

# INFLUENZA A AND FLAVIVIRUS MANIPULATION OF CELL DEATH

By

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for degree of Doctor of Philosophy.

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# **INFLUENZA A AND FLAVIVIRUS MANIPULATION OF CELL DEATH**

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Advisor: Dr. Zahra Zakeri

## **Abstract**

Viruses employ a variety of strategies to manipulate cell fate and maximize their replication potential during infection. Interacting specifically with cell death pathways allows viruses direct control over the cellular decision to survive or die during infection.

Influenza A virus, of the family Orthomyxoviridae, encodes proteins that interact with the cell at several points along the apoptosis pathway to induce apoptosis. We demonstrate that caspase activation through pro-apoptotic Bax, a downstream target of Bcl-2, is a critical determinant of the nature of cell death induced by Influenza A infection. Influenza A virus cannot establish an apoptotic response without functional Bax, and instead elicits autophagy after infection. Efficient virus replication is dependent upon Bax-mediated apoptosis as Bax KO also causes a 99% reduction in Influenza A viral titer.

In stark contrast, cells infected with Dengue-2 or Modoc virus of the family Flaviviridae do not die, even at high MOI. Instead, infection with either of these flaviviruses generates an autophagy-dependent protection against several toxins. Autophagy upregulation following infection is also critical to maximum flavivirus replication. Expression of NS4A from either virus is uniquely sufficient to elicit both autophagy and protection against death similar to whole virus infection. As autophagy upregulation is essential for maximum flavivirus replication, flavivirus NS4A is therefore identified as a potential target for the development of specific anti-viral therapy for flavivirus infection.

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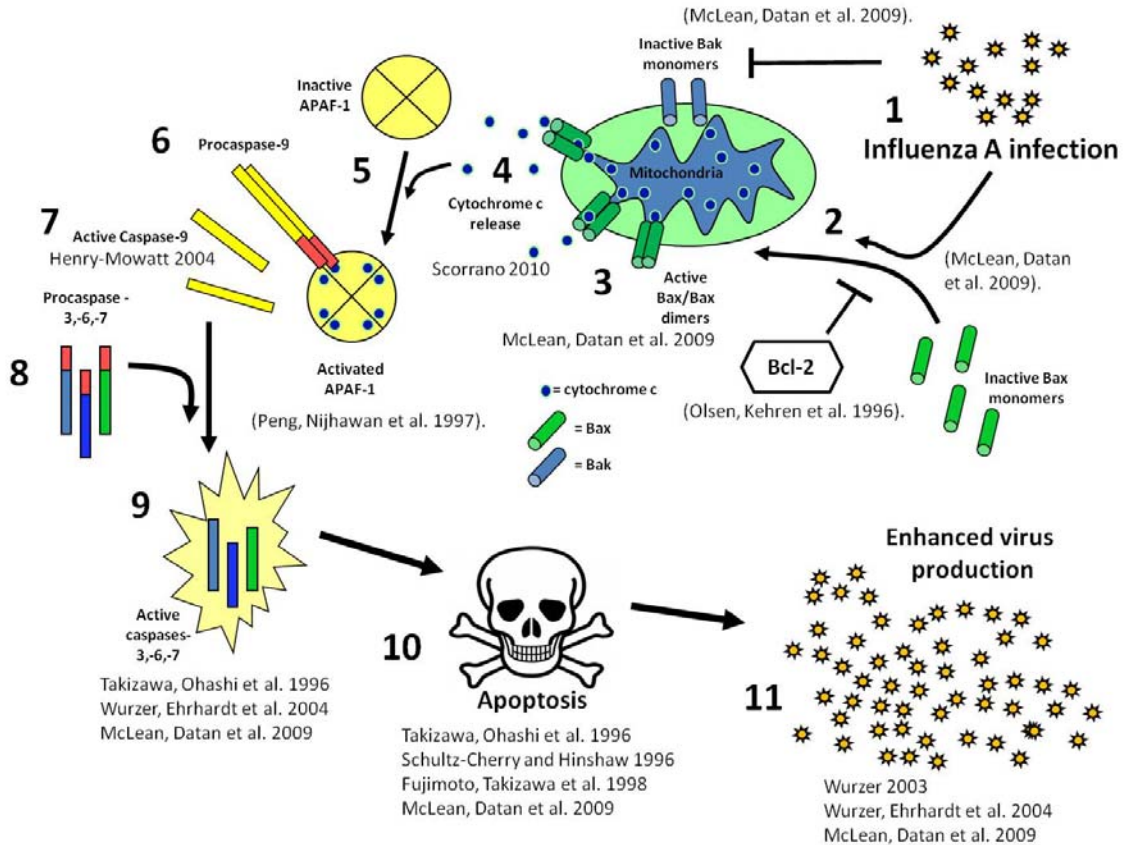
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## Overview

The success of any viral infection can be measured by the number of infectious progeny produced from an infected cell. To survive and reproduce, the virus must evade destruction by tissues. Redundancy of anti-viral signaling pathways in eukaryotic cells makes it nearly impossible for an invading virus to go unnoticed, but the evolution of anti-viral innate immunity has led to concurrent evolution in viral evasive strategies. By selectively either inducing cell death or inhibiting it, viruses improve their chances to propagate and disseminate. Many unrelated viruses modulate host anti-viral responses by manipulating cell death, underscoring the importance of cell death biochemistry in innate immunity. In this thesis we examine how two unrelated RNA viruses manipulate cell death: Orthomyxoviridae (Influenza A virus) and Flaviviridae (Dengue-2 and Modoc viruses), and how their modulation of cell death affects their replication.

Ectopic overexpression of Bcl-2 restricts both influenza A replication and virus-induced apoptosis in MDCK cells, suggesting that Bcl-2 family members normally limit replication. Here we report that influenza A virus cannot establish an apoptotic response without functional Bax, a downstream target of Bcl-2, and that both Bax and Bak are directly involved in influenza A virus replication and virus-induced cell death (see Diagram 1). As we will further demonstrate, Bax is activated and translocates from the cytosol to the mitochondria following Influenza A infection; this activation is required for the efficient induction of apoptosis and virus replication. Knockout of Bax in mouse embryonic fibroblasts blocks the induction of apoptosis, restricts infection-mediated activation of executioner caspases, and inhibits viral propagation. Bax knockout cells die but by an alternative death pathway displaying characteristics of autophagy, similar to



**Diagram 1. Influenza A induces death by activating Bax during infection.** Infection with Influenza A (1) triggers downregulation of Bak expression and simultaneous Bax translocation to the outer mitochondrial membrane (2), where it forms homodimers and Bax/Bax homodimers (3) which in turn allow cytochrome c release from the inner mitochondrial space to the cytoplasm (4). Cytoplasmic cytochrome c binds and activates APAF-1 (5). Activated APAF-1 then interacts with procaspase-9 (6), facilitating its autocleavage and yielding active caspase-9, an initiator caspase (7). Active caspase-9 then cleaves procaspases -3, -6 and -7 (8), activating these executioner caspases (9). Executioner caspase activity then induces apoptosis (10). Bax-mediated executioner caspase signaling during Influenza A infection promotes virus replication and assembly (11). Further references are provided in the Background section and Chapter one of this thesis.

our previous observation that influenza A virus infection in the presence of a pan-caspase inhibitor leads to an increase in autophagy (Datan et al., *in preparation*). Knockout of Bax causes retention of influenza A virus NP within the nucleus.

In contrast, Bak is substantially downregulated during influenza A virus infection in MDCK cells, and the knockout of Bak in mouse embryonic fibroblasts yields a

dramatic rise in the rate of apoptotic death and a corresponding increase in viral replication, suggesting that Bak suppresses both apoptosis and virus replication and that in normal influenza A virus infection the virus suppresses Bak. We conclude that appropriately timed apoptosis is important for the replication of influenza A virus and that the cell and virus struggle to control apoptosis and autophagy. The details of our findings are published (Mclean, Datan et al. 2009) and presented in Chapter One of this report.

Cellular response to infection varies with both type of virus and host cell. Manipulation of cell death seems to play an important role in the delivery of genomic segments from sites of replication within the nucleus to cytoplasmic viral assembly centers during replication of Influenza A, a negative sense RNA virus. However, most positive-sense RNA viruses do not carry out genomic replication within the nucleus, and therefore may not require apoptosis during infection for maximal virus yield. To investigate this possibility, we expanded our study to include an unrelated group of positive-sense RNA viruses, the *Flavivirus* genus of the family Flaviviridae.

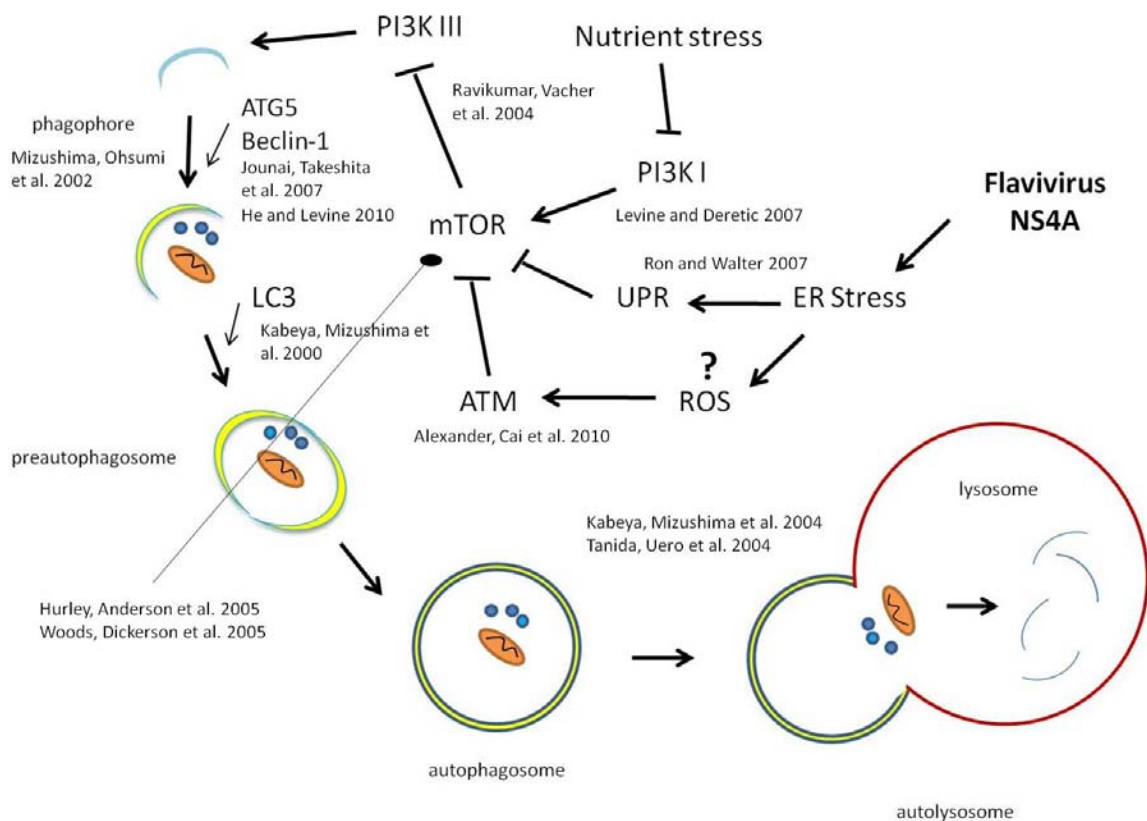
Flaviviruses include some of the most prevalent and medically challenging viruses in the world. Flaviviruses naturally infect many cell types, including neurons, macrophages, hepatocytes, epithelial cells and fibroblasts (Davis, Hardy, et al. 1974; Lum, Lum et al. 1996; Jessie, Fong et al. 2004). Macrophages, which die after Dengue infection, have been identified as a primary site of viral replication and have been a main focus of studies on the etiology of dengue-related disease (Gulati, Chaturvedi et al. 1982; Halstead 1989; Despres, Flamand et al. 1996; Jan, Chen et al. 2000; Espina, Valero et al. 2003). However, the establishment of persistent infections following host recovery from

acute illness *in vivo* indicates the existence of a subset of host cells that survive infection and continue to shed virus. Persistence of flaviviruses in infected individuals has been attributed to persistent infection of hepatocytes and renal epithelial cells that do not undergo cell death following infection (Johnson 1970; Davis, Hardy et al. 1974; Pogodina, Folova 1983; Sharma, Mathur et al. 1991; Gitsun, Frolova et al. 2003; Mathur, Arora et al. 2005; Tesh, Siirin et al, 2005; Tonry, Xiao et al. 2005; Murray; Siirin, Duan et al. 2007; Walker et al. 2010).

Here we report that infection with Dengue-2 virus or Modoc virus (a murine flavivirus) kills primary murine macrophages, but protects epithelial cells and fibroblasts from death induced by several cell death inducers. Further, we identify flavivirus NS4A as the sole viral determinant of flavivirus-induced protection in epithelial cells. In addition, we demonstrate that upregulation of autophagy following flavivirus infection enhances viral replication in MDCK renal tubular epithelial cells. Thus we conclude that flavivirus NS4A-mediated upregulation of autophagy in these cells serves the dual function of providing both a well protected host and the appropriate intracellular conditions for maximal flavivirus replication.

Flavivirus-induced protection against cell death results from an ATM-and PI3K-dependent upregulation of autophagy in these cells (see Diagram 2). We conclude this because inhibition of autophagy by inhibiting either ATM or PI3K nullifies the protection conferred by flavivirus infection, while upregulation of autophagy by either starvation or inactivation of mTOR leads to protection similar to that produced by viral infection. Further, as ATM mediates upregulation of autophagy following ER stress our results indicate that infection-induced ER stress may play a major role in the upregulation of

autophagy and protection during flavivirus infection. These effects are entirely replicable by expression of flavivirus NS4A, indicating that this protein is sufficient to induce ATM and PI3K-dependent autophagy and protect cells against death. Expression of other Dengue-2 genes fails to both induce autophagy or protect cells against various insults. Further, inhibition of autophagy by inhibition of PI3K limits replication of both Dengue-2 and Modoc virus. As autophagy enhances flavivirus replication in these cells, NS4A activity is therefore identified as a determinant of flavivirus replication and presents a novel target for the development of flavivirus anti-viral drugs. The details of our findings are presented in Chapter Two of this report.



**Diagram 2. Flavivirus NS4A induces autophagy in epithelial cells.** Flavivirus NS4A induces ATM and PI3K-dependent autophagy and protection from death. This is possibly due to NS4A-induced ER stress, which signals mTOR inactivation via ATM activity. Flavivirus NS4A-induced protection is dependent upon ATM, PI3K III, ATG5 and Beclin1. Further references are provided in the Background section and Chapter 2 of this thesis.

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## **Background: Pathways to Cell Death**

### **1.1 Cell death in health and disease**

Cell death is essential to proper development, tissue homeostasis and immune defense (Barisic 2003; Rossi 2003; Debatin 2004). Several metabolic pathways converge on key pro- and anti-death signaling molecules within the cell (Depre and Taegmeyer 2000; Danial and Korsmeyer 2004; Nutt, Margolis et al. 2005; Ying, Alano et al. 2005; Jin, DiPaola et al. 2007; Degterev and Yuan 2008; Kim, Xu et al 2008; Khaliullina, Panakova et al. 2009, Fulda, Gorman et al 2010). By actively regulating the survival and demise of cells, the organism maintains appropriate tissue-specific cell turnover (Szabadkai and Duchon 2009; Snow, Pandiyan et al. 2010), sculpts organs (Conradt 2009; Kato, Awasaki et al 2009) and destroys pathogen-infected cells (Labbe and Saleh 2008). Regulating the timing and means of cell death is a requirement for tissue health, and a high degree of refinement in death signaling has evolved.

Once thought of as a purely passive process whereby cells meet their demise through overwhelming insult, most cell death is now understood to be an active process, requiring the synthesis or processing of particular proteins and leading to the specific fate of an organized death. In healthy tissues, a delicate balance exists between cell proliferation and cell death. Any perturbation or disturbance of either process can lead to pathogenesis (Danial and Korsmeyer 2004). Failure to induce cell death is observed in all cancers (Kerr, Winterford et al. 1994; Ambrosini, Adida et al 1997; Evan and Vousden 2001; Johnstone, Ruefli et al. 2002) and many autoimmune disorders (Stassi and DeMaria 2002; Sharpe, Wherry et al. 2007; Ortona, Margutti et al. 2008). Conversely, excessive cell death is the major route of pathogenesis due to ischemic injury during strokes

(Degterev, Huang et al. 2005; Schinzel, Takeuchi et al. 2005), and is seen in several neurodegenerative disorders, including Parkinson's disease (Mochizuki, Goto et al. 1996) and Alzheimer's disease (Bossy-Wetzel 2004).

Dying cells typically adopt one of three major distinct morphologies: apoptosis, autophagy or necrosis. These death pathways require active cell metabolism and protein synthesis (Schultz, Weller et al. 1996; Lockshin and Zakeri 2004), and each differs in its appearance, biochemical signaling and the eventual method of cellular destruction. There seems to be significant crosstalk between necrosis, autophagy and apoptosis signaling, implying a high degree of regulation in the cell's decision over which fate to choose. However, much work remains to elucidate the interactions governing this choice (Linette, Li et al. 1996; Roy and Nicholson 2000; Neumar, Xu et al. 2003; Chandra, Choy et al. 2004; Boya, Gonzalez-Polo 2005).

Cell death has been observed in tissue sections since the 19<sup>th</sup> century (Clarke and Clarke 1996). However, early descriptions of cell death were strictly morphological and little effort was made to elucidate the causes or regulation of these phenomena (Zakeri and Lockshin 2008). The active induction of particular biochemical pathways leading to cell death was first surmised in the 1960's (Kerr 1972; Lockshin 2001). Introducing the term "programmed cell death" (PCD) to indicate the deliberate self-destruction of cells, Lockshin and Williams emphasized cell demise as a purposeful cell fate (Lockshin and Williams 1964, 1965a, b). It was not until the 1980's that evidence of a specific biochemistry behind cell death was produced, when DNA fragmentation was realized as a typical feature of apoptosis (Wyllie, Morris et al. 1984). Several forms of programmed cell death have since been characterized, each carrying with it particular changes in cell

morphology and gene expression in response to extrinsic or intrinsic signals (Fulda, Gorman et al. 2010). Although every programmed cell death pathway eventually leads to the common endpoint of death, the mechanisms behind each are very different. The characterization of caspases (discussed below) in the 1990's brought a rapid expansion of knowledge regarding apoptosis, and with it came new treatments for many diseases as discussed below (Buytaert, Dewaele et al. 2007; Chowdhury and Lieberman 2008; deBruin and Medema 2008; Maker, Trisler et al. 2008; Sarfarez, Adhami et al. 2008; Khan, Adhami et al. 2010; Szlosaerk, Delage et al. 2010).

Advances in our understanding of apoptosis have already provided an ever-expanding arsenal against many diseases. Manipulating the molecular machinery of cell death has proven successful in treatment of several cancers, genetic disorders and neurodegenerative diseases. Disruptions in Bcl-2 family protein signaling are linked to the development of cancer and present a challenge to successful anti-cancer treatment strategies (Adams 2007). Bcl-2 antagonists have proven successful in the treatment of lymphoma and prostate cancer, especially in combination with apoptosis-inducing drugs (Mason, Vandenberg et al. 2008; Pro, Leber et al. 2008; Bray, Chen et al. 2009; Kang and Reynolds 2009; Tabrizi, Niuro et al. 2009). Crohn's disease, an inflammatory bowel disease marked by the proliferation of T-cells, is regularly treated with anti-TNF antibodies, which bind the TnF receptor of T-cells and induce apoptosis, regulating their proliferation and reducing symptoms (Mudter and Neurath 2007; Van den Brande, Koehler et al. 2007). Cardiovascular disease is marked by the progressive loss of cardiomyocytes by stress-induced apoptosis, which results in poor tissue health and eventual heart failure (Sosnovik, Nahrendorf et al. 2009). Treatment of cardiovascular

disease with anti-apoptosis drugs is cardioprotective, preventing stress-induced apoptosis in cardiomyocytes and maintaining heart tissue health, prolonging survival (Lee and Gustafsson 2009). The neurodegenerative disorders Alzheimer's and Parkinson's disease are both marked by excessive apoptosis of terminally differentiated neurons, causing eventual tissue failure and the subsequent symptoms of disease. Treatments focused on inhibiting apoptosis in affected tissues have proven successful in slowing progression of these diseases (Ho, Rideout et al. 2009; Sheng, Gong et al. 2009, Lukiw and Bazan 2010).

