a favorable environment for ZIKV to replicate. Generation of a gene knockout of ATG12, which is crucial for the formation of the autophagophore, has demonstrated the significance of autophagy in enhancing ZIKV replication in trophoblast cells. Our observations confirm previous studies conducted with a different autophagy-related gene, AT16L1. Additionally, our findings further strengthen the current understanding regarding autophagy mechanism and ZIKV replication.

Autophagy is a crucial mechanism of cell survival. ZIKV infections take advantage of the autophagosome formation. ZIKV has been known to induce autophagy once the NS4A and NS4B are translated. NS4A and NS4B play a role in inducing autophagy in cells (Ma et al., 2018). Our experiments have expanded our knowledge and understanding regarding autophagy induction. We induced autophagy using a drug known as rapamycin. Rapamycin is an mTOR inhibitor and an autophagy inducer. Autophagy induced with rapamycin resulted in higher ZIKV viral load in WT trophoblast cells. Our results have confirmed that

autophagy formation facilitates the viral replication of ZIKV. The effect of rapamycin on ZIKV replication was abolished in ATG12 deficient trophoblast cells, which was expected due to the absence of autophagophore formation. Rapamycin may induce autophagy, but the cells were not able to form a robust autophagophore structure that surrounds the ZIKV. Therefore, ZIKV was not able to replicate efficiently.

Due to the significant impact of ZIKV on placental and neural cells, therapeutic interception is required to limit viral replication of ZIKV in the fetus and neuronal cells. Our study demonstrates an alternative approach to limiting the viral replication of ZIKV in trophoblast cells through autophagy regulation. Currently, multiple studies have demonstrated promising results using drugs to limit the ZIKV replication in placental trophoblast cells (Bayer et al., 2016; Delvecchio et al., 2016; Elfiky, 2016; Sacramento et al., 2017; Xu et al., 2016; Zmurko et al., 2016). Combining these drugs with autophagy inhibitors might provide a stronger suppression to the viral replication of ZIKV and may limit the possibility of maternal-placental transmission. More studies are required to investigate the combinational therapies using these promising anti-ZIKV drugs with autophagy blockade drugs that target essential autophagy-related genes at both in vitro and in vivo settings.

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