In the early 2000's a similar rapid expansion in the understanding of ATG proteins (discussed below) and their relation to autophagy led to another advance in the understanding of cell death and the recognition of the role of autophagy in pathogenesis (Bröker, Kruyt et al 2005; Levine and Deretic 2007; Maiuri, Zalckvar et al 2007; Kourtis and Tavernarakis 2008; Kroemer and Levine 2008; Mizushima, Levine et al. 2008; Groth-Pedersen and Jäätelä 2010). Another rapid expansion of knowledge regarding cell death is currently underway with the discovery of specific activation of pathways leading to necrosis (discussed below). New advances in the field of cell death will bring the development and refinement of targeted approaches to chemotherapy for diseases of all kinds, including anti-cancer and anti-viral drugs.

## **1.2 Types of Cell Death**

### **1.2.1 Necrosis**

Uncontrolled cell death resulting from overwhelming injury is termed necrosis. Necrosis has historically been considered an unregulated passive process, due to its low requirement for energy or synthesis of new proteins. However, an ordered series of

intracellular events typically occurs during necrotic death, implying regulation (Golstein and Kroemer 2007). Necrotic cell death is accompanied by distension of the mitochondria and endoplasmic reticulum, random degradation of nuclear DNA, and swelling of the cell until plasma membrane rupture (Syntichaki 2002, McCall 2010). Loss of plasma membrane integrity leads to rapid spillage of pro-inflammatory cellular contents into the intercellular space, damaging nearby viable cells and confounding the immune system's ability to clear the cell corpse (Fink 2005). Necrotic cells typically invoke an inflammatory response, which has been linked to the pathogenesis of several viruses (Li 2000). Early signs of necrosis include mitochondrial swelling and production of Reactive Oxygen Species (ROS), ATP depletion, failure of calcium homeostasis, perinuclear clustering of organelles and the activation of proteases including calpains and cathepsins. Later signs of necrosis include lysosomal rupture, plasma membrane rupture and spillage of the cell contents (Golstein and Kroemer 2007). Recent research in *C. elegans* and *Drosophila* have led to a model in which stresses promote increased cytoplasmic  $Ca^{2+}$ , which in turn activates existing proteolytic calpains. Calpain activation causes lysosomal rupture and the release of cathepsin proteases, rapidly inducing cell demise through uncontrolled digestion of cell proteins (Syntichaki 2002). Others have shown necrosis to be related to interactions of Fas-associated death domain (FADD) with tumor necrosis factor receptor 1 (TNF-R1), leading to tumor necrosis factor receptor type 1-associated death domain (TRADD)-induced activation of RIP1 and subsequent necrotic death (Festjens, Van den Berghe et al. 2006). Activation of Toll-like receptor (TLR) 3 and TLR4 also leads to necrotic cell death via activation of kinase receptor interacting protein 1 (RIP1) and subsequent necrosis. Necrosis has been linked

to the DNA damage response via JNK-mediated mitochondrial ROS production (Festjens, Van den Berghe et al. 2006). A common feature of necrosis signaling is the activation of kinase receptor interacting protein-1 (RIP1), and RIP1 deficient cells have deficiencies in cell necrosis (Holler, Zaru et al. 2000). Further investigation revealed that RIP1 signaling is crucial for programmed cell necrosis (Vandenebeebe 2010), and that RIP1-RIP3 complex formation promotes pro-necrosis signaling (Cho 2009). Interactions with proteins activated during apoptosis and autophagy may disrupt necrosis signaling. One example of this is the cleavage of RIP1 by apoptosis-related protease caspase-8 during early apoptosis, inhibiting pro-necrosis signaling and allowing apoptosis to proceed unhindered (Chan, Shisler et al. 2003, Galluzzi 2009). Necrosis has been observed in several systems after inhibition of apoptosis and/or autophagy during exposure of cells to different cell stressors (White 2008). This indicates that necrosis may be a default death triggered as a backup when apoptosis and autophagy are not available or correctly activated.

### **1.2.2 Apoptosis**

Apoptosis, or type I programmed cell death, is an integral part of the innate immune response to infection (Lee and Esteban 1994; Griffith, Brunner et al. 1995; Zhou, Paranjape et al. 1997), as well as an essential process for development and tissue homeostasis (Lockshin and Zakeri 2004; Penaloza, Lin et al. 2006). Death by apoptosis is mediated by the caspase family of cysteine proteases (discussed below). Once triggered, the caspase cascade is an irreversible and powerfully autocatalytic process that rapidly causes nuclear destruction, degradation of cell contents and release of signals to provoke uptake by macrophages (Elmore 2007; Taylor, Cullen et al. 2008). Apoptosis

plays a major role in the development and maintenance of tissues (Taylor, Cullen et al. 2008; Conradt 2009; Kato, Awasaki et al. 2009; Szabadkai and Duchon 2009; Snow, Pandiyan et al. 2010). It is also involved in the removal of potentially dangerous mutated cells (Letai 2008; Cotter 2009). Apoptosis is a prominent feature of organogenesis during embryonic development, and organisms with defects in apoptosis-related genes often die *in utero* (Galluzzi, Joza et al. 2008; Joza, Pospisilik et al. 2009; Zakeri and Lockshin 2009). Those that survive display extensive difficulties in maintaining appropriate levels of tissue growth leading to many problems including cancer (Debatin 2004; Henry-Mowatt 2004) and organ failure (Zhou 1997). Apoptosis is also a primary tool of T-killer cells that target virus-infected cells, activating apoptosis in the target cells (Shi, Kraut et al. 1992; Kashii, Giorda et al. 1999; Sato, Hida et al. 2001), and is responsible for much of the pathogenesis seen during influenza A (Brydon, Morris et al. 2005; Kash, Tumpey et al. 2006; Uprasertkul, Kitphati et al. 2007) and flavivirus infections (Kashii, Giorda et al. 1999; Solomon 2004; Quaresma, Barros et al. 2006). Apoptosis also serves a central role in the innate anti-viral response of some cells, killing infected cells before viral replication is maximized and thus preventing spread of the invading pathogen.

#### **1.2.2.1 Cellular morphology during apoptosis**

Apoptosis is characterized by blebbing of the cell membrane, degradation of nuclear chromatin, dysfunction of mitochondria with cytochrome c release, and activation of several proteases whose activity lead to cell fragmentation (Taatjes 2008). As fragmentation occurs, the cytosol, condensed chromatin and organelles are packaged into membrane-bound structures called apoptotic bodies, which are then engulfed by

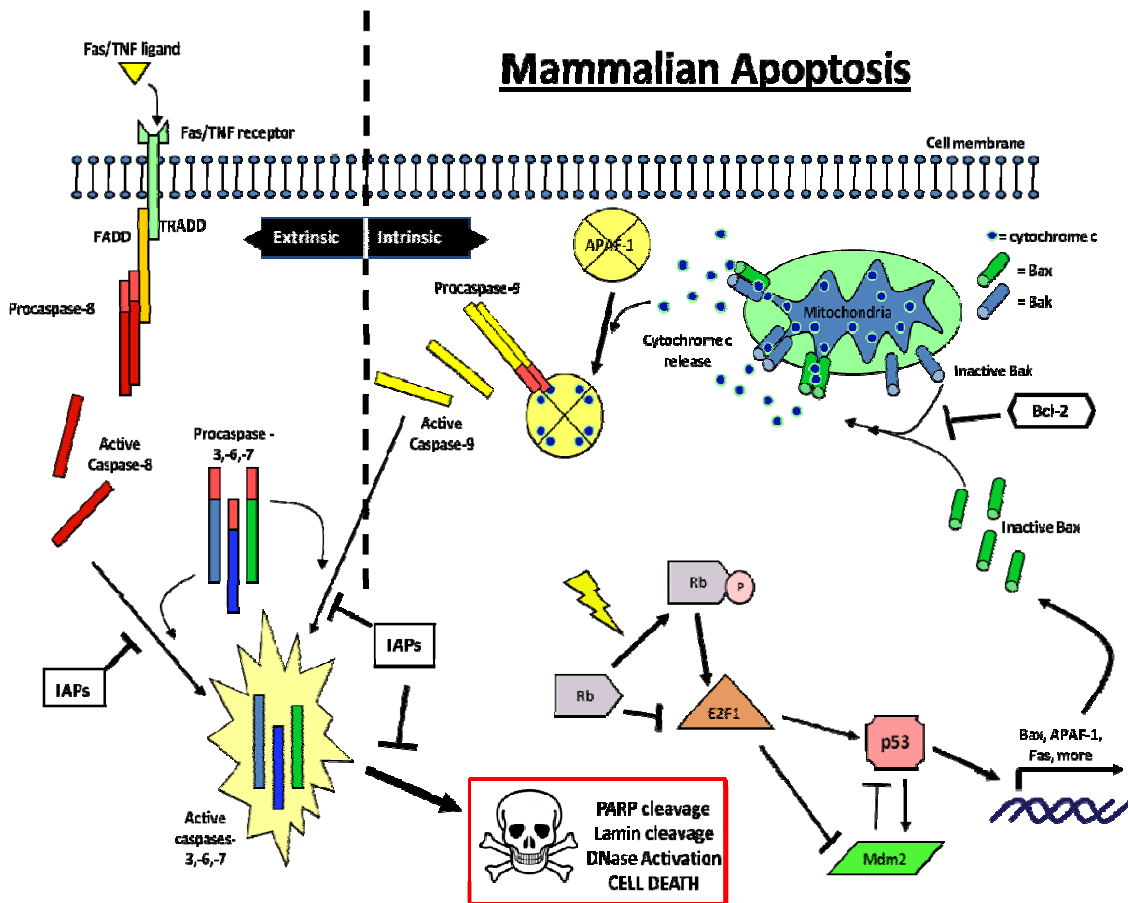
phagocytes (Kerr 1972). During early apoptosis phosphatidylserine, a phospholipid normally associated with the inner leaflet of the plasma membrane, becomes exposed on the outside of these cell fragments (Schutters 2010). The newly exposed phosphatidylserine molecules act as receptors for macrophages and dendritic cells, which then engulf the fragments and release several cytokines that inhibit inflammation, including IL-10 and TGF- $\beta$  (Fink 2005). At the later stages of apoptosis, nuclear chromatin condenses, followed by nuclear fragmentation, which is easily observed and quantified using fluorescent Hoechst staining. Powerful endonucleases activated during apoptosis cleave DNA between histones, producing different sized fragments that form a characteristic ladder pattern under DNA gel electrophoresis. The cleaved DNA can also be observed *in situ* using TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) staining (Loo 2011).

Several proteases are activated during apoptosis that carry out cell execution, including calpains, cathepsins and caspases (Squier 1994; Altnauer 2004). Protease activation results in actin depolymerization and the cleavage of several cytoskeletal elements, causing the dying cell to lose its shape, detach from its neighbors, and subsequently shrink as the cytoplasmic contents are digested (Breere 2005, Doonan 2008). Cytoplasmic blebbing gives apoptotic cells a characteristic morphology that differs from healthy cells or cells dying by autophagy or necrosis.

#### **1.2.2.2 Molecular Mechanisms Leading to Apoptosis**

Apoptosis may be triggered intrinsically by several intracellular signals, including DNA damage (Roos and Kaina 2006), nutrient deprivation (Yang, Yang et al. 2007), increased intracellular reactive oxygen species (ROS's) (Perez-Galan, Roue et al. 2006),

excessive protein misfolding (Soto and Estrada 2008), hypoxia (Zhu, Chen et al. 2006) and heat shock (Hardwick 2001) via the intrinsic (mitochondria-mediated) apoptosis pathway (discussed below). It can also be induced by extrinsic signals through the activity of specific death receptors on the cell surface (Green 1997) via the extrinsic (receptor-mediated) apoptosis pathway (discussed below). Whether initiated intrinsically



**Diagram 3. Mammalian apoptosis.** Cell death by apoptosis is initiated by two pathways: extrinsic, receptor mediated and intrinsic, mitochondrial mediated signaling. Each involves specific cellular proteins to activate initiator caspases (discussed in text). Both extrinsic and intrinsic pathways converge on executioner caspases -3, -6, and -7, which kill the cell by stimulating proteolysis and chromatin degradation. For further discussion and references, see text.

or extrinsically, apoptosis is mediated primarily by the caspase (cysteine-aspartic-acid-protease) family of cysteine proteases (Huai 2010) (See Diagram 3).

### **1.2.2.3 Caspases**

To date, eleven caspases have been described in humans, each with specific activity and substrate specificity (Lavrik 2005, Taylor 2008). Caspases are constitutively expressed in healthy cells as inactive precursor procaspases, which are cleaved upon activation to expose their enzymatic domain. Post-translational regulation of caspases allows the rapid induction of apoptosis upon receipt of death signals (Rossi 2003, Huai 2010). The several caspases involved in apoptosis are categorized into two groups: initiator and executioner caspases. Initiator caspases, such as caspase-2, caspase-8, caspase-9, and caspase-10, have a large prodomain region containing either a caspase recruitment domain (CARD) or a death effector domain (DED), which allow their interaction with various death signals in the cell. Once death stimuli are received initiator caspases aggregate, bringing their proteolytic domains in close association with each other. This allows the activation of initiator caspases (Fuentes-Prior 2004), which then go on to cleave the prodomain of executioner caspases, caspase-3 and caspase-7, via interactions between the specific death domains of each. Once activated, executioner caspases rapidly destroy cytosolic proteins and organelles while simultaneously activating endonucleases that cleave genomic DNA (Lavrik 2005). Executioner caspases also target naïve initiator caspases, causing a rapid upregulation of caspase activity through the positive feedback cascade that ensues (Hardwick 2001, Doonan 2008, Taylor 2008). Most intrinsic and extrinsic apoptosis triggers converge on caspase-3, an

executioner caspase that delivers much of the damage during the cell's programmed demise (Lakhani 2006, Huai 2010).

#### **1.2.2.4 The Intrinsic (Mitochondria-mediated) Pathway to Apoptosis**

Many intracellular apoptosis-inducing stimuli signal through the mitochondrial pathway, where cytochrome c release from the inner mitochondrial matrix to the cytosol causes the activation of caspase-9 (Henry-Mowatt 2004, Guerrero 2008). This is achieved by the formation of the apoptosome complex, primarily composed of apoptotic protease activating factor 1 (APAF-1), forming a ring-like structure that is activated by cytochrome c binding. Upon activation, APAF-1 forms a scaffold to which procaspase-9 binds and forms dimers. The aggregation of procaspase-9 dimers at the apoptosome allows their cleavage and activation (Peng, Nijhawan et al. 1997, Bratton 2010). As an initiator caspase, caspase-9 then cleaves several executioner caspases, triggering the caspase cascade.

Cytosolic cytochrome c is a potent inducer of apoptosis (Scorrano 2010). Mitochondrial membrane permeabilization, or MMP, allows the translocation of cytochrome c and other important pro-apoptotic stimuli from the inner mitochondrial space to the cytosol. It also diminishes mitochondrial membrane potential causing mitochondrial dysfunction and failure. MMP is induced through both intrinsic and extrinsic signals and two distinct mechanisms for the MMP have been identified. In many dying cells a Permeability Transition Pore (PTP) forms in the inner membrane of the mitochondria when pro-apoptotic stimuli are received. The mitochondrial matrix then swells until the membrane ruptures, leaking cytochrome c and other apoptotic stimuli from the inner mitochondrial space to the cytosol. This is accompanied by a rapid loss of

mitochondrial membrane potential, effectively shutting down mitochondrial function (Breckenridge 2004, Tait 2010). In other dying cells a different mechanism is responsible, allowing cytochrome c release without the loss of mitochondrial membrane potential, regulated by the Bcl-2 family proteins through the formation of channels in the mitochondrial membrane (Hardwick 2003).

The Bcl-2 protein family is subdivided by the number of homology domains found in each protein. The BH3-only proteins, containing only one homology domain, include Bid and Bim. Apoptotic stimuli converge on these BH3-only proteins, activating them by cleavage. Upon activation, the BH3-only protein BID is cleaved to form tBID, which activates pro-apoptosis Bcl-2 members including Bax and Bak through interaction with their homology domains. In healthy cells, Bak is constitutively associated with the mitochondrial membrane, and is held in an inactive state by its association with another mitochondrial membrane protein, VDAC-2. Bax is a cytosolic protein, and is held inactive near the nucleus through its interaction with cytosolic Ku-70 and humanin peptides (Cheng 2003; George, Targy et al. 2010). Activated BH3-only proteins such as tBID activate Bax and Bak by inducing allosteric conformational changes upon interaction with their homology domains. The resulting conformational change causes Bax and Bak to break from their inhibitory chaperones. Bax and Bak form both homodimers and heterodimers with each other. Upon dimer formation, these complexes translocate to the outer mitochondrial membrane. This interaction forms large pores in the mitochondrial membrane that facilitate MMP and cytochrome c release. This model is supported by experiments in which Bax, along with tBID, was shown to induce the formation of channels in reconstituted liposomes through which dextrans of up to 2kDa

could pass (Eskes 2000). Bax also interacts with VDAC in the outer mitochondrial membrane, and ANT in the inner mitochondrial membrane to facilitate VDAC/ANT channel formation and cytochrome c release (Shimizu 2000). Several lines of evidence now indicate that Bak and Bax are also constituents of the putative cytochrome c release channel MAC in the outer mitochondrial membrane. The MAC channel is a high conductance, voltage independent channel that forms upon translocation of Bax to the outer mitochondrial membrane. MAC formation corresponds with the release of cytochrome c (Dejean 2005).

The channels formed by VDAC/ANT and Bax/Bak (putative MAC) in the outer mitochondrial membrane facilitate cytochrome c translocation from the inner mitochondrial space to the cytosol, where it binds with APAF-1 to form the apoptosome. Apoptosome formation facilitates the apoptosis by interacting with procaspase-9 and cleaving it to form activated caspase-9, which then activates several executioner caspases including caspase-3 that carry out cell execution. Apoptosis Initiating Factor (AIF) and EndoG, both endonucleases, are also released from the mitochondria upon MMP, and translocate to the nucleus where they cause DNA fragmentation and chromosome condensation. The inner mitochondrial protein Smac/DIABLO is also released from the mitochondria during MMP. It binds to Inhibitor of Apoptosis Proteins (IAP's), which normally inhibit caspase activity in healthy cells. Upon binding to IAP, Smac/DIABLO releases caspases from IAP inhibition. Activated caspases then kill the cell by means of their proteolytic activity (Fink 2005).

Aside from their involvement in cytochrome c release and mitochondrial-mediated apoptosis, Bax and Bak also play a prominent role in mitochondrial morphology through

their interactions with other mitochondrial proteins. Bax activity upregulates mitochondrial fusion in healthy cells by activating the assembly of GTPase mitofusin-2 (mfn2) (Karbowski, Norris et al. 2006). During apoptosis, mfn2 colocalizes with Bax and Bak in distinct foci at sites of mitochondrial fusion (Neuspiel, Zunino et al. 2005; Karbowski, Norris et al. 2006). Mitochondrial fusion in turn inhibits apoptosis (Sugioka, Shimizu et al. 2004; Perfettini, Roumier et al. 2005) and reduces mitochondrial susceptibility to depolarization (Neuspiel, Zunino et al. 2005). Overexpression of mfn2 inhibits apoptosis, presumably by increasing the rate of mitochondrial fusion (Lee, Jeong et al. 2004).

Bax/Bak mediated outer mitochondrial membrane permeabilization (OMP) also allows the protein translocase of inner mitochondrial membrane 8 (TIMM8a) to be released from the mitochondria to the cytosol, where it interacts with dynamin-like GTPase Dynamin-related protein-1 (Drp1) (Wasiak, Zunino et al. 2007). This causes Drp1 redistribution and subsequent Drp1-mediated mitochondrial fission, resulting in fragmented mitochondria, often resulting in mitochondrial failure and apoptosis (Arnoult, Rismanchi et al. 2005; Wasiak, Zunino et al. 2007). While a detailed mechanism of the interaction between Bax, Bak, mfn2, drp1 and other proteins is still lacking, Bax and Bak play a prominent role in both mitochondrial morphology and function. Perturbations in related signaling pathways are therefore likely to affect the ability of the cell to regulate mitochondrial dynamics and cell death during viral infection.

#### **1.2.2.5 The Extrinsic (Receptor-mediated) Pathway to Apoptosis**

Apoptosis can be induced by extracellular factors through several death receptors on the cell surface. The cell death inducing activity of T-killer cells relies on the

extrinsic pathway of cell death to remove dangerous or pathogenic cells. Extrinsic induction of apoptosis occurs primarily via ligand binding of the transmembrane death receptors Fas and Tumor Necrosis Factor Receptor (TNFR) (Ashkanazi 2008). In both cases ligand binding causes receptor trimerization, which brings together the death domains found in their cytoplasmic tails. These death domains interact with other death domain-containing cytoplasmic proteins, including FADD, TRADD and RIP (Albeck 2008). Fas is a death receptor expressed primarily on the surface of activated T cells and interacts directly with FADD upon ligand binding to cause the death of targeted cells. FADD contains a death effector domain that, upon dimerization with Fas, interacts with procaspase-8 and causes its autoproteolytic cleavage (Hymowitz 2010). Activated caspase-8 initiates the caspase cascade in part by cleaving BID into tBID, which then activates the pro-apoptotic mitochondrial proteins Bax and Bak (Fink 2005). TNFR activates caspase-8 as well, but recruits FADD indirectly through its interaction with TRADD (Cao 2010). As described above, the mitochondria are key regulators of cell death and release of their contents to the cytoplasm is a critical trigger of apoptosis. By regulating this process, Bax and Bak also regulate induction of apoptosis from both intrinsic and extrinsic death signals through the mitochondrial pathway (Alvarado-Kristensson 2004).

### **1.2.3 Autophagy**

Autophagy is a normal process in healthy cells by which cellular organelles and proteins are isolated and degraded (Kroemer and Levine 2008). Constitutive low levels of autophagy recycle cell contents by organelle and bulk protein turnover (Lockshin and Zakeri 2004; Komatsu, Wang et al. 2007). Autophagy is upregulated in response to

stress including nutrient deficiency (Soto, Tsuchihara et al. 2007), growth factor withdrawal (Li, Capan et al. 2006) and ER stress (Ogata, Hino et al. 2006; Yorimitsu, Nair et al. 2006). Autophagy is also a key component of the innate immune response to pathogens including viral infection (Levine 2005; Levine and Deretic 2007; Mizushima, Levine et al. 2008). Perturbations in autophagy signaling are related to certain cancers and several neurodegenerative diseases (Shintani and Klionsky 2004; Levine and Kroemer 2008).

Autophagy is characterized by an increase in cytoplasmic vacuolarization and the formation of distinct double-membrane structures called autophagosomes. During autophagy, the cytoplasm that contains the organelles, proteins or invading pathogens to be degraded are first surrounded by an isolation membrane that encloses the material to form the double-membraned autophagosome (Mizushima, Ohsumi et al. 2002). Completely formed autophagosomes containing cytoplasmic cargo then fuse with endosomes to become amphisomes. Amphisomes fuse with lysosomes to form autolysosomes where lysosomal hydrolases degrade the contents of each vesicle. As discussed below, the formation of autophagic vacuoles is tightly regulated (Kabeya, Mizushima et al. 2000; Tanida, Ueno et al. 2004; Bampton, Goemans et al. 2005).

Excessive autophagy and the associated autophagosome-mediated degradation of cellular components is the cause of autophagic cell death. During autophagic cell death, the dying cell continues to accumulate autophagic vesicles until the cell contents are almost completely digested at which time the cell, exhausted of its internal resources, dies. Dying and dead cells may be phagocytosed by neighboring cells, completing their destruction and recycling the components of dead cells (Fink 2005).

### **1.2.3.1 Regulation of Autophagosome Formation**

During autophagy, portions of the cytosol, damaged organelles or invading pathogens are first sequestered in an isolation membrane (also called a phagophore or preautophagosome). Initiation of this process is largely controlled by the serine/threonine kinase mammalian target of Rapamycin (mTOR) (Ravikumar, Vacher et al. 2004) and the class I and class III phosphoinositide 3-kinases (PI3Ks) (Levine and Deretic 2007, Yu 2010). During periods of stress a complex of Beclin-1 and the class III PI3K VSP34 is formed that activates downstream ATG signaling. ATG signaling then leads to two equally important ubiquitin-like events in the formation of mature autophagosomes: ATG12 conjugation and LC3 modification (discussed below). The presumptive autophagosomal membrane becomes enlarged by addition of new membrane (the origin of which remains unclear), and eventually surrounds the cytosol, organelles or pathogens targeted for digestion, sealing it into a double-membraned autophagosome (Itakura 2010). This process is controlled by the autophagy-related-genes (ATG) family of proteins. Initiation of isolation membrane formation is induced by ATG5 insertion into the presumptive autophagosomal membrane. Targeting of additional ATG proteins to these membranes is accomplished through a series of ubiquitin-like interactions between several members of the ATG family (Hanada, Noda et al. 2007, Mari 2010). The process is initiated when ATG7, an E1-activating enzyme, interacts with ATG12. ATG7 transfers ATG12 to the E2-conjugating enzyme ATG10. This ATG10-ATG12 intermediate then presents ATG12 to ATG5 for conjugation. The newly formed ATG5-ATG12 conjugate is stabilized non-covalently by ATG16, and stimulates ATG5 oligomerization on the outside membrane of the growing autophagosome (Jounai,

Takeshita et al. 2007; Levine and Deretic 2007; Levine and Kroemer 2008). The insertion of ATG5 into the outer autophagosomal membrane facilitates LC3II insertion and completion of autophagosome formation (Fujita, Itoh et al. 2008, Wrighton 2010).

As construction of the autophagosomal membrane is initiated by ATG5, a second ubiquitin-like pathway, LC3 (ATG8) modification, is triggered that completes the formation of a mature double-membraned autophagosome (Fujita, Itoh et al. 2008). LC3 is the mammalian ortholog of yeast ATG8 (Tanida, Ueno et al. 2004). The inactive form of LC3, ProLC3, is constitutively expressed in cells. Upon expression, most ProLC3 is cleaved to LC3I, which localizes to the cytosol. Once activated, ATG7 interacts with LC3I and transfers it to ATG3, which facilitates the insertion of LC3I into the autophagosomal membrane, generating LC3II (the lipidated form of LC3I) (Kabeya, Mizushima et al. 2000). Insertion of LC3 into the autophagosomal membrane facilitates the closure of the isolation membrane to form a fully mature autophagosome (Tanida, Ueno et al. 2004). Upon closure of the autophagosome, both the ATG5-ATG10-ATG16 complex and the LC3II on the outer membrane are delipidated by ATG4 and subsequently recycled. The LC3II on the inner membrane persists until it is degraded by lysosomal proteases along with the inner autophagosomal membrane following autophagosome fusion with lysosomes (Kabeya, Mizushima et al. 2004; Tanida, Uero et al. 2004).

### **1.2.3.2 Autophagy As a Consequence of ER Stress**

Several viruses, including the flaviviruses, use host endoplasmic reticulum (ER) membranes for replication. The ER is an extensive membranous network within eukaryotic cells that is the principal site of synthesis, folding and posttranslational

modification of proteins. The ER lumen is a highly oxidative environment, a critical feature for the formation of disulfide bonds between amino acids and proper protein folding (Malhotra and Kaufmann 2007). In addition, the ER is also a major site of lipid biogenesis, regulating steroid, cholesterol and other lipid production (Zhang and Kaufman 2008). The ER is dependent upon the activity of several calcium-dependent molecular chaperones including Grp78 (Li, Ni et al. 2008), Grp94 (Mao, Wang et al. 2010) and calreticulin (Michalak, Groenendyk et al. 2009), which promote proper protein folding by stabilizing protein folding intermediates (Jonikas, Collins et al. 2009). The ER is highly sensitive to imbalances in cell homeostasis, excessive protein production, accumulation of mutant proteins, loss of calcium homeostasis and inhibition of N-linked glycosylation. These stresses within the ER often lead to its malfunction, collectively called ER stress (Todd, Lee et al. 2008). Therefore, as virus replication proceeds during infection, accumulated virus proteins and aberrant membranes of the ER trigger ER stress which often leads to autophagy (discussed below).

During ER stress, resident ER chaperone proteins become occupied and release transmembrane ER signaling proteins as they interact with and stabilize protein folding intermediates (discussed below) (Hosokawa 2008). Once released, these proteins trigger an evolutionarily conserved means of alleviating ER stress called the Unfolded Protein Response (UPR) (Ron and Walter 2007). The UPR has three main signaling pathways that are triggered as misfolded proteins accumulate in the ER during stress. This accumulation occupies resident ER chaperone proteins, causing the release of ER transmembrane signal transducers RNA-dependent Protein kinase-like ER kinase (PERK), Inositol-required enzyme 1 (Ire1) and Activating transcription factor 6 (ATF6)

(Wolfson, May et al. 2008). Liberated IRE1, PERK and ATF6 stimulate the transcription of specific genes that enhance protein folding capacity, promote ER-associated protein degradation, and temporarily degrade mRNA to reduce synthesis of new proteins (Wu and Kaufman 2006).

Importantly, if the initial UPR response is insufficient to relieve the cell of aberrant protein accumulation, one of the many functions of PERK, IRE1 and ATF6 release is to upregulate autophagy, enlisting the destructive power of autophagosomes to assist in the degradation of misfolded proteins and damaged organelles during periods of ER stress (Bernales, McDonald et al. 2006; Yorimitsu, Nair et al. 2006), while simultaneously promoting pro-survival signaling (Ogata, Hino et al. 2006).

The ER lumen contains the highest levels of calcium in the cell due to  $\text{Ca}^{2+}$ -ATPase mediated active transport of calcium ions (Høyer-Hansen and Jäätelä 2007). Although not a UPR element but rather a symptom of ER stress, aberrant intracellular  $\text{Ca}^{2+}$  levels also upregulate autophagy. Calcium transport channels at the ER membrane pump calcium ions into the ER lumen (Høyer-Hansen and Jäätelä 2007) rendering the ER the chief intracellular  $\text{Ca}^{2+}$  storage compartment. One of the consequences of ER stress is the release of sequestered calcium to the cytoplasm. Calcium-activated calmodulin-dependent kinase kinase- $\beta$  (CaMKK $\beta$ ) is stimulated by the increase in cytosolic calcium and activates AMP activated protein kinase (AMPK). AMPK inhibits the mTORC1 complex which in turn negatively regulates autophagy (Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). As stated above, downregulation of mTORC1 is sufficient to stimulate autophagy.

Thus the UPR relieves ER stress by inducing several intracellular biochemical pathways that promote the proper folding of proteins while degrading those proteins that are mutated or rendered unreparable by aberrant synthesis and folding. A common feature of the signaling pathways used for this process is the recruitment of autophagosomes to assist in degradation when these pathways are overwhelmed. While much focus has been paid to the UPR as the key mediator of this process, recent research has revealed a UPR-independent, Ataxia-Telangiectasia-mutated (ATM)-mediated pathway that serves a similar purpose.

### **1.2.3.3 Ataxia-Telangiectasia Mutated (ATM): A Link from ER Stress to Autophagy**

Ataxia-Telangiectasia is a severe, autosomal recessive genetic disorder characterized by a broad range of symptoms that include small stature, hypogonadism (Stanovic, Kidd et al. 1998), progressive neuronal degeneration (Shi, Venkataraman et al. 2004; Biton, Dar et al. 2006), severe immunodeficiency (Xu, Ashley et al. 1996), insulin resistance (Yang and Kastan 2000), a predisposition to cancer (Stanovic, Kidd et al. 1998; Meyn 2001; Shiloh and Kastan 2001; DiTullio, Mochan et al. 2002; Renwick, Thompson et al. 2006) and premature aging (Wong, Maser et al. 2003) in both human patients and in mouse models of ataxia-Telangiectasia.

The mutation causing this disorder has been mapped to human chromosome 11 (Bullrich, Rasio et al. 1999), and is appropriately named Ataxia Telangiectasia-Mutated (ATM). ATM is a 370 kDa (Lavin 1999) protein the expression of which is conserved from yeast to humans (Brush, Morrow et al. 1996; Sanchez, Desany et al. 1996; Aoki, Sato et al. 2000; Takata, Kanoh et al. 2004; Pusapati, Rounbehler et al. 2006), indicating its essential role in cellular homeostasis. ATM mutations result in increased oxidative

stress (Sasaki, Ikeda et al. 2008; Yan, Shen et al. 2008; Yoshida, Shiojima et al. 2009; Alexander, Cai et al. 2010), increased ER stress (He, Kim et al. 2009; Schleicher, Moretti et al. 2010), defective apoptosis (Callen, Jankovic et al. 2009) and deregulated cell cycle control (Tanaka, Halicka et al. 2007; Lim, Jocelyn et al. 2009). These lead to chromosomal instability (Teoh, Dan et al. 2008), telomere end-to-end fusions (Dimitrova and de Lange 2009), cell cycle checkpoint defects occurring at G1/S and G2/M (Lavin and Kozlov 2007; Yong, Bao et al. 2007; Hitomi, Yang et al. 2008), as well as cytoskeletal and plasma membrane defects (Kastan and Lim 2000). Those with a family history of AT are at increased risk for B-cell lymphocytic leukemia (the most common adult leukemia) (Bullrich, Rasio et al. 1999; Austen, Skowronska et al. 2007) and breast cancer (Renwick, Thompson et al. 2006) due to heterozygosity for the causative mutation (discussed below). ATM is a Class IV phosphatidyl inositol 3-kinase (PI3K), grouped with mTOR, ATR and DNA-PK (Foster, Traer et al. 2003). ATM-like proteins in all organisms are similar in size and all contain a C-terminal PI3K catalytic domain. Once activated, ATM autophosphorylates (Bakkenist and Kastan 2003) and subsequently phosphorylates a variety of intracellular substrates, allowing the coordination of a broad range of biochemical responses.

Until recently, research regarding ATM has focused specifically on its activity as a nuclear stress sensor, as it is activated after double-stranded DNA breaks and functions to induce p53-dependent cell cycle arrest, allowing DNA repair enzymes the time needed for their work (Bishop, Hollander et al. 2003). Mechanistically, double-strand DNA breaks activate ATM, which activates p53 by inducing phosphorylation on p53 at Ser15 and Ser20 (Zheng, Li et al. 2005). ATM directly phosphorylates p53 at Ser15. ATM

also activates CHK2 by phosphorylation at T69, and activated CHK2 in turn phosphorylates p53 at Ser20 (Cao, Kim et al. 2006). Activated p53 then upregulates p21<sup>WAF</sup> expression (Xu, Yang et al. 1998), which in turn downregulates Cyclin E expression leading to cell cycle arrest at G1/S (Barlow, Brown et al. 1997).

While the nuclear stress sensing activities of ATM are clearly established, its activity as a cellular metabolic stress sensor has only recently been uncovered. Increased numbers of lysosomes are a common cellular feature of AT; high levels of trophic factors are required for *in vitro* growth of cells from AT patients (Vinięra 2004); and extended incubation times with death stimuli are required to kill AT cells *in vitro* (Alexander, Cai et al. 2010). These reports suggest that deregulation of autophagy may also be a critical determinant of pathology associated with ATM mutations. Recently direct evidence of ATM-dependent upregulation of autophagy due to cytoplasmic stress been reported. In response to increased ROS production due to ER stress, ATM has been shown to mediate the downregulation of mTOR activity both by direct phosphorylation and through activation of TSC2, which in turn phosphorylates mTOR further. This double punch to mTOR activity effectively induces the derepression of autophagy signaling and allows the upregulation of autophagosome formation (Alexsander, Cai et al. 2010). ATM also upregulates AKT activity, which in turn inhibits pro-apoptosis BAD signaling allowing cell survival while cytoplasmic stress is relieved by autophagosomal degradation of aberrant cellular structures (Vinięra 2004). ATM therefore functions as an intracellular stress sensor, and its activation by either nuclear or cytoplasmic stresses causes cell cycle arrest, upregulation of major antioxidants (including superoxide dismutase, catalase,

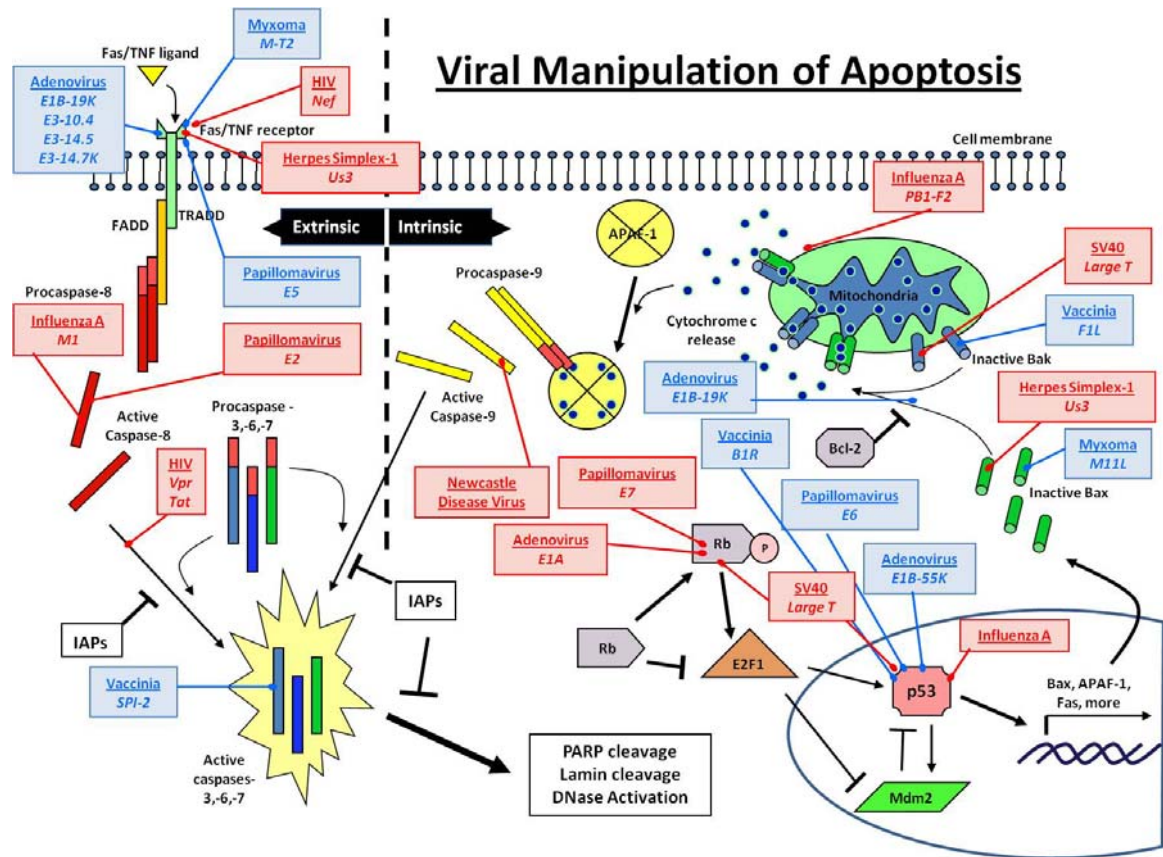
glutathione reductase and glutathione peroxidase (Biton, Dar et al. 2006) and the upregulation of pro-survival (anti-apoptosis) signaling among other effects.

### **1.3 Viral Manipulation of Cell Death**

The success of any viral infection can be measured by the number of infectious progeny produced from an infected cell. Several redundant anti-viral pathways have evolved in eukaryotic cells that trigger host cell death in an effort to minimize tissue damage due to viral infection while quickly clearing the invading pathogen. Redundancy of anti-viral signaling pathways in eukaryotic cells makes it nearly impossible for an invading virus to go unnoticed, and the evolution of anti-viral innate immunity in host tissues has necessitated a concurrent evolution in viral strategies to evade these mechanisms. A primary aim of this thesis is to demonstrate that two different RNA virus families, Influenza A (family Orthomyxoviridae) and Dengue-2 (family Flaviviridae), utilize particular aspects of the cell death machinery during infection to maximize virus replication.

While manipulating components of cell death pathways is not unique to these viruses, the methods of cell death regulation during infection are specific to each virus type and in many cases also cell-type dependent. Regardless of virus or cell type, viral manipulation of cell death is often exceedingly complex, as virus-host interactions determine the outcome at both cell and tissue levels (Irusta 2003). Consequences of viral infection vary by virus and range from rapid killing of the cell to complete blockage of host-induced cell death (See Diagram 4) (Casella 1999; Roulston 1999; Hardwick 2001; Kumar, Delmolino et al. 2002; Wurzer 2003; Hilleman 2004; Wurzer 2004, Ehrhardt et al. 2004; Aoyagi, Zhai et al. 2007). As many viruses depend on their ability to

manipulate cell death-related proteins to replicate, assemble and/or escape from the cell, targeting these pathways within infected cells presents a novel strategy for development of anti-viral therapeutics.



**Diagram 4. Viral Manipulation of Apoptosis.** Several viruses have evolved strategies for manipulating the cell death program during infection, by either inducing or inhibiting the activity of key proteins along the apoptosis biochemical pathway. Red = apoptosis induction, blue = apoptosis inhibition. For further discussion and references, see text.

Whether inducing cell death or inhibiting it, many viruses have evolved strategies to manipulate cell death-related biochemistry to avoid immune detection and maximize virus replication. Viral induction of cell death aids in dissemination of some viruses through cell lysis (Roulston 1999) and also allows other viruses to provoke the destruction of immune-specific cells, lowering the host's ability to clear the invading

pathogen (Casella 1999). Still others, including Influenza A, take advantage of specific cellular proteases upregulated during the death response to further their maturation (Wurzer 2003; Wurzer, Ehrhardt et al. 2004).

Virus targets for inducing cell death vary and include p53 (Forte and Luftig 2009), mitochondrial cytochrome c release (McLean, Datan et al. 2009), and increased extrinsic cell death signaling through increased Fas and TNFR expression (Gasper-Smith, Crossman et al. 2008). Induction of cell death can also be facilitated through direct caspase activation or indirectly by disrupting intracellular caspase inhibitors (IAPs) (Clarke and Tyler 2009). Induction of membrane instability (Salsman, Top et al. 2005), syncytium formation (Guo, Shen et al, 2009), or host interferon-mediated immune responses also stimulate death in infected cells (Fraietta, Mueller et al. 2010). Influenza A is one example of a virus that induces apoptosis following infection, and is discussed below.

In contrast to those that induce cell killing, many viruses strongly inhibit cell death, and some of the most effective caspases inhibitors are viral in origin. This inhibition allows more time for viral replication before the death of their host cell, maximizing viral propagation (Kumar, Delmolino et al. 2002). Other viruses, particularly those without their own replicative machinery, accidentally engage the death response as a side effect of cell cycle inhibition. As a result, many of these concurrently encode proteins that specifically inhibit death signaling, avoiding the cell death response that typically accompanies cell cycle inhibition.

Viruses are able to inhibit or delay cell death directly by suppressing cell death proteases, preventing release of cytochrome c from the mitochondria, or by inhibition of p53-mediated apoptosis. Manipulation of cell death receptors like Fas and TNFR by viral proteins through competitive binding, receptor mimicry, and transcriptional downregulation also aid in enforced cellular survival. Many viruses inhibit the death response by encoding proteins that specifically target intracellular death components for inactivation by competitive binding, increased turnover by ubiquitination, or downregulation of cellular death protein transcription (discussed further below). This typically allows more time for viral replication before host death.

Virus-induced inhibition of cell death also shields infected cells from killing by both innate and adaptive immune mechanisms, allowing viral replication to continue despite the host's attempts to clear the virus (DeFilippis, Goodwin et al. 2003). Some viruses avoid the host immune response to infected cells primarily by downregulating or competitively binding extrinsic death receptors, including Fas and TNFR (Ray, Black et al. 1992; Dobbelstein and Shenk 1996). Many viruses employ a combination of these strategies, encoding several proteins designed to maximize viral replication while avoiding the immune consequences of infection (Ray, Balck et al. 1992; Mizushima, Levine et al. 2008). Some viruses, including flaviviruses (discussed further below), provide protection only to certain cell types following infection. This allows virus-induced destruction of immune responders while simultaneously inhibiting death in cells playing host to virus replication.

The life or death consequences of infection vary both by virus and by cell type, ranging from rapid killing of host cells to complete blockage of host-induced cell death (Hilleman 2004). Thus viruses appear to be able to intervene at all steps of eukaryotic cell death, either to prevent death or to provoke it. Viral manipulation of these pathways is often essential to the effective propagation and dissemination of infectious progeny (Hardwick 2001; Irusta 2003). By unraveling the specific details surrounding virus-host interactions, we may reveal novel therapeutic targets and aid in improved antiviral and vaccine design, as well as gain a clearer insight into the complex machinery of eukaryotic cell death. In this thesis, we present results from the study of two unrelated RNA virus families, Influenza A and the flaviviruses, focusing on their differing interactions with mammalian apoptosis and autophagy pathways. We demonstrate that both Influenza A and Flaviviruses engage the host cell death machinery to maximize virus replication. As Influenza A induces apoptosis while Flaviviruses suppress it, the specific mechanisms engaged by each virus are very different. We identify specific viral and cellular components governing replication-enhancing interactions with host cell death pathways for both virus families, detailed in Chapters One and Two of this thesis.

## **2 Materials and Methods**

### **2.1 Cell Culture**

#### **2.1.1 Madin Darby Canine Kidney (MDCK) epithelial cells.**

MDCK cells (catalog no. CCL-34, ATTC) are immortalized, adherent canine kidney epithelial cells that were originally isolated by S.H. Madin and N.B. Darby from a female cocker spaniel in 1958. MDCK cells support the replication of a wide range of animal viruses. These cells were kindly provided by Dr. Anastasia Gregoriades of Queens College, New York City.

MDCK cells were cultured and maintained in Dulbecco's modified Eagle medium® (DMEM) (catalog no. 12800-017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSFBS-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®; catalog no. 15140-122; Gibco) and incubated at 37°C under a humidified 5% CO<sub>2</sub> atmosphere.

MDCK cells were passaged 1:10 every 3-4 days by aspirating old media, washing once with 1x phosphate-buffered saline (PBS) (see reagents section for formula) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with trypsin solution at 37°C until they began to detach from the bottom of the culture dish. Warmed DMEM® was added to neutralize trypsin activity and cells were collected and centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in warmed DMEM®.

### **2.1.2 Vero African Green Monkey Kidney Fibroblast cells**

The Vero cell line was first isolated from the kidney of a normal adult African green monkey in 1962 by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan. Vero cells are permissive to infection by a variety of viruses, and are widely used in virology and vaccine development research. Vero cells were purchased from the ATCC (catalog no. CCL-81; ATCC).

Vero cells were maintained in DMEM® (catalog no. 12800- 017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LFSB-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®, catalog no. 15140-122; Gibco) and incubated at 37°C under a humidified 5% CO<sub>2</sub> atmosphere.

Vero cells were passaged 1:10 every 2-3 days by aspirating old media, washing once with 1x phosphate-buffered saline (PBS) (see reagents section) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with trypsin solution at 37°C until they began to detach from the bottom of the culture dish. Warmed DMEM® was added to neutralize trypsin activity and cells were collected and centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in warmed DMEM®.

### **2.1.3 293T Human Kidney cells**

293T cells are derived from HEK (Human Embryonic Kidney) cells and were originally isolated from a healthy aborted human fetus by Alex Van der Eb of the Netherlands in 1972 and transformed with adenovirus by another member of Van der Eb's lab, Frank Graham. The HEK cell line was then transformed with SV40 large T antigen, creating the 293T cell line. The name comes from the fact that this was Graham's 293<sup>rd</sup> experiment, and that the T antigen was added to transform the cells. 293T cells were a generous gift of Adolfo Garcia-Sastre, Mt. Sinai Medical School, New York City.

293T cells were maintained in DMEM® (catalog no. 12800-017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSFBS-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®, catalog no. 15140-122; Gibco) and incubated at 37°C under a humidified 5% CO<sub>2</sub> atmosphere.

293T cells were passaged 1:10 every 3-4 days by aspirating old media, washing once with 1x phosphate-buffered saline (PBS) (see reagents section) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with trypsin solution at 37°C until they began to detach from the bottom of the culture dish. Warmed DMEM® was added to neutralize trypsin activity and cells were collected and centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in warmed DMEM®.

#### **2.1.4 HeLa Human Cervical Carcinoma cells**

HeLa cells were originally isolated from the epitheloid cervix carcinoma of 31 year old in 1951. The name HeLa is derived from the patient's name, Henrietta Lacks. HeLa was the first continuously cultured cell line, and have been widely used for cancer and virological research. HeLa cells were purchased from ClonTech (catalog no. 632110; ClonTech).

HeLa cells were cultured and maintained in RPMI-1640® medium (catalog no. 23400-013, Gibco) supplemented with 10% FBS and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep, catalog no. 15140-122; Gibco) at 37°C under a 5% CO<sub>2</sub> humidified atmosphere. HeLa cells were passaged 1:5 every 3-4 days by aspirating old media, washing once with 1x phosphate-buffered saline (PBS) (see reagents section) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with trypsin solution at 37°C until they began to detach from the bottom of the culture dish. Warmed RPMI-1640® was added to neutralize trypsin activity and cells were collected and centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in warmed RPMI-1640®.

#### **2.1.5 Swiss Webster and C57Black Primary MEF's**

Primary mouse embryonic fibroblasts were obtained from whole embryo squashes of 10 day pre-natal pathogen-free Swiss Webster and C57Black embryos. Primary MEF cells were cultured and maintained in DMEM® (catalog no. 12800- 017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSF-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter

sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®, catalog no. 15140-122; Gibco) and incubated at 37°C under a humidified 5% CO<sub>2</sub> atmosphere. Cells were harvested from embryos and collected into tissue culture tubes. The cells were centrifuged at 1000 x g for 5 minutes and the supernatant was aspirated. The cell pellet was washed by resuspension in warmed complete maintenance media. Cells were then seeded into 100mm tissue culture dishes, covered with 7 mL of warmed complete maintenance medium and incubated for 48 hours before experimentation. Cells were then collected by aspirating old media, washing once with 1x phosphate-buffered saline (PBS) (see reagents section) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with trypsin solution at 37°C until they began to detach from the bottom of the culture dish. Warmed DMEM® was added to neutralize trypsin activity and cells were collected and centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in warmed DMEM®. For experimentation, primary MEF cells were counted on a hemocytometer and 10<sup>5</sup> cells were seeded in 1mL of complete maintenance medium per well onto 12 well tissue culture plates and allowed to attach overnight at 37°C before infection and/or treatment.

#### **2.1.6 Swiss Webster Primary Macrophages**

Primary murine macrophages were obtained by peritoneal collection from pathogen-free Swiss Webster Mice. Cells were cultured and maintained in RPMI-1640® medium (catalog no. 23400-013; Gibco) supplemented with 10% FBS and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®, catalog no. 15140-122; Gibco) 50

U/mL PenStrep at 37°C under a 5% CO<sub>2</sub> humidified atmosphere. Cells were harvested into tissue culture tubes and centrifuged at 1000 x g for 5 minutes. The supernatant was aspirated and the cell pellet was washed by resuspension with 1x PBS. The cells were centrifuged again at 1000 x g for 5 min., the supernatant was aspirated, and the cell pellet was resuspended in complete RPMI maintenance medium. Cells were counted under a hemocytometer and 10<sup>5</sup> cells were seeded in 1mL of complete maintenance medium per well onto 12 well tissue culture plates and incubated for 48 hours at 37°C before experimentation.

### **2.1.7 C636 *Aedes albopictus* mosquito cells**

C636 mosquito cells were first isolated from the hatched larvae of *Aedes albopictus*. C636 cells are commonly used for flavivirus growth and research due to their ease of culture, permissiveness to flavivirus infection and the necessary human-mosquito interspecies infection cycle that occurs in many natural infections. C636 cells were a generous gift from Adolfo Garcia-Sastre, Mt. Sinai University, New York City.

C636 mosquito cells were cultured and maintained in EMEM®, 10%FBS, 1mM sodium pyruvate (catalog no. S8636, Sigma-Aldrich), 1% non-essential amino acids (catalog no. M7145, Sigma-Aldrich), 2mM L- glutamine (catalog no. 25030-081; Gibco), 25U/mL Fungizone (catalog no. 15290-018, Gibco) and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®, catalog no. 15140-122; Gibco) at 28°C, 5% CO<sub>2</sub>.

C636 cells were passaged 1:5 every 3-4 days by aspirating old media, washing once with 1x phosphate-buffered saline (PBS) (see reagents section) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with Trypsin/EDTA

solution at 25°C until they began to detach from the bottom of the culture dish. Complete maintenance media was added to neutralize trypsin activity and cells were collected and centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in complete maintenance media.

### **2.1.8 Baby Hamster Kidney (BHK) cells**

BHK cells were originally isolated from 5 unsexed, 1 day old hamsters in 1961 by I.A. Macpherson and M.G.P. Stoker at Glasgow University in Glasgow, Scotland. BHK cells are commonly used for virological research and vaccine development, and are commonly used in flavivirus research. BHK cells were purchased from ATCC (catalog no. CCL-10, ATCC).

BHK cells were cultured and maintained in DMEM® (catalog no. 12800- 017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSF-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®, catalog no. 15140-122; Gibco) and incubated at 37°C under a humidified 5% CO<sub>2</sub> atmosphere.

BHK cells were passaged 1:5 every 2-4 days by aspirating old media, washing once with 1x phosphate-buffered saline (PBS) (see reagents section for formula) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with trypsin solution at 37°C until they began to detach from the bottom of the culture dish. Warmed DMEM® was added to neutralize trypsin activity and cells were collected and

centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in warmed DMEM®.

### **2.1.9 Di-4G2-4-15 hybridoma cells (for production of anti-Dengue-2 E protein antibody)**

Di-4G2-4-15 hybridoma cells produce mouse monoclonal anti-Dengue-2 E protein antibodies for use as primary antibody in immunocytological and FACS staining (this antibody does not work well with western blotting). Di-4G2-4-15 cells were purchased from ATCC (catalog no. HB-112; ATCC). These cells grow in suspension, and were maintained in 150cm<sup>2</sup> tissue culture flasks at 37°C in Hybri-Care® media (catalog no. 46-X; ATCC) media supplemented with 10% FBS, 2mM L- glutamine (catalog no. 25030-081; Gibco) and 50 U/ml penicillin plus 50 mg/ml streptomycin (PenStrep®, catalog no. 15140-122; Gibco) under a humidified 5% CO<sub>2</sub> atmosphere. Cells were passaged 1:5 every 3-4 days. To collect antibody, cell media was collected and centrifuged at 1000 x g for 5 min. to remove the cells. The supernatant was transferred to a new tube and stored at 4°C. The presence of antibody in the supernatant was tested for IgG protein accumulation. 30µL of supernatant was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (see reagents section). Following electrophoresis, the gel was removed and stained with Coomassie blue (see reagents section) for 10 minutes at room temperature. The Coomassie blue was then removed, and the gel was cleared with destain solution (see reagents section) for 30 minutes. A strong band at 60 kDa by Coomassie staining indicates the presence of IgG.

## **2.2 Virus Culture**

### **2.2.1 Influenza A/WSN/33 (H1N1)**

Influenza A/WSN/33 is a mouse-adapted neurotropic virus derived by William Smith from human Influenza A first isolated in 1933. Its name is an abbreviation of William Smith (Neurotropic)/1933.

Influenza A/WSN/33 virus was cultured in 8-11 day embryonated specific-pathogen free chicken eggs (Charles River SPAFAS, North Franklin, CT). Eggs were candled to ensure viability and to mark the position of the shell membrane/air sac interface, the embryo and any major blood vessels. An injection site was marked just above the shell membrane/air sac interface avoiding the embryo and blood vessels. The injection site was swabbed with ethanol and the shell was thinned by scraping with a sharp dissecting probe before injecting 100 $\mu$ L of a 10<sup>-3</sup> dilution of Influenza A/WSN/33 stock in Influenza diluting media (see reagents section for formula) using a 1cc syringe. The hole at the injection site was then covered with wax and the eggs were incubated at 35-37°C for 48 hours. The infected eggs were then incubated overnight at 4°C to kill the embryo and coagulate the blood. The tops of the eggs were removed and the infectious allantoic fluid was collected in sterile tubes on ice. Infectious allantoic fluid was centrifuged at 1000g for 5 minutes to remove blood and other debris and aliquoted into sterile cryotubes for storage at -80°C.

## **2.2.1.1 Quantitation of Influenza A/WSN/33 virus**

### **2.2.1.1.1 Influenza Plaque Assay**

The plaque assay is one of the most sensitive infectivity assays for measuring viral titer. Plaques are zones of cell lysis formed when infection induces cytopathic effect on a monolayer of cells. For Influenza A plaque assay,  $10^6$  MDCK cells were seeded into 35mm tissue culture plates and allowed to grow to a tight monolayer for 2 days at 37°C before infecting with serial dilutions of Influenza A virus in Influenza diluting media (see reagents section). Cells were inoculated with 100  $\mu$ L of virus dilution ( $10^{-1}$  to  $10^{-6}$  dilutions of virus were typically applied), and incubated at room temperature for 1 hour, with shaking every 20 minutes to ensure even spread of virus. After incubation, the inoculum was aspirated and cells were covered with 2mL of freshly prepared Influenza plaquing agar medium (see reagents section). Once the agar medium hardened, plates were inverted and incubated upside-down at 37°C for 36-72 hours to allow plaque formation. The agar medium was then gently removed from each plate with a cell scraper, and the cells were stained and fixed by incubating with methanol/crystal violet stain (see section on reagents) for 10 minutes at room temperature. The stain was decanted and the plates were gently rinsed with tap water before counting plaques. Plaques appear as clear patches on a dark purple background. Viral infectivity titer is expressed as the number of plaque forming units per mL (pfu/mL) and is calculated by this formula: (number of plaques) X (dilution factor)<sup>-1</sup> X (inoculation volume in mL)<sup>-1</sup>. For statistical significance, only plates containing 20-100 plaques were included in calculations.

#### **2.2.1.1.2 Influenza Hemagglutination Assay (HA assay)**

Influenza viruses aggregate red blood cells (RBCs) via interactions between RBC surface receptors and the Influenza HA protein, a phenomenon termed hemagglutination. The hemagglutination assay is an indirect measure of the number of virus particles in a sample, and requires very high numbers of virus particles to induce a positive sample. As such, this assay is one of the least sensitive methods of quantifying Influenza viral titer.

50  $\mu$ L of undiluted virus was added to the first two wells of a V-bottomed 96 well plate. 2-fold serial dilutions of the virus were made across the plate, and 50 $\mu$ L of 0.5% chicken red blood cells (RBCs) (catalog no. 30-903J, BioWhittaker) was added to all wells. The bottom of each well was rubbed with a toothpick to mix the samples, and the tray was incubated at 4°C for 1 hour before analysis. Samples positive for hemagglutination were indicated when the RBCs coated the side of the well as a result of influenza-induced aggregation. Negative samples formed well-defined RBC pellets at the bottom of the well. Viral titer was determined by endpoint titration of the highest dilution causing complete hemagglutination, and the HA titer of each sample was calculated using the equation (highest dilution causing complete haemagglutination)<sup>1</sup>/(inoculation volume) = HA units/mL.

#### **2.2.1.1.3 qRT-PCR for Influenza A**

To determine the amount of total influenza A virus RNA released during infection, viral titer was determined by collecting cell supernatant samples at 12, 24 and

48 hours post infection (hpi) and subjecting them to quantitative reverse transcription-PCR. Total viral RNA was extracted using a viral RNA extraction® kit (catalog no. 52904; Qiagen) according to the manufacturer's protocol. cDNA was then generated from extracted viral RNA using a Superscript III first-strand synthesis® kit (catalog no. 18080-400; Invitrogen) according to the manufacturer's protocol. One microgram of cDNA was amplified by quantitative reverse transcription-PCR in 20- $\mu$ L reaction mixtures using a LightCycler FastStart DNA Master Sybrgreen 1® kit (catalog no. 03515869001; Roche) with forward primer TACACCCAGTCACAATAGGAGAGTG and reverse primer CCATGCATTCATTGTCACACTTGTGG for the influenza A virus hemagglutinin (HA) gene using a Roche LightCycler 2.0 real-time PCR machine under the following conditions: 95°C for 10 min, followed by 50 cycles of 10 min at 95°C, 5 min at 69°C, and 22 min at 72°C. Samples of known viral titers were also analyzed to develop a standard curve of influenza A virus particles released/mL, to which experimental samples were normalized. Relative influenza A virus particle release in each cell type was compared to that for mock-infected cells and presented as a ratio of infected/mock-infected cells.

#### **2.2.1.2 Influenza A/WSN/33 virus Infection Procedure**

Cells were seeded into tissue culture plates and allowed to attach overnight at 37°C using cell-type appropriate complete maintenance media with full serum (typically 10% FBS).  $2 \times 10^6$  cells were seeded into 100mm tissue culture plates,  $10^6$  cells into 60mm plates,  $4 \times 10^5$  cells into 35mm plates or  $10^5$  cells into 12-well plates, depending on the requirements of the experimental protocol. The medium was aspirated, cells were washed once with warmed 1x PBS, then inoculated at a multiplicity of infection (MOI) of

5 for most experiments. An MOI of 0.1 was used for experiments studying influenza replication. Mock-infected cells received only influenza diluting media without virus. The MOI is the number of virus particles added per cell to the culture dish during inoculation. 0.5mL inoculation volume was used for 100 mm plates, 0.3mL for 60mm plates and 0.1mL for 35mm plates. Stock virus was diluted in influenza diluting media (see reagents section) to the appropriate inoculation volume, and calculated using the equation:  $C_1V_1=C_2V_2$ , where  $C_1$  is the concentration of stock virus,  $V_1$  is the unknown amount of stock virus to add to the virus dilution,  $C_2$  is the desired final viral dilution concentration, and  $V_2$  is the desired total volume of inoculum for the experiment. Inoculated cells were incubated for 1 hour at room temperature, with shaking every 20 minutes to ensure even spread of virus. After incubation, cells were washed once with warmed 1x PBS and covered with reduced serum maintenance medium (medium serum typically reduced to 5% FBS). Cells were then incubated at 37°C under a humidified 5% CO<sub>2</sub> atmosphere until collection at the desired time.

### **2.2.2 Flaviviruses (Dengue-2 and Modoc viruses)**

Dengue virus belongs to the family Flaviviridae, and is related to West Nile Virus, Yellow Fever Virus and Japanese encephalitis virus, among others. Dengue virus infections are among the most common and deadliest vector borne human diseases in the world. At present, there is no cure or vaccine for Dengue infection, and treatment is generally restricted to supportive care until recovery. There are four strains of Dengue (Dengue-1,-2,-3,-4), and infection with multiple strains increases the risk of developing Dengue Hemorrhagic Fever, a severe complication of infection (See introduction:

flaviviruses). Dengue-2 virus was generously provided by Adolfo Garcia-Sastre of Mt. Sinai Medical School in New York City.

Modoc virus is a mouse flavivirus related to Dengue virus that has been proposed as a research model for flavivirus infections due to its similarity of pathogenesis with other flaviviruses, comparative safety and ease of culture (Leysen, Van Lommel et al. 2001). It was originally isolated from a small boy who fell ill after playing with a sick mouse in Modoc County, California in 1958 (Davis 1973). Modoc virus is endemic in the small mammal population throughout the northwestern United States and western Canada (Zarnke and Yuill 1985). However, human Modoc infections are rare. Modoc virus strain M544 used in these studies was originally collected from the lactating mammary gland of an infected deer mouse (*Peromyscus maniculatus*) in 1958. Modoc virus strain M544 was purchased from ATCC (catalog no VR-415; ATTC).

For expansion of Dengue stocks for all strains, sub-confluent C636 mosquito cells were infected with virus at MOI=1 and incubated at 28°C for 6 days. The culture medium of the infected plates was agitated and collected, then mixed 1:1 with flavivirus freeze medium (see reagents section), aliquotted into 300µL portions and stored at -80°C. For expansion of Modoc virus stocks, the virus was applied at MOI=1 to sub-confluent Vero cells in 150cm<sup>2</sup> filter-top tissue culture flasks and incubated at 37°C for 7 days in a minimum of complete maintenance medium. The culture medium of the infected plates was agitated and collected in culture tubes on ice, then mixed 1:1 with flavivirus freeze medium (see reagents section), aliquotted into 300µL portions and stored at -80°C.

Viral titer of frozen Dengue-2 and Modoc stocks were assessed by plaque assay as described below.

### **2.2.2.1 Quantitation of flaviviruses (Dengue virus and Modoc virus)**

#### **2.2.2.1.1 Flavivirus Plaque assay**

Viral load of Modoc and Dengue-2 stock solutions was determined by plaque assay. Vero cells in 35mm plates were infected with serial dilutions of virus samples ( $10^0$  to  $10^{-5}$ ) and covered with flavivirus plaquing medium I (see reagents section), followed by incubation at 37°C, 5% CO<sub>2</sub> for 5 days. On day five, the medium was aspirated and replaced with flavivirus plaquing medium II containing agar and vital stain (see reagents section). After the medium solidified, plates were inverted and incubated for an additional 1-2 days to allow plaque development and staining.

Due to the labor intensive nature of the method described above, after preliminary experiments we changed our plaque assay method to the following procedure.  $1.0 \times 10^5$  BHK cells were plated in 1mL of complete DMEM culture medium per well in 12 well plates and allowed to attach overnight. Cells were then aspirated and washed twice with 1x PBS before application of serial dilutions of virus samples ( $10^0$  to  $10^{-5}$ ), which were generated using complete DMEM culture medium and pulse-vortexed immediately prior to application of 200µL of each sample to cells. Cells were incubated for 2 hours at 37°C, then covered with flavivirus plaquing medium III containing agar (see reagents section). After the medium solidified, plates were inverted and incubated at 33°C for 6 days to allow plaque development.

For both plaque assay techniques, plaques were counted and viral titer was then calculated as plaque-forming units per mL (pfu/mL) using the equation:  $\text{pfu/mL} = (\# \text{ plaques}) \times (\text{inoculation volume})^{-1} \times (\text{dilution factor})^{-1}$ .

#### **2.2.2.1.2 qRT-PCR for Flaviviruses**

To determine the amount of total flavivirus RNA released during infection, viral titer was determined by collecting cell supernatant samples at 48, 96 and 144 hours post infection (hpi) and subjecting them to quantitative reverse transcription-PCR. Cell culture media samples of Modoc and Dengue-2 infected cells were collected at various times post-infection and total viral RNA was extracted using a viral RNA extraction® kit (catalog no. 52904; Qiagen), following manufacturer's protocol. cDNA was then generated from extracted viral RNA using a Superscript III first strand synthesis® kit (catalog no. 18080-400; Invitrogen), following manufacturer's protocol. 1µg of cDNA was amplified by qRT-PCR in 20 µl reactions using a LightCycler FastStart DNA Master SyberGreen 1® kit (catalog no. 03515869001; Roche), with primers for Modoc (fwd: GATTCAGGATGGCCCAAGAATC, rvs: AGTAGGAAGGTGGACAGAATGA), and Dengue-2 (fwd: TTAGAGGAGACCCCTCCC, rvs: TCTCCTCTAACCTCTAGTCC), using a Roche LightCycler 2.0 real-time PCR machine. Approximate viral titer (pfu/mL) was determined by quantifying viral RNA in serial dilutions of stock virus at a known concentration and generating a standard curve for comparison analysis. Relative RNA release in each cell type was compared to mock infected cells and presented as (infected – mock).

### **2.2.2.2 Flavivirus Infection Procedure**

Cells were seeded into tissue culture plates and allowed to attach overnight at 37°C using cell-type appropriate complete maintenance media with full serum. Typically, 2 x 10<sup>6</sup> cells were seeded into 100mm tissue culture plates, 10<sup>6</sup> cells into 60mm plates, 4 x 10<sup>5</sup> cells into 35mm plates or 10<sup>5</sup> cells into 12-well plates, depending on the requirements of the experimental protocol. The medium was aspirated, cells were washed once with warmed 1x PBS, then inoculated at a multiplicity of infection (MOI) of 10 for all experiments except were otherwise noted. Mock-infected cells received only flavivirus diluting medium (see reagents section) without virus. For all experiments, 0.5mL inoculation volume was used for 100 mm plates, 0.3mL for 60mm plates and 0.1mL for 35mm plates, 6 well trays and 12-well trays. Stock virus was diluted in flavivirus diluting medium (see reagents section) to the appropriate inoculation volume. Inoculated cells were incubated for 1.5 hours at 37°C, with shaking every 20-30 minutes to ensure even viral spread. After incubation, cells were washed once with warmed 1x PBS and covered with complete maintenance media. Cells were then incubated at 37°C until collection at the desired time.

### **2.2.2.3 Transfection of Individual Flavivirus Genes**

Individual Dengue-2 genes (DEN2 E, C, prM, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) were expressed in mammalian cells from a pCAGGS-HA plasmid vector (kindly provided by Adolfo Garcia-Sastre, Mount Sinai Medical School, New York City), and individual Modoc genes (NS1, NS2A, NS4A, NS4B) were expressed in mammalian cells from a TOPO plasmid vector (donated by Stephane Boissinot, Queens College, New

York City). For transfection of individual Modoc and Dengue-2 genes, cells were plated to approximately 85% confluency and allowed to attach overnight in PenStrep-free cell-type appropriate culture media supplemented with 10% FBS. Cells were transfected with the individual viral genes of interest using the Lipofectamine 2000® system (catalog no. 52887; Invitrogen). Lipofectamine 2000 reagent® and DNA were mixed to appropriate proportions (depending on plate size) in virgin DMEM® (0% FBS, 0% PenStrep®) as determined by manufacturer's protocol and incubated at room temperature for 30-60 minutes to allow Lipofectamine 2000®-DNA complex formation. Cells were then washed several times with warmed 1x PBS to remove residual FBS. Lipofectamine 2000®-DNA mixture was applied to the cells and incubated at 37°C for 5 hours, with shaking every 30 minutes to ensure even spread of the mixture and to avoid drying. Empty plasmid vector was used as a control, and transfection efficiency was checked by observation under fluorescent or confocal microscope. Experiments with a transfection efficiency of less than 60% were not analyzed further. Cells were then washed once with warmed 1x PBS, covered with warmed PenStrep®-free culture media supplemented with 10% FBS. Cells were then incubated at 37°C for at least 16 hours before collection or treatment to allow plasmid expression.

## **2.3 Death, Apoptosis and Autophagy Assays**

### **2.3.1 Trypan Blue Exclusion Assay**

Dead and dying cells experience a loss of plasma membrane integrity, allowing vital dyes such as trypan blue to enter the cell and stain cytoplasmic proteins while the intact plasma membrane of living cells excludes these dyes (Tennant 1964). Staining

with trypan blue thus leaves living cells clear and dead cells blue under microscopic examination, making this a convenient and reliable method for quantifying the number of living and dead cells in a population. After infection and/or treatment, cells were collected at the desired time point and centrifuged at 1000 x g for 5 minutes. The medium was aspirated and the cell pellet was resuspended gently but thoroughly in an appropriate volume of warmed 1x PBS. A 100 $\mu$ L sample of this cell suspension was mixed with an equal volume of 0.4% trypan blue solution (see reagents section) and incubated at room temperature for 5 minutes. Cells were then observed and counted on a hemocytometer under a light microscope. Each assay was run in triplicate. Data was typically expressed as percent cell death and was calculated using the equation: percent cell death = ((number of dead cells)/(total number of dead and living cells)) x (100%).

### **2.3.2 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a soluble, bright yellow compound that is reduced to insoluble purple formazan in metabolically active cells by the succinate-tetrazolium reductase system within the mitochondrial respiratory chain (Twentyman 1987). MTT can also be used to measure changes in mitochondrial activity. To measure the viability of a cell population after infection and/or treatment,  $1.5 \times 10^6$  cells were seeded into 35mm tissue culture dishes and allowed to attach overnight at 37°C in complete maintenance medium. Cells were then infected and/or treated before incubation at 37°C in the appropriate medium until the desired time point. Medium from each plate was then collected and centrifuged at 1000 x g for 5 min. to collect and pellet any dead, floating cells. These cells were resuspended in

a small amount of medium and put back into the cell culture plate. 1mL of a 1:10 dilution of 5mg/mL MTT stock was then applied to each plate and incubated at 37°C for 2 hours to allow formazan formation. 1mL of acidic isopropanol (0.04M HCl in absolute isopropanol) was then added to each plate and mixed thoroughly to solubilize the newly formed formazan. All cells and medium were then collected from each plate and centrifuged at 14,000rpm for 5 min. to remove cellular debris. The supernatant from each sample was then transferred into a plastic spectrophotometer cuvette and the absorbance of the converted dye was measured at a wavelength of 570nm with a background subtraction at 650nm. A decrease in absorbance indicates a decrease in mitochondrial activity and/or death.

### **2.3.3 Treatment with Death Inducers**

To determine the effect of whole flavivirus infection or individual expression of flavivirus proteins on ectopically-induced cell death,  $10^5$  MDCK cells were seeded per well onto a 12-well tissue culture plate in 1 mL of complete maintenance medium and allowed to attach overnight at 37°C. Cells were then infected with Dengue-2 or Modoc virus at MOI=10, or transfected with individual flavivirus genes as described above, followed treatment with one of the following death inducers at 24 hours post infection/transfection (hpi/hpt): Camptothecin (CPT) (catalog no. C9911, Sigma-Aldrich), a topoisomerase I inhibitor that causes apoptotic cell death through a p53-mediated DNA damage response, was applied at concentrations ranging from 70-100 $\mu$ M. Staurosporine (STS) (catalog no. S5921, Sigma-Aldrich), a kinase inhibitor that drives apoptotic death through blockade of several key biochemical pathways, was applied at 10 $\mu$ M.

Cyclohexamide (CHX) (catalog no. 01811, Sigma-Aldrich), a protein synthesis inhibitor that induces apoptosis via an unknown mechanism at high concentrations, was applied at 150 $\mu$ M. Influenza A, a segmented RNA virus that causes apoptosis through both extrinsic and intrinsic mechanisms, was applied to cells at MOI=5, incubated for 1 hour at room temperature, washed once with 1x PBS and covered with maintenance medium. Following treatment with death inducer, cells were incubated at 37°C for 24 hours in complete maintenance medium to allow cell killing. Cells were then collected by trypsinization and centrifuged at 1000 x g. The supernatant was aspirated, and the cell pellets were resuspended in 200-500 $\mu$ L of 1x PBS. Each sample was thoroughly resuspended immediately before taking a 100 $\mu$ L sample for the trypan blue exclusion assay (described above) and counting on a hemacytometer under a light microscope. Each assay was run in triplicate. Data was expressed as percent cell death and was calculated using the equation: percent cell death =  $((\text{number of dead cells}) / (\text{total number of dead and living cells})^{-1}) \times 100\%$ . Protection was indicated when percent death by inducer was less in virus-infected samples than in mock-infected samples.

#### **2.3.4 Measurement of Autophagy Induction by LC3-GFP assay**

During autophagy, cytoplasmic LC3 is cleaved to form LC3II, which translocates and collects at the autophagosomal membranes. Transient expression of LC3 that contains a GFP tag offers a convenient method of assessing the degree of autophagy upregulation during infection and/or treatment, as LC3-GFP cleavage and translocation is observable under confocal microscopy. A diffuse, evenly distributed cytosolic GFP expression pattern indicates healthy cells with little autophagy, as LC3 remains cytoplasmic. During

upregulation of autophagy, this GFP pattern shifts to dense, punctate dots throughout the cytoplasm, as LC3 collects on newly formed autophagosomal membranes.

LC3-GFP was transiently expressed by transfection with a C1-LC3-EGFP construct (provided by Guido Kroemer, Institut Gustave-Roussy, Villejuif, France) using Lipofectamine 2000® (catalog no. 52887; Invitrogen). A total of  $4 \times 10^5$  MDCK cells was plated onto heat-sterilized glass coverslips in 35-mm plates and allowed to attach overnight in PenStrep-free DMEM® supplemented with 10% FBS. Medium was then aspirated, and cells were washed several times with 1x PBS to remove residual FBS. 1.4 µg C2-LC3-EGFP DNA was mixed with 10µL of Lipofectamine 2000 reagent® in a total volume of 500µL minimal maintenance medium (0% FBS, 0% PENsTREP®) and incubated at room temperature for 30 minutes to allow DNA/Lipofectamine 2000® complex formation. The DNA/Lipofectamine 2000® mixture was then applied to each culture dish and incubated at 37°C for 5 hours, with shaking every 30 minutes to ensure an even spread of DNA and to keep the cells from drying. Cells were then washed once with warmed 1x PBS and covered with PenStrep®-free DMEM® supplemented with 10% FBS. Transfected cells were incubated for 16-18 hours to allow LC3-GFP expression. Cells were then infected and/or treated and incubated for 24 hours at 37°C as described above. At 24hpi, cells were fixed with ice-cold 3.5% paraformaldehyde (catalog no. 04042; Fisher) for 10 min and rinsed once with 1x PBS. Cells were then embedded by gel mount (catalog no. M-01; Biomed) and slides were sealed with clear nail polish and allowed to dry in the dark at room temperature. Mounted cells were observed by confocal microscopy for the expression pattern of LC3-GFP. A transfection efficiency of at least 50% was confirmed prior to quantitation of cells displaying diffuse

and punctate GFP expression. Mock-infected cells were also analyzed to ensure that LC3-GFP expression alone did not cause an autophagy response. At least 400 GFP-positive cells were counted per sample, and each sample was run in triplicate. Data was expressed as the percent of GFP positive cells that displayed punctate LC3-GFP distribution. Autophagy was also assessed by western blot for cleaved LC3 as described below.

### **2.3.5 FACS analysis of Intracellular Lysosomal Volume**

Lysosomal swelling is common during extreme cellular stress, as lysosomes and autophagosomes become engorged with damaged cellular material. To determine the overall lysosomal volume after infection and/or treatment,  $10^6$  cells were seeded into 60mm tissue culture dishes and allowed to attach overnight in complete maintenance media at 37°C. The cells were then infected and/or treated and incubated at 37°C until the desired time. Immediately prior to collection, cells were incubated with 1 $\mu$ L/mL LysoTracker Red DND-99® (catalog no. L7528; Invitrogen) for 30 min at 37°C. Cells were then rinsed once with warmed 1x PBS, collected by trypsinization and centrifuged at 1000 x g for 5 min.. The supernatant was aspirated, and the stained cell pellet was resuspended in 1x PBS. Cells were then analyzed by FACS using a Beckman-Dickson FACScaliber flow cytometer equipped with Cell Quest® software. 50,000 events per sample were analyzed. The mean LysoTracker Red fluorescence of each population was used as a measure of overall lysosomal volume, and calculated using the equation: (mean fluorescence of infected/treated cells) – (mean fluorescence of mock/control cells).

### **2.3.6 FACS Analysis of Mitochondrial Activity**

During intrinsic mitochondria-mediated apoptosis, mitochondrial respiration declines as a result of the loss of mitochondrial membrane potential, permeabilization of the outer mitochondrial membrane and the breakdown of the mitochondrial respiratory chain including cytochrome c release to the cytosol. The uptake of MitoTracker Red CMXRos® is dependent upon mitochondrial membrane potential. To determine overall mitochondrial activity after infection and/or treatment,  $10^6$  cells were seeded into 60mm tissue culture dishes and allowed to attach overnight in complete maintenance media at 37°C. The cells were then infected and/or treated and incubated at 37°C until the desired time point. Immediately prior to collection, cells were incubated with 2  $\mu$ L/mL MitoTracker Red CMXRos® (catalog no. M7512; Invitrogen) for 30 min at 37°C. Cells were then rinsed once with warmed 1x PBS, collected by trypsinization and centrifuged at 1000 x g for 5 minutes. The supernatant was aspirated, and the stained cell pellet was resuspended in 1x PBS. Cells were then analyzed by FACS using a Beckman-Dickson FACScaliber flow cytometer equipped with Cell Quest® software. 50,000 events per sample were analyzed. The mean MitoTracker Red fluorescence of each population was used as a measure of overall mitochondrial activity, and calculated using the equation: (mean fluorescence of infected/treated cells) – (mean fluorescence of mock/control cells).

### **2.3.7 Protein analysis**

#### **2.3.7.1 Protein Extraction**

To obtain whole-protein lysates from mock/control and infected and/or treated cells for protein analysis,  $2 \times 10^6$  cells were seeded into 100mm tissue culture dishes and

allowed to attach overnight in complete maintenance media at 37°C. The cells were then infected and/or treated and incubated until the desired time point. Cells were scraped from the culture dish using a cell scraper, transferred to an ice-cold tube and centrifuged at 4°C, 1000 x g for 10 minutes. The supernatant was aspirated, and the cell pellet was washed by resuspension with ice-cold 1x PBS. Cells were then centrifuged again at 4°C, 1000 x g for 10 min., the supernatant was aspirated and the pellet was resuspended in a minimal volume (50-200µL) of ice-cold RIPA buffer supplemented with protease inhibitor cocktail (see reagents section). Cells were incubated on ice for 30 minutes, and centrifuged at 4°C, 12,000 x g for 20 min.. The supernatant containing the whole-protein lysate was then transferred to a new ice-cold tube and stored at -20°C.

#### **2.3.7.2 western blotting**

For analysis by western blot, equal quantities of proteins (30µg) were loaded onto a polyacrylamide gel and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10, 12.5 or 15% gels (depending on the size of the protein of interest). Proteins were then transferred onto a nitrocellulose membrane (catalog no. RPN-303E; Amersham) overnight at 4°C. Nonspecific adsorption of primary antibody was blocked using 4% milk solution for 1 hour before addition of primary antibody [anti-cleaved caspase-7; 1:500 dilution (catalog no. 9491s; BD Pharmingen), anti-active caspase-3; 1:500 dilution (catalog no. 559565; Sigma), anti-Bcl-2; 1:200 dilution (catalog no. sc492; Santa Cruz Biotech), anti-influenza A virus NP; 1:1000 dilution (provided by Adolfo Garcia- Sastre, Mt. Sinai University and Medical Center), and anti-β-tubulin 1:500 dilution as a loading control (catalog no. sc9104; Santa

Cruz Biotech)]. Positive signals were detected using ECF (catalog no. RPN5787; GE Healthcare) according to the manufacturer's protocol and visualized using a Storm 860 scanner and software (GMI, Inc., MN) or by ECL (catalog no. RPN2132; GE Healthcare) and visualized using Amersham Hyperfilm ECL photoradiographic film (catalog no. 28906835; GE Healthcare).

### **2.3.7.3 Immunocytochemistry**

For immunocytochemical analysis,  $4 \times 10^5$  cells were seeded onto flame-sterilized glass coverslips in 35mm tissue culture dishes and allowed to attach overnight in complete maintenance media at 37°C. Cells were washed with warmed 1x PBS and infected and/or treated and incubated at 37°C until the desired time. Cells were washed once with 1x PBS and 400µL of ice-cold 3.5% paraformaldehyde was applied to each sample. Cells were incubated at room temperature in the dark for 10 min., then washed once with 1x PBS, covered with 1x PBS and stored in the dark at 4°C until staining. To label the proteins of interest, nonspecific binding sites were blocked using 1x PBS containing 18 µg/ml bovine serum albumin for 1 hour before immunoblotting with the rabbit antibodies anti-Bax; 1:200 dilution (catalog no. sc493; Santa Cruz Biotech), anti-active caspase-3; 1:500 dilution (catalog no. 559565; Sigma), anti-influenza A/WSN virus; 1:1000 dilution or anti-influenza A virus NP; 1:1000 dilution (Influenza antibodies provided by Adolfo Garcia-Sastre, Mt. Sinai University and Medical Center, New York, NY); 1:1000 dilution of anti-dengue-2-E protein (Di-4G2-4-15; see cell culture section). Cells were then rinsed three times in 1x PBS, 5 to 10 minutes each. This was followed by incubation with anti-rabbit immunoglobulin G-AlexaFluor 488 secondary antibody;

1:1000 dilution (catalog no. A11008; Molecular Probes) or anti-rabbit immunoglobulin G-AlexaFluor 555 at 1:1000 dilution (catalog no. A-21430; Molecular Probes) solution for 1.5 hours at room temperature in the dark. Cells were then rinsed twice with 1x PBS before further staining and mounting. Polymerized actin was stained with phalloidin-tetramethyl rhodamine isocyanate (phalloidin-TRITC; catalog no. P1951; Sigma) and nuclei were stained with 10 µg/ml of 4',6'-diamidino-2-phenylindole (DAPI) (catalog no. D8417; Sigma). Lysosomal localization was determined by staining cells with 1 µL/mL LysoTracker Red DND-99® (catalog no. L7528; Invitrogen) for 30 min immediately prior to fixation. Mitochondrial localization was determined by staining cells with 2µL/mL MitoTracker Red CMXRos® (catalog no. M7512; Invitrogen) for 30 minutes immediately prior to fixation. Cells were then mounted with el mount (catalog no. M-01; Biomed) and sealed with clear nail polish. Mounted cells were visualized by confocal microscopy (Leica, Germany).

## **2.3.8 Reagents**

### **2.3.8.1 Eukaryotic Cell Culture**

#### **2.3.8.1.1 10x Phosphate Buffered Saline (10x PBS)**

The following were dissolved in 800mL ddH<sub>2</sub>O: 80g NaCl, 2g KCl, 14.4g Na<sub>2</sub>HPO<sub>4</sub>, 2.4g KH<sub>2</sub>PO<sub>4</sub>. Total volume was brought to 1000mL with ddH<sub>2</sub>O, and pH was adjusted to 7.4 w/ HCl. The solution was sterilized by autoclave prior to use.

#### **2.3.8.1.2 1x Phosphate Buffered Saline (1x PBS)**

100 mL 10x PBS (discussed above) was brought to a total volume of 1000mL with ddH<sub>2</sub>O. This is equal to 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O and 1.4 mM KH<sub>2</sub>PO<sub>4</sub> in dH<sub>2</sub>O. The pH was adjusted to 7.3 and sterilized by autoclave prior to use.

#### **2.3.8.1.3 1x Phosphate Buffered Saline Tween-20 (1x PBST)**

2mL Tween-20 was added to 998mL 1x PBS. The pH was adjusted to 7.2.

#### **2.3.8.1.4 2.5% Trypsin EDTA (100mL)**

10mL 25% Trypsin (=10x stock) was mixed with 10mL 10x PBS, 10mL 10mM EDTA, 0.5mL 5% NaHCO<sub>3</sub> and 69.5mL ddH<sub>2</sub>O. All reagents were sterile prior to mixing.

#### **2.3.8.1.5 Dulbecco's Minimum Essential Media (DMEM)**

Dulbecco's modified Eagle medium® (DMEM) (catalog no. 12800- 017; Gibco) was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSFb-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (catalog no. 15140-122; Gibco)

#### **2.3.8.1.6 Eagle Modified Essential Medium (EMEM)**

Eagle Modified Essential medium® (EMEM) (catalog no. 12-668E; BioWhittaker) was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSFb-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-

Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (catalog no. 15140-122; Gibco).

#### **2.3.8.1.7 C636 Culture Media**

EMEM® was supplemented with 10%FBS, 1mM sodium pyruvate (catalog no. S8636, Sigma-Aldrich), 1% non-essential amino acids (catalog no. M7145, Sigma-Aldrich), 2mM L- glutamine (catalog no. 25030-081; Gibco), 25U/mL Fungizone® (catalog no. 15290-018, Gibco) and 50 U/ml penicillin plus 50 mg/ml streptomycin (catalog no. 15140-122; Gibco).

#### **2.3.8.2 pH adjustment**

##### **2.3.8.2.1 10N NaOH**

40g NaOH was dissolved in 100mL ddH<sub>2</sub>O. As the solution gets very hot as it dissolves, protective gloves were used to handle the bottle until it cooled.

#### **2.3.8.3 Chemical manipulation of mammalian biochemical pathways**

##### **2.3.8.3.1 Wortmannin® (20 μM)**

Wortmannin is a PI3K class I and III inhibitor used for autophagy inhibition. 1mg Wortmannin was dissolved in 467μL Dimethylsulfoxide (DMSO) to make a 100mM stock and stored at -20°C. 5μL stock/mL culture media was used to make a 20μM final concentration.

##### **2.3.8.3.2 3-methyl- adenine (5 mM)**

3-methyl adenine (3MA) is a PI3K class III inhibitor used for autophagy inhibition. 100mg 3MA was dissolved in 6.7mL DMSO to make a 100mM stock. The solution was stored at room temperature in the dark. 50μL stock/mL culture media was used to make a 5mM final concentration.

#### **2.3.8.3.3 Caffeine (20 $\mu$ M)**

Caffeine was used as an ATM inhibitor. 39mg was dissolved in 1mL ddH<sub>2</sub>O on shaker at room temperature to make a 200 $\mu$ M stock. The solution was stored at 4°C in the dark. 10 $\mu$ L stock/mL culture media was used for a 20 $\mu$ M final concentration.

#### **2.3.8.3.4 Rapamycin (50 nM)**

Rapamycin was used to inhibit mTOR. A 50mM stock in DMSO was stored at -20°C. 1 $\mu$ L stock/mL culture media was used to make a 50 $\mu$ M final working concentration.

#### **2.3.8.4 Fixation of mammalian cells**

##### **2.3.8.4.1 3% Paraformaldehyde**

Under a fume hood, 300mg paraformaldehyde was added to 10mL 1xPBS supplemented with 5 $\mu$ L 10N NaOH. The solution was heated to 55-65°C (not above 65°C) to dissolve the paraformaldehyde. The solution was filtered and stored at -20°C.

#### **2.3.8.5 Bacterial Cell Culture**

##### **2.3.8.5.1 LB Broth**

The following was dissolved in 1L ddH<sub>2</sub>O 20g Tryptone, 5g Yeast extract, 0.5g NaCl. The solution was sterilized by autoclave prior to use.

##### **2.3.8.5.1.1 Luria-Bertani (LB) agar**

LB broth (discussed above) was supplemented with 2% bacteria agar (catalog no. BD1426, Fisher). The solution was autoclaved, and plates were poured before the solution cooled.

#### **2.3.8.5.1.2 Super Optimal Culture (SOC) buffer**

The following was dissolved in 1L ddH<sub>2</sub>O: 20.0g tryptone, 5.0g yeast extract, 0.5g NaCl, 0.5g KCl. The pH was adjusted to 7.0. The solution was sterilized by autoclave. 2.5mL 2M Mg<sup>2+</sup> Stock\* and 2.5mL 1M glucose was added immediately before use.

#### **2.3.8.5.1.3 CaCl<sub>2</sub> Solution**

3.3g PIPES was dissolved into 425mL ddH<sub>2</sub>O. The pH was adjusted to 7.0. 3.33g CaCl<sub>2</sub> was added (for dihydrate 4.41g was added), and 75mL glycerol was added. The solution was sterilized by filtration or autoclave prior to use.

#### **2.3.8.5.1.4 1x TE Buffer**

The following were dissolved in 990mL ddH<sub>2</sub>O: 10mL 1M Tris-HCl (pH 8) and 400uL 0.25M EDTA.

### **2.3.8.6 Protein Extraction and Analysis – western blotting and FACS**

#### **2.3.8.6.1 Ripa buffer**

A solution containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5 mM EDTA) was made in 10mL ddH<sub>2</sub>O. One protease inhibitor cocktail® tablet (catalog no. 11836-170-001; Roche) was dissolved into 10mL solution. The solution was aliquotted at 1mL/each and stored at -20°C. (For 100mL: 0.12g Tris was dissolved in 96.72mL ddH<sub>2</sub>O, the pH was adjusted to 7.4 before adding 0.88g NaCl, 1mL Triton-X 100®, 1g deoxycholic acid, 0.1g SDS, and 0.1861g EDTA).

#### **2.3.8.6.2 2x Sample Buffer**

The following was dissolved in 4.5mL ddH<sub>2</sub>O : 6.5mL 0.5M Tris/SDS (pH 6.8), 6.25mL 80% glycerol, 5mL 10% SDS, 2.5mL BromoPhenol Blue (1mg/mL) and 0.5mL β-mercaptoethanol

#### **2.3.8.6.3 30:08 Acrylamide**

To make a 30% acrylamide/0.8% bisacrylamide solution, 150g Acrylamide and 4g N,N'-methylene bisacryl was dissolved in 500mL ddH<sub>2</sub>O. The solution was stored at 4°C.

#### **2.3.8.6.4 Bromophenol Blue**

1mg bromophenol blue was dissolved in 3mL depH<sub>2</sub>O and 7mL glycerol was added.

#### **2.3.8.6.5 10mM Tris, pH 7.6**

0.12g Tris powder was dissolved in 1L ddH<sub>2</sub>O. The pH was adjusted to 7.6 using HCl.

#### **2.3.8.6.6 4x Tris/SDS, pH 8.8**

91g Tris base, 20mL 10% SDS was dissolved in 500mL ddH<sub>2</sub>O. The pH was adjusted to 8.8 using 6N HCl.

#### **2.3.8.6.7 0.5M Tris/SDS, pH 6.8**

4mL 10% SDS was added to 100mL 0.5M Tris (pH 6.8).

#### **2.3.8.6.8 5x SDS electrophoresis running buffer**

7.53g Tris, 36g glycine and 2.5g SDS were dissolved in 500mL ddH<sub>2</sub>O. The solution was diluted to 1x before running gels.

#### **2.3.8.6.9 10% SDS**

10g sodium dodecyl sulfate was dissolved in 100mL ddH<sub>2</sub>O.

#### **2.3.8.6.10 Coomassie Blue Solution**

2g Coomassie Blue were dissolved in 250mL ddH<sub>2</sub>O. 75mL of glacial acetic acid and 500mL of ethanol were added before bringing the final volume to 1000mL with ddH<sub>2</sub>O. The final concentrations of this solution are: 0.2% Coomassie Blue, 7.5% acetic acid and 50% ethanol.

#### **2.3.8.6.11 Destain Solution**

50mL methanol and 100mL acetic acid were mixed and the final solution volume was brought to 1000mL with ddH<sub>2</sub>O.

#### **2.3.8.6.12 FACS incubation buffer**

0.5g of Bovine Serum Albumin Fraction V was dissolved into 100mL of 1xPBS. The solution was made fresh just prior to use.

### **2.3.8.7 Influenza A Culture**

#### **2.3.8.7.1 Influenza A diluting media**

0.6 ml 35% BSA and 1 ml 100x CaMg solution (1g CaCl<sub>2</sub>, 1g MgCl<sub>2</sub> dissolved in 100mL ddH<sub>2</sub>O) were dissolved in 10mL 10xPBS. CaMg was mixed in last to avoid precipitate formation.

#### **2.3.8.7.2 Influenza A plaquing media**

0.25mL 1% DEAE Dextran, 0.5mL 5% NaHCO<sub>3</sub> and 2.5mL ddH<sub>2</sub>O were added to 14mL plaquing MEM Mix (2x EMEM, subtract 172μL/mL from

final desired volume, 20 $\mu$ L/mL 1.0M HEPES, 20 $\mu$ L/mL 200mM L-glutamine, 48 $\mu$ L/mL 5% NaHCO<sub>3</sub>, 84 $\mu$ L/mL 5% BSA, 10 $\mu$ L/mL PenStrep). The solution was mixed before the addition of 7.5mL warmed 2% high purity agar. All reagents were sterile and warm before mixing. The solution was allowed to cool slightly before adding to cells.

### **2.3.8.7.3 Crystal Violet Staining Solution**

4.0 mL of 1% crystal violet solution in H<sub>2</sub>O and 8 mL methanol were added to 30 mL ddH<sub>2</sub>O. The solution was made fresh just prior to use.

### **2.3.8.8 Flavivirus Culture**

#### **2.3.8.8.1 Flavivirus dilution media**

For 500mL of flavivirus diluting media, 3.5g NaCl, 1.55g H<sub>3</sub>BO<sub>3</sub>, 3.75g Bovine Serum Albumin fraction V was dissolved in 500mL ddH<sub>2</sub>O. The solution was filter-sterilized into autoclaved 100mL bottles and stored at 4°C). The final concentrations are : 0.75% Bovine serum albumin fraction V in 0.12 M NaCl and 0.05 M H<sub>3</sub>B0<sub>3</sub>.

#### **2.3.8.8.2 Flavivirus plaquing media I**

1.5% methyl cellulose (catalog no. 274429; Sigma-Aldrich) in EMEM® (catalog no. 12-668E; BioWhittaker) was supplemented with 2% heat-inactivated fetal bovine serum (FBS) (catalog no. LSF0500; Equitech-Bio), 50U/mL Penicillin-Streptomycin (PenStrep) (catalog no. 15140-122; Gibco) and 1.5 g/liter sodium bicarbonate (catalog no. S8875; Sigma-Aldrich). All media components were sterile before mixing.

#### **2.3.8.8.3 Flavivirus plaquing media II**

1.0% purified agar (catalog no. LP0028; Oxoid) in EMEM® (catalog no. 12-668E; BioWhittaker) supplemented with 2% heat-inactivated fetal bovine serum (FBS) (catalog no. LSF0500; Equitech-Bio), 50U/mL Penicillin-Streptomycin (PenStrep®) (catalog no. 15140-122; Gibco), 1.5 g/liter sodium bicarbonate (catalog no. S8875; Sigma-Aldrich) and 1:36000 neutral red. All media components were sterile before mixing.

#### **2.3.8.8.4 Flavivirus plaquing media III**

1.0% low melting point Agar (catalog no. V2111, Promega) in EMEM® (catalog no. 12-668E; BioWhittaker) was supplemented with 5% heat-inactivated fetal bovine serum (FBS) (catalog no. LSF0500; Equitech-Bio) and 50U/mL Penicillin-Streptomycin (PenStrep®) (catalog no. 15140-122; Gibco). The solution was allowed to cool slightly before applying to cells.

### **3 Chapter 1. Influenza A Manipulation of Cell Death**

While specific anti-virals exist to combat Influenza A infection, the rapid evolution of resistant (the recent H1N1 pandemic) and deadly (the recent H5N1 epidemics in Southeast Asia) strains of the virus underline the importance of identifying novel biochemical targets for anti-viral drug development. Apoptosis and subsequent caspase-3 activity is required for maximum Influenza A replication, suggesting that the biochemical components of this pathway may be manipulated to minimize virus replication in infected individuals. Influenza A induces apoptosis by a number of mechanisms, and encodes viral proteins that promote the activity of apoptosis-related pathways, including NS1 and PB1-F2. In this chapter, we present results identifying Bax, an intracellular protein that modulates apoptosis by regulating cytochrome c release, as required for maximum Influenza A replication, and present a mechanism explaining the Bax-dependence of Influenza A replication.

#### **3.1 The Global Importance of Influenza A virus**

Diseases associated with Influenza A (of the viral family Orthomyxoviridae), are distributed worldwide and continue to threaten the lives of hundreds of thousands of people per year despite extensive research, vaccines and treatment options. Influenza A virus affects both humans and animals with high mortality and morbidity. Several pandemics have occurred with this virus in the past century, including the 1918 pandemic that killed over 20 million people worldwide. Influenza infection and influenza-related complications such as pneumonia claim the lives of tens of thousands of people each year. Over 80% of these deaths occur in individuals over the age of 65 (Tompkins 2004).

Another pandemic (*e.g.* the recent H5N1 Asian flu crisis) could occur in the near future since antigenic shifts in the virus occur readily (Chen 2004). Currently, Influenza A is responsible for illness in millions of people worldwide, and annually causes ~20,000 deaths in the United States (Tompkins 2004).

Influenza A belongs to the family Orthomyxoviridae, a group that contains several related influenza viruses. Influenza A is the most virulent human pathogen of the group, infecting a range of vertebrate hosts, including humans, horses, ferrets, pigs and birds. Influenza B virus is closely related to Influenza A, and also causes disease in mammals, but it is much less severe and has only one known serotype. Much less is known about Influenza C viruses, which are not known to cause disease and are morphologically and genetically distinct from the other Influenza viruses (Shaw 1992).

## **3.2 Influenza A Genome and Replication Cycle**

### **3.2.1 Influenza A virus Genome**

Influenza A is an enveloped, negative strand RNA virus. The Influenza A genome consists of eleven genes organized into eight discrete genome segments. All genome segments are associated with viral NP and PB1, PB2, and PA proteins, creating ribonucleoprotein (RNP) complexes that translocate from the nucleus (where they originate) to virus assembly centers in the cytosol for packaging into progeny virions (Tao and Ye 2010). The genome segments are identified by their relative size, and are enumerated largest to smallest, 1-8. The largest genome segment (first segment) encodes the viral polymerase PB2, a component of the virus replication complex (Steel, Lowen et al. 2009). The next largest genome segment (second segment) encodes the viral polymerase PB1, another component of the virus replication complex (Wunderlick,

Juozapaitis et al. 2011). This segment also encodes a second protein, PB1-F2, expressed due to a reading frame shift during translation (McAuley, Zhang et al. 2010). Upon expression, PB1-F2 localizes to the mitochondria and sensitizes infected cells to apoptosis. The third segment encodes viral polymerase PA, another component of the virus replication complex. The fourth segment encodes the surface glycoprotein hemagglutinin (HA), which is a major antigenic determinant and essential for the virus binding to the host cell receptor (Langley, Thoennes et al. 2009). The fifth segment encodes the nucleocapsid protein (NP), which binds negative sense virus RNA and forms the ribonucleoprotein (RNP) complexes that function as genome segments (Coloma, Valpuesta et al. 2009). The sixth segment encodes the neuraminidase (NA) protein, another antigenic determinant that is involved in virus release from infected cells (Lai, Chan et al. 2011). The seventh segment encodes two proteins, Matrix Protein 1 (M1) and M2 (Schwahn, Wong et al. 2010). The eighth RNA segment encodes two non-structural virus genes: non-structural protein 1 (NS1) and NS2. This segment itself encodes only for NS1, and NS2 is a splice derivative of NS1 mRNA (Robb, Smith et al. 2009). Specific mechanisms of function for each virus protein are discussed below.

### **3.2.1.1 Influenza A proteins**

#### **3.2.1.1.1 Influenza A structural proteins**

Nine of the eleven Influenza A virus proteins are found as structural components of the Influenza A virion, and are thus termed structural proteins. Although these nine proteins play an important role in the physical structure of the virus, they also have essential roles in the manipulation of host cell function during infection. Their

interaction with both virus and host cell factors during infection drives viral replication and release.

#### **3.2.1.1.1 RNA-dependent RNA polymerase complex (PB2-PB1-PA)**

The three virus proteins that compose the virus replication complex were originally named based on their behavior on an isoelectric focusing gel. Two were basic, thus named PB proteins, and the other was acidic, PA. Upon viral entry and decapsidation, targeting sequences contained in the viral nucleocapsid protein (discussed below) induce the translocation of viral RNPs to the nucleus, where transcription of the genome begins. PB2 attaches to the 5' m7G cap of host cell RNAs, and PB1 cleaves this cap. The host RNA cap remains associated to PB2 and serves as a primer for limited PB2 polymerase activity. Once PB2 has added several nucleotides to the stolen RNA cap it dissociates. PB1, together with PA, then complete transcription of the genome segment, leading to (+) sense vRNA strands (Steinhauer 2002). Two types of (+) sense vRNA are transcribed from each segment. Incomplete, polyadenylated (+) sense vRNA strands are created due to a polyadenylation signal at the 5' end of the template vRNA. These incomplete vRNA's translocate to the cytoplasm and serve as mRNA's for viral protein synthesis. Read-through of this polyadenylation signal causes the transcriptional completion of template vRNA. Non-polyadenylated, (+) sense vRNA's serve as cRNA templates for synthesis of progeny (-) sense RNA genome segments within the nucleus (Krug 2001).

### 3.2.1.1.1.2 Hemagglutinin (HA)

Hemagglutinin is one of two surface proteins that coat the Influenza A virion (along with neuraminidase, discussed below). Hemagglutinin binds Influenza A virions to the cell surface via associations with terminal neuraminic acid residues on sialic acid molecules that coat target cells (Gubareva, Kaiser et al. 2000, Langley, Thoennes et al. 2009)). Once bound, viral particles typically stimulate clathrin-mediated endocytosis and are subsequently exposed to the low pH of cellular endosomes. The low pH within endosomes is neutralized by the viral M2 protein (discussed below), which is activated upon endosomal entry and serves as a viral proton pump. Low pH stimulates cleavage of the viral HA protein, forming HA<sub>1</sub> and HA<sub>2</sub>. HA cleavage causes an activating conformational change in the trans-membrane HA<sub>2</sub> subunit, fusing the viral membrane with the endosomal membrane, allowing viral uncoating and genome release into the host cytosol (Shaw 1992, Langley, Thoennes et al. 2009).

There are 15 known HA serotypes and 9 known NA serotypes of Influenza A (Govorkova, Tien et al. 2005). The interplay between infections in different vertebrate hosts allows the recombination of these serotypes, leading to new viral strains every year. Avian and mammalian strains of Influenza A have different HA serotypes due to amino acid position 226 of the HA molecule, presenting an effective species barrier against cross-infection. However, pigs may be infected with both strains simultaneously. This leads to a dangerous “melting pot” effect in which both human and avian strains may recombine to form novel strains of the virus for which there is no protective immunity. For this reason many pandemics have originated in rural areas where animal and human hosts are in close contact (Zhou 1999, Smith, Bahl et al. 2009).

### **3.2.1.1.1.3 Nucleocapsid protein (NP)**

Upon translation, Influenza A NP protein translocates to the nucleus where it associates with newly replicated viral progeny genome segments (Ozawa, Fujii et al. 2007), as do components of the viral replication complex: PB1, PB2 and PA. The NP gene encodes a polypeptide that is 498 amino acids long (Shu, Bean et al. 1993). Multiple NP's bind to each strand of newly synthesized (-)-sense viral ssRNA within the nucleus, forming viral ribonucleoprotein complexes (RNP's) (Martin-Benito, Area et al. 2001). Each of these RNPs is then bound to a PB1-PB2-PA virus replication complex to form a complete genome segment.

The generation of Influenza A genome segments and their successful incorporation into progeny virions is wholly dependent upon NP interactions with viral ssRNA (vRNA) and other proteins, as well as NP homo-oligomerization. The N-terminal third of the NP protein serves as an RNA binding domain and it allows NP association with vRNA. NP-vRNA binding occurs with high affinity, but the interaction has little vRNA sequence specificity (Elton, Medcalf et al. 1999). NP binds to the phosphate backbone of vRNA (Baudin, Bach et al. 1994). vRNA then becomes extensively wrapped around NP molecules at an approximate ratio of 24 nucleotides per 1 NP molecule (Ortega, Martin-Benito et al. 2000; Martin-Benito, Area et al. 2001). Several NP's bind to each vRNA during RNP formation, and subsequent NP homo-oligomerization stabilizes the structure of RNPs during assembly. There are two specific binding regions for NP-NP interactions, one between amino acids 189-358, and another between amino acids 371-465 (Portela and Digard 2002). RNA-free NPs associate to form structures morphologically similar to RNPs (Ruigrok and Baudin 1995), indicating that NP-NP

associations determine the overall morphological structure of Influenza A viral genome segments.

Association of NP-vRNA to the virus polymerase complex (PB2-PB1-PA) is also dependent upon NP interactions. The binding of NP to the virus replication complex is dependent upon separate NP interactions with the PB1 and PB2, but not PA proteins (Biswas, Boutz et al. 1998; Medcalf, Poole et al. 1999; Martin-Benito, Area et al. 2001). These interactions tether the replication complex to the RNP, completing the formation of the viral genome segment. The amount of viral NP protein in the nucleus is thought to control the proportion of viral mRNA to cRNA produced, suggesting that viral NP is an important regulator of initiation of viral assembly. The Influenza A M1 protein (discussed below) then associates with vRNPs via both RNA and NP interactions. Targeting sequences on M1 then stimulate vRNP transport to cytoplasmic viral assembly centers where each genome segment is incorporated into newly synthesized progeny virions.

#### **3.2.1.1.1.4 Neuraminidase (NA)**

Neuraminidase is one of two Influenza A surface proteins (along with HA, described above), and is a major antigenic determinant. The variation of HA and NA between Influenza A strains allows the development of virus resistance to the immune system and necessitates the formulation of new seasonal Influenza vaccines each year. NA destroys the  $\alpha$ -ketosidic bond in the Influenza binding receptor sialic acid, between the sialic acid glycoprotein and its terminal neuraminic acid residue. This cleaves neuraminic acid from sialic acid, thereby destroying the region of the molecule supporting HA association, removing Influenza A binding sites (Gubareva, Kaiser et al.

2000). As progeny virions mature in the endosomes, the HA molecule becomes active and binds to sialic acid binding receptors on the host cell membrane, thereby inhibiting release of viral progeny if surface receptors are not removed. NA-mediated cleavage of these receptors ensures that new virions being produced do not bind to the host cell membranes during exocytosis (Kash 2004).

Influenza A binding receptors are also found in the mucus surrounding the airway epithelia. Receptors in the mucosa bind Influenza A HA, stopping the virus before it reaches the tissue surface for infection. NA-mediated destruction of these receptors by neuraminic acid cleavage allows penetration of virions through the mucosa to the cell surface to allow infection (Moscona 2005). Newly formed HA molecules also possess neuraminic acid residues. NA cleaves these residues to inhibit self-aggregation. The Influenza A antivirals Zanamivir<sup>®</sup> and Oseltamivir<sup>®</sup> inhibit NA activity, thus causing progeny virions to bind to host cell membranes, to receptors in the mucosa, causing self-aggregation of viral progeny and therefore inhibiting viral release.

#### **3.2.1.1.1.5 The Matrix Proteins (M1 and M2)**

The Influenza A M1 protein is the most abundant structural protein within the Influenza virion. Newly expressed M1 proteins are transported to the nucleus early in infection, and bind to vRNPs (Rey and Nayak 1992). Multiple M1 domains participate in its association with vRNPs, including an RNA binding region and several NP interacting regions (Bui, Whittaker et al. 1996; Baudin, Petit et al. 2001; Huang, Liu et al. 2001). M1 then mediates vRNP transport from the nucleus and incorporation of genomic segments into progeny virions by interacting with Influenza NS2 (discussed below). Once in the cytoplasm, M1 collects at intracellular membranes causing them to thicken.

Newly expressed HA and NA proteins incorporate into these thickened membrane regions (Whittaker, Kemler et al. 1995; Bui, Wills et al. 2000). vRNP genome segments are thus packaged into the budding viral particle as it protrudes from the membrane. In a mature virion, the M1 protein forms a shell at the internal surface of the virus membrane (Nermut 1972; Schulze 1972), between the membrane and vRNP genomic segments. This occurs primarily through electrostatic interactions with the virus membrane, and does not rely on membrane insertion of M1 (Ruigrok, Barge et al. 2000).

A minor component of the membrane of mature virions, the M2 protein is 97 amino acid transmembrane protein that exists as a homotetramer in the viral membrane (Sugrue and Hay 1991). M2 is a membrane-bound proton pump activated upon virus entry and incorporation into endosomes (Lamb, Zebedee et al. 1985). The low pH of the endosomes activates M2 prior to virus-host membrane association, and the acidification of the internal compartment of the virus disrupts the association of M1 and vRNPs, allowing their release into the cytosol (Pinto, Holsinger et al. 1992). M2 also prevents premature low-pH induced HA cleavage and subsequent binding to host cell membranes late in infection by equilibrating the pH of trans-Golgi compartments to that of the cytosol (Ciampor, Bayley et al. 1992; Henkel, Gibson et al. 2000).

Influenza anti-viral drugs Amantidine<sup>®</sup> and Rimantidine<sup>®</sup> target M2, reducing its functionality as a proton pump and therefore inhibiting the decapsulation of infecting viruses, stopping the replication cycle before it begins (Hay, Wolstenholme et al. 1985; Cady and Hong 2008). Several amantidine-resistant strains of Influenza A have evolved and currently circulate in North America and Asia (Bright, Medina et al. 2005; Saito, Suzuki et al. 2008). This resistance has been mapped to mutations in the transmembrane

domain of M2 (Wang, Takeuchi et al. 1993), indicating that M2 proton pump activity is essential for Influenza A replication.

#### **3.2.1.1.1.6 Nuclear Export Protein (NEP)**

The nuclear export protein was originally termed non-structural protein 2 (NS2) because it was not initially detected in Influenza A virions. However, the protein was eventually found associated with M1 within the interior of the virus (Richardson and Akkina 1991; Yasuda, Nakada et al. 1993) and the name was changed (O'Neill, Talon et al. 1998). NS2 associates with vRNPs in the nucleus via interactions with M1 (Yasuda, Nakada et al. 1993; Akarsu, Burmeister et al. 2003) and subsequently facilitates the export of virus RNPs from the nucleus via associations with both vRNPs and the host nuclear export machinery. Although NEP contains a nuclear export signal (NES), the disruption of this domain does not inhibit vRNP export from the nucleus. However, NEP interacts with cellular export proteins including CRM1, which is sufficient to transport the vRNP-M1-NS2 complex to the cytoplasm (Neumann, Hughes et al. 2000). NEP also interacts directly with cellular nucleoporins, serving as an adaptor molecule for nucleoporin-vRNP interactions and facilitating rapid nuclear export of viral genomic segments (O'Neill, Talon et al. 1998).

#### **3.2.1.1.2 Influenza A non-structural proteins (NS1 and PB1-F2)**

Influenza A non-structural protein 1 (NS1) and PB1-F2 are abundant in infected host cells, but are not detectable in actual virions, hence their designation as nonstructural proteins. The NS1 protein is an important virulence factor (Li, Jiang et al. 2006) and contributes to the evasion of innate immunity by inhibiting the activation of several

Interferon  $\alpha/\beta$  -induced antiviral pathways (Fernandez-Sesma, Marukian et al. 2006; Kochs and Garcia-Sastre 2007). NS1 binds dsRNA replication intermediates, sequestering them from interaction with cellular dsRNA sensors (Wang, Riedel et al. 1999; Donelan, Basler et al. 2003) including retinoid-inducible gene-1 (RIG1) (Mibayashi, Martinze-Sobrido et al. 2007) and preventing the activation of transcription factors including interferon regulatory factor-3 (IRF3) (Talon, Horvath et al. 2000), PKR (Bergmann, Garcia-Sastre et al. 2000), Jun N-terminal kinase (JNK) (Ludwig, Wang et al. 2002) and nuclear factor  $\kappa$ B (NF- $\kappa$ B) (Wang, Li et al. 2000). NS1 sequestration of dsRNA also inhibits dsRNA-stimulated OAS/RNaseL (Min and Krug 2006) and suppresses the induction of RNA interference by host cells (Li, Li et al. 2004). NS1 also blocks nuclear export of polyadenylated host mRNAs to the cytoplasm (Fortes, Beloso et al. 1994; Qian, Alonso-Caplen et al. 1994; Qiu and Krug 1994) and inhibits pre-mRNA splicing (Fortes, Beloso et al. 1994; Lu, Qian et al. 1994), effectively shutting down the translation of host gene products. NS1-induced retention of host mRNA's in the nucleus also makes these transcripts available to PB2-PB1 for cleavage and mRNA cap association with vRNA (Chen, Li et al. 1999). In addition, NS1 forms a complex with eukaryotic translation initiation factor 4G1 (eIF4G1) and poly-(A) binding protein 1 to stimulate translation of viral RNA (Burgui, Aragon et al. 2003).

The viral PB1-F2 protein is an 87 amino acid, pro-apoptotic influenza A viral protein (Chen, Calvo et al. 2001). PB1-F2 protein is translated from an alternate reading frame in the genome segment that encodes the viral PB1 protein. PB1-F2 contains a mitochondrial targeting sequence (Yamada, Chounan et al. 2004). The interaction of PB1-F2 with proteins in the inner mitochondrial membrane (ANT3) and the outer

mitochondrial membrane (VDAC-1), results in changes to mitochondrial permeability during apoptosis, allowing cytochrome c release and subsequent caspase activation (Zamarin, Garcia-Sastre et al. 2005). This sensitizes infected cells to other cell death stimuli, contributing significantly to the pathogenesis of the disease (Zamarin 2005).

Although promoting cell death may seem counterintuitive to viral propagation, the activation of the caspase cascade actually promotes the processing and maturation of influenza virus (Wurzer 2003). Also, sensitization to apoptosis reduces the amount of cytokines an infected cell may release, limiting the effectiveness of intercellular antiviral signaling through the TNF $\alpha$  pathway (Levy 2001). The finding that influenza PB1-F2 protein induces cell death more readily in human monocytes than other cell types indicates that influenza eludes host defenses by killing them preferentially, leaving non-immune cells alive for continued viral propagation (Chen 2001). Infection with recombinant virus lacking this protein reduces the rate of apoptosis in lymphocytes (Chen, Calvo et al. 2001). ANT3 and VDAC1, which both associate with PB1-F2 at the mitochondrial membrane, are expressed in varying levels according to cell type, which may explain why some cells are more prone to PB1-F2 mediated apoptosis induction than others (Stepien 1992, Mitzner, Dudek et al. 2009, LeGoffic, Bouguyon et al. 2010).

### **3.2.2 Influenza A and cell death**

Influenza A is a major respiratory pathogen, affecting both humans and domestic animals with high morbidity and mortality. Influenza A infection usually results in fever, muscle ache, headache and dry cough (Georgantopoulos, Bergquist et al. 2009). However, several highly lethal pandemics have occurred with this virus in the past century, including the infamous 1918 pandemic that killed over 50 million people

worldwide (Kash, Tumpey et al. 2006). Currently, Influenza A is responsible for illness in millions of people worldwide, and causes ~20,000 deaths in the United States annually (Tompkins 2004; Thompson, Moore et al. 2009), 80% of which occur in individuals over the age of 65 (Tompkins 2004). Another pandemic (e.g. the recent H5N1 Asian bird flu and H1N1 swine flu crises) could occur in the near future because antigenic shifts in the virus occur readily (Chen 2004; Malik-Peiris, Poon et al. 2009; Wang and Palese 2009).

Despite the worldwide prevalence of Influenza A, the directed induction of death of infected cells by the virus was not realized until the early 1990's, when Takizawa, Matsukawa et al demonstrated that Influenza A infection of HeLa and MDCK cells results in DNA laddering characteristic of apoptosis (Takizawa, Matsukawa et al. 1993). Furthermore, Hinshaw, Olsen et al demonstrated that influenza A induced severe lymphocyte depletion both *in vivo* and *in vitro*, with infected lymphocytes displaying DNA laddering and morphological changes characteristic of apoptosis, including membrane blebbing and nuclear condensation (Hinshaw, Olsen et al. 1994). Further *in vivo* research demonstrated that infection of mice leads to death of bronchial and alveolar epithelial cells (Mori, Komatsu et al. 1995). Although the specific virus-host interactions leading to Influenza A-induced apoptosis were not revealed during this time, these early experiments paved the way for further research that has since identified several Influenza A manipulations of both extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) apoptosis-related signaling pathways.

Influenza A induces apoptosis in cultured cells as well as *in vivo* (Ito, Kobayashi et al. 2002). Apoptosis following Influenza A infection is mediated by the caspase family of

cysteine proteases (discussed above) (Lin, Holland et al. 2002; Wurzer 2003). The consequences of this activation for virus replication or host cell defense are still under debate (Lowy 2003; Ludwig, Pleschka et al. 2006). Caspase-3 expression is required for efficient Influenza A virus propagation (Wurzer 2003; Wurzer, Ehrhardt et al. 2004; McLean, Datan et al. 2009). However, caspase-mediated cleavage of the Influenza A nucleoprotein (NP) limits the amounts of viral protein required for proper assembly (Zhirnov, Konakova et al. 1999). Furthermore the Influenza A Matrix-1 (M1) protein specifically binds to caspase-8 and weakly to caspase-7, suggesting involvement of M1 in caspase-8 mediated apoptosis (Zhirnov, Konakova et al. 2002). Pharmaceutical inhibition of caspases does not result in a severe change in cell death (Matassov 2004), although virus replication is strongly impaired in the presence of caspase inhibitors or where caspase-3 is partially knocked-down by siRNA (Wurzer 2003). Consistent with these findings, poor Influenza A replication in caspase-deficient cells is ameliorated by expression of ectopic caspase-3 (Wurzer, Ehrhardt et al. 2004). Mechanistically, the block in virus propagation caused by loss of caspases is due to the retention of viral RNP complexes in the nucleus (Wurzer 2003; McLean, Datan et al. 2009). Caspase activation also inhibits active nuclear export by cleavage of cellular transport proteins. This suggests an alternative strategy by which caspases may regulate RNP export (Faleiro and Lazebnik 2000; McLean, Datan et al. 2009). Furthermore, isolated NP or RNP complexes (discussed above) can partially translocate to the cytoplasm when caspases are activated by other apoptosis inducers (Wurzer 2003). Additionally, overexpression of the anti-apoptotic protein Bcl-2 results in impaired virus production due to a misglycosylation of the viral surface protein HA. Upon infection of Bcl-2

overexpressing cells, viral RNP complexes are retained in the nucleus, resulting in repressed virus titers (Olsen, Kehren et al. 1996). Thus Influenza seems to take advantage of the early events of apoptosis, racing the impending death of host cells to minimize the host immune response while maximizing viral production.

Initial exploration of the mechanisms surrounding Influenza A-induced caspase activation and apoptosis was focused on the extrinsic (receptor-mediated) pathway of apoptosis. Transforming growth factor-beta (TGF- $\beta$ ) is upregulated *in vitro* in MDCK cells as well as *in vivo* in mice infected with the virus (Schultz-Cherry and Hinshaw 1996) resulting in increased apoptosis following Influenza A infection. TGF- $\beta$  upregulation was shown to be under the influence of the viral neuraminidase (NA) protein (Schultz-Cherry and Hinshaw 1996), and NA inhibitors attenuate the apoptosis phenotype during infection (Morris, Price et al. 1999). It also attenuates the development of immune-specific cells following infection in neonatal mice (Hoskins, Lines et al. 2010).

The functionally related Tumor Necrosis Factor-Alpha (TNF- $\alpha$ ) is also upregulated following Influenza A infection, promoting the destruction of macrophages and B-cells via the extrinsic apoptosis pathway (Nain, Hinder et al. 1990; Peschke, Bender et al. 1993; Sedger, Hou et al. 2002). Additionally, expression of Influenza A non-structural protein 1 (NS1) also increases expression of Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) (Schultz-Cherry, Dybdahl-Sissoko et al. 2001; Brincks, Kucaba et al. 2008), providing further means of apoptosis induction following infection while concurrently promoting virus replication (Wurzer, Ehrhardt et al. 2004). NS1 also

prevents antiviral interferon signaling by inhibiting the activation of interferon regulatory factor 3 (IRF-3), a key regulator of IFN  $\alpha/\beta$  gene expression (Talon, Horvath et al. 2000; Garcia-Sastre 2004). Type I Interferons are inducers of influenza A-induced apoptosis (Balachandran, Roberts et al. 2000), and interferon-mediated apoptosis following infection limits the release of pro-inflammatory cytokines, further reducing the presence of immune cells in infected tissues (Brydon, Smith et al. 2003).

In addition to TGF- $\beta$  and TNF- $\alpha$  upregulation following infection, the Fas/FasL apoptosis inducing receptor/ligand system is expressed in a PKR-dependent manner in Influenza A infected cells (Peschke, Bender et al. 1993; Takizawa, Ohashi et al. 1996; Fujimoto, Takizawa et al. 1998), contributing to virus-induced cell death through the FADD/caspase 8-dependent pathway (discussed above) (Balachandran, Roberts et al. 2000). Inhibition of intracellular protein trafficking by Brefeldin A inhibits Influenza A-induced cell death in MDCK cells, prompting the hypothesis that death receptor expression and transport to the cell surface are necessary for the virus to kill the cell (Saito, Tanaka et al. 1996).

Although cell death following Influenza A infection occurs in part through the extrinsic apoptosis pathway, several host intracellular proteins have been identified that induce apoptosis via the intrinsic apoptosis pathway during infection. Viral activation of mitogen-activated kinases (MAPK's) has been linked to the onset of apoptosis. In cultured MAPK-kinase (MAPKK)-deficient mouse embryonic fibroblast cells, Influenza A-induced p38 and JNK activation are inhibited concomitantly with inhibition of caspase 3 activation, resulting in reduced apoptosis (Maruoka, Hashimoto et al. 2003, Hui et al.

2009). Furthermore, during infection with neurovirulent Influenza A JNK (but not p38) activity correlates with the induction of apoptosis in the brain tissue of infected mice (Mori, Goshima et al. 2003).

The transcription factor Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) also promotes Influenza A-induced apoptosis and replication, an effect that is dominant over the typical antiviral activity of NF- $\kappa$ B (Grandvaux, Tenover et al. 2002; Tsuchida, Kawai et al. 2009). This is likely due to NF- $\kappa$ B induction of expression of proapoptotic factors, such as TRAIL or FasL, which are important mediators of virus replication (Wurzer, Ehrhardt et al. 2004). Moreover, the viral requirement for NF- $\kappa$ B activity suggests that antiviral drugs could target this pathway. It has recently been demonstrated that inhibitors of NF- $\kappa$ B act as antiviral therapeutics *in vivo* (Ludwig, Planz et al. 2003). p53 expression and activity are also up-regulated during influenza infection (Marriott and Dockrell 2010), and p53 is required for Influenza A-induced apoptosis (Turpin, Luke et al. 2005). Moreover, a decrease in p53 activity prevents interferon activation, resulting in an increase in Influenza A replication that may be a direct result of a decreased interferon response (Turpin, Luke et al. 2005).

The Influenza A PB1-F2 protein is a pro-apoptotic protein (Chen, Calvo et al. 2001) translated from an alternate reading frame in the genome segment that encodes the viral PB1 protein (discussed above). PB1-F2 induces apoptosis via the intrinsic mitochondrial pathway when expressed ectopically, and infection with recombinant Influenza A lacking this protein reduces the rate of apoptosis in lymphocytes (Chen, Calvo et al. 2001). PB1-F2 contains a mitochondrial targeting sequence and localizes to the outer mitochondrial

membrane following infection (Yamada, Chounan et al. 2004; Zell, Krumbholz et al. 2007). The interaction of PB1-F2 with proteins in the inner mitochondrial membrane (ANT3) and the outer mitochondrial membrane (VDAC-1), results in changes to mitochondrial permeability during apoptosis, allowing cytochrome c release and subsequent caspase activation (Zamarin, Garcia-Sastre et al. 2005; Chevalier, Al Bazzal et al. 2010). However, this effect is specific to immune cells, promoting the hypothesis that PB1-F2 induction of apoptosis has evolved specifically to clear tissues of immune responders during infection (Zamarin, Ortigoza et al. 2006).

While research has mainly focused on cell death by apoptosis as a result of Influenza A infection, involvement of other types of cell death cannot be ruled out. The fact that cell death following Influenza A infection is not affected *in vitro* by the presence of caspase inhibitors (Matassov 2004) suggests that other types of cell death (*i.e.* autophagy) may also play a role in the induction of cell death by the virus (Ehrhardt and Ludwig 2009; McLean, Datan et al. 2009; Zhou, Jiang et al. 2009; Lu, Masic et al. 2010). A major goal of the research presented Chapter 1 of this thesis is to identify additional intracellular factors that may promote apoptosis through the intrinsic apoptosis pathway following Influenza A infection.

### **3.3 Results, part 1: Published Results: “Lack of Bax Prevents Influenza A Virus-Induced Apoptosis and Causes Diminished Viral Replication.”**

McLean, J., E. Datan, D. Matassov, Z. Zakeri. (2009). "Lack of Bax Prevents Influenza A Virus-Induced Apoptosis and Causes Diminished Viral Replication." Journal of Virology 83(16): 8233-8246.

## **Lack of Bax Prevents Influenza A Virus-Induced Apoptosis and Causes**

### **Diminished Viral Replication**

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## **Lack of Bax Prevents Influenza A Virus-Induced Apoptosis and Causes Diminished Viral Replication**

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### **ABSTRACT**

The ectopic overexpression of Bcl-2 restricts both influenza A virus-induced apoptosis and influenza A virus replication in MDCK cells, thus suggesting a role for Bcl-2 family members during infection. Here we report that influenza A virus cannot establish an apoptotic response without functional Bax, a downstream target of Bcl-2, and that both Bax and Bak are directly involved in influenza A virus replication and virus-induced cell death. Bak is substantially downregulated during influenza A virus infection in MDCK cells, and the knockout of Bak in mouse embryonic fibroblasts yields a dramatic rise in the rate of apoptotic death and a corresponding increase in levels of virus replication, suggesting that Bak suppresses both apoptosis and the replication of virus and that the virus suppresses Bak. Bax, however, is activated and translocates from the cytosol to the mitochondria; this activation is required for the efficient induction of apoptosis and virus replication. The knockout of Bax in mouse embryonic fibroblasts blocks the induction of apoptosis, restricts the infection-mediated activation of executioner caspases, and inhibits virus propagation. Bax knockout cells still die but by an alternative death pathway displaying characteristics of autophagy, similarly to our previous observation that influenza A virus infection in the presence of a pan-caspase inhibitor leads to an increase in levels of autophagy. The knockout of Bax causes retention of influenza A virus NP

within the nucleus. We conclude that the cell and virus struggle to control apoptosis and autophagy, as appropriately timed apoptosis is important for the replication of influenza A virus.

## INTRODUCTION

The pathology of influenza A virus infection usually arises from acute lymphopenia and inflammation of the lungs and airway columnar epithelial cells (Mori, Komatsu et al. 1995; Nichols, Xu et al. 2001). Influenza A virus induces apoptotic death in infected epithelial, lymphocyte, and phagocytic cells, and apoptosis is a source of tissue damage during infection (Schultz-Cherry, Krug et al. 1998; Brydon, Morris, et al. 2005; McLean, Ruck, et al. 2008) and increased susceptibility to bacterial pathogens post-infection (Ramphal, Small et al. 1980). While the induction of apoptosis by influenza A virus has been well documented (Takizawa, Fukuda et al. 1995; Schultz-Cherry, Krug, et al. 1998; Nichols, Niles, et al. 2001; Brydon, Smith et al. 2003; Lowy 2003; Ludwig, Planz et al. 2003; Ludwig, Pleschka et al. 2006), the mechanisms of this interaction are not well understood. Two viral proteins, NS1 and PB1-F2, have been associated with viral killing of cells. NS1, originally characterized as being proapoptotic (Schultz-Cherry, Dybdahl-Sissoko et al. 2001), was later identified as being an interferon antagonist, inhibiting the activation of several key antiviral responses and restricting the apoptotic response to infection (Garcia-Sastre, Egorov et al. 1998; Bergmann, Garcia-Sastre et al. 2000; Wang, Zhang et al. 2000; Zhirnov, Konakova et al. 2002; Seo, Hoffman et al. 2004; Li, Min et al. 2006; Kochs, Garcia-Sastre et al. 2007). In contrast, PB1-F2 induces apoptosis primarily by localizing to the outer mitochondrial membrane, promoting cytochrome *c* release, and triggering the apoptotic cascade (Zamarin, Garcia-Sastre et al. 2005). This effect, however, is typically restricted to infected monocytes, leading to the hypothesis that PB1-F2 induces

apoptosis specifically to clear the landscape of immune responders (Chen, Calvo et al. 2001; Zamarin, Ortigoza et al. 2006). Although PB1-F2 activity does not directly manipulate virus replication or virus induced apoptosis, PB1-F2 localization to the mitochondrial membrane during infection potentiates the apoptotic response in epithelial and fibroblastic cells through tBID signaling with proapoptotic Bcl-2 family protein members Bax and Bak (Zamarin, Garcia-Sastre et al. 2005; Zamarin, Ortigoza et al. 2006; McLean, Ruck et al. 2008).

The Bcl-2 protein family consists of both pro- and antiapoptotic members that regulate cytochrome *c* release during mitochondrion-mediated apoptosis through the formation of pore-like channels in the outer mitochondrial membrane (Hinshaw, Olsen et al. 1994; Kroemer, Galluzzi et al. 2007). During the initiation of mitochondrion-mediated apoptosis, cytoplasmic Bid is cleaved to form tBID. This, in turn, activates proapoptotic Bax and Bak (Wei, Zong et al. 2001), which drive cytochrome *c* release and subsequent caspase activation. Bak is constitutively associated with the mitochondrial membrane, whereas inactive Bax is primarily cytosolic, translocating to the outer mitochondrial membrane only after activation (Cheng, Sheiko et al. 2003). The activation of Bax and Bak results in homo- and heterodimer formation at the outer mitochondrial membrane, generating pores that facilitate mitochondrial membrane permeabilization and cytochrome *c* release (Karbowski and Youle 2003; Li, Backer et al. 2003), leading to caspase activation and the apoptotic cascade (Feldstein, Wernenburg et al. 2006). Anti-apoptotic members of the Bcl-2 protein family, including Bcl-2, inhibit the activation of proapoptotic Bax and Bak primarily by sequestering inactive Bax and Bak monomers via interactions between their BH3 homology domains (Chipuk and Green 2008). Bcl-2 expression has been linked to decreased viral replication rates (Nencioni, DeChiara et al. 2009). Bcl-2 overexpression inhibits influenza A virus induced cell death and reduces the titer and spread of

newly formed virions (Olsen, Kehren et al. 1996). The activation of caspase-3 in the absence of sufficient Bcl-2 is critical to the influenza A virus life cycle. Both Bcl-2 expression and the lack of caspase activation during infection lead to the nuclear accumulation of influenza virus ribonucleoprotein (RNP) complexes, thereby leading to the improper assembly of progeny virions and a marked reduction in titers of infectious virus (Zhrinov, Konakova 1999; Wurzer 2003; Wurzer, Ehrhardt et al. 2004; Nencioni, DeChira et al. 2009).

Here we show that influenza A virus induces mitochondrion-mediated (intrinsic-pathway) apoptosis signaled specifically through Bax and that this Bax signaling is essential for the maximum efficiency of virus propagation. In contrast, Bak expression is strongly downregulated during infection. Cells lacking Bak (while expressing Bax) display a much more severe apoptotic phenotype in response to infection and produce infectious virions at a higher rate than the wild type (WT), suggesting that Bak, which can suppress viral replication, is potentially downregulated by the virus. Our results indicate essential and opposing roles for Bax and Bak in both the response of cells to influenza A virus infection and the ability of the virus to maximize its own replicative potential.

## **MATERIALS AND METHODS**

**Cell culture and experimental treatment.** MDCK cells were supplied by Anastasia Gregoriades, Queens College, Queens, NY. Simian virus 40-immortalized, adherent WT, Bax<sup>-/-</sup> (Bax knockout [KO]), Bak<sup>-/-</sup> (Bak KO), and Bax/Bak double-KO (DKO) mouse embryonic fibroblasts (MEFs) were a generous gift of the late Stanley Korsmeyer of Harvard University, Boston, MA. All cells were maintained in Dulbecco's modified Eagle medium (DMEM) (catalog no. 12800-017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS)

(catalog no. LSF0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin plus 50 mg/ml streptomycin (catalog no. 15140-122; Gibco) and incubated at 37°C under a humidified 5% CO<sub>2</sub> atmosphere. All experiments were run in triplicate. For all experiments except plaque assay, exponentially growing cells were seeded at 4 x 10<sup>5</sup> cells per plate and allowed to attach overnight before influenza A/WSN/33 virus was delivered to rinsed cells at a multiplicity of infection (MOI) of 5 for 1 h at room temperature (RT). The cells were covered with DMEM containing 5% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin and incubated at 37°C until collection. Bax replacement in KO cells was achieved through the transient transfection of plasmid C2-Bax-GFP under the control of a human cytomegalovirus promoter (a generous gift of the late Stanley Korsmeyer, Harvard University) by use of Lipofectamine 2000 reagent (catalog no. 52887; Invitrogen) according to the manufacturer's protocol. In brief, plasmid DNA was mixed with Lipofectamine 2000 reagent and applied to cells for 4 h at 37°C. The cells were then rinsed, covered with DMEM containing 10% FBS, and incubated at 37°C for 15 h before viral infection. A transfection efficiency of at least 50% was confirmed using fluorescence microscopy (data not shown).

**Viral growth and titer determination.** Influenza A/WSN/33 virus was cultured in 10-day-old specific-pathogen-free embryonated chicken eggs (Charles River SPAFAS, North Franklin, CT) for 2 days at 35°C. Infected egg albumen was collected and cleared by centrifugation at 1,000 rpm for 10 min. The supernatant was collected and stored at -80°C. Virus titer was determined by plaque assay. Briefly, monolayers of MDCK cells were incubated overnight in DMEM containing 10% FBS and 1% penicillin-streptomycin. This was followed by infection with 10-fold serial dilutions of virus suspension for 1 h at RT. Cells were then covered with warmed

Eagle's minimum essential medium (catalog no. 12-668E; BioWhittaker) containing 0.1% DEAE-dextran (catalog no. D9885; Sigma-Aldrich) and 1% purified agar (catalog no. LP0028; Oxoid). This agar medium was allowed to solidify at RT and incubated for 2 days at 37°C to promote plaque development. Immediately prior to plaque analysis, the solidified agar was removed, and cells were fixed and stained with a methanol-crystal violet solution. Plaques were counted, and the virus titer was expressed as PFU/ml. For experimental determinations of virus replication, cells were infected with influenza A/WSN/33 virus at an MOI of 0.01, and at 24 h intervals, cell supernatant samples were collected and plaques were assayed.

**Assessment and characterization of cell death.** The effect of Bax and Bak KO during influenza A virus-induced cell death was assessed using a Trypan blue exclusion assay. Cells were infected with influenza A virus as described above and detached from culture plates by trypsin digestion. Washed cell pellets were resuspended in 1x phosphate-buffered saline (PBS), and samples were mixed 1:1 with 0.4% Trypan blue (catalog no. 302643; Sigma-Aldrich) in 1x PBS solution and incubated at RT for 5 min. Trypan blue is taken up by dead cells and excluded from living cells by their intact cell membranes. Cells were counted by use of a hemocytometer. In all cases, percent cell death was calculated as the ratio of dead cells to the total number of cells. The percent cell death of mock-infected cells was subtracted as background, and data are expressed as percent cell death greater than death in mock-infected cells. The effect of Bax and Bak KO on Influenza A virus-induced cell death was further characterized by Hoechst staining, which is a reliable indicator of chromatin condensation and nuclear fragmentation, markers of mid- to late-stage apoptotic cell death. Cells were infected and collected as described above. Cell pellets were washed once with ice-cold 1x PBS and resuspended in ice-cold 3.5% paraformaldehyde (catalog no. 04042; Fisher) for 10 min. Fixed cells were stained on ice with 16

µg/ml Hoechst 33258 bis-benzimide stain (catalog no. 2883; Sigma-Aldrich) for 30 min, rinsed once with 1x PBS, and analyzed for chromatin condensation by fluorescence microscopy (Leitz, Germany). Healthy and apoptotic nuclei were counted, and in all cases, the count of mock-infected apoptotic nuclei was subtracted from the count of influenza A virus-infected apoptotic nuclei, and data are expressed as percent apoptotic nuclei greater than mock. Executioner caspase activity in mock- and influenza A virus-infected cells was quantified as the cleavage of fluorogenic caspase-3/7 substrate rhodamine 110, bis(*N*-carbobenzyloxy-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide [Z-DEVD-R110]), using the Apo-One homogenous caspase-3/7 assay kit (catalog no. G7792; Promega) according to the manufacturer's protocol.

**Quantification of viral RNA release.** The amount of total influenza A virus RNA released during infection in WT, Bax KO, Bak KO, and Bax/Bak DKO cells was determined by collecting cell supernatant samples at 48 h postinfection (hpi) and subjecting them to quantitative reverse transcription-PCR. Total viral RNA was extracted at 12 hpi and 24 hpi using a viral RNA extraction kit (catalog no. 52904; Qiagen) according to the manufacturer's protocol. cDNA was then generated from extracted viral RNA using a Superscript III first-strand synthesis kit (catalog no. 18080-400; Invitrogen) according to the manufacturer's protocol. One microgram of cDNA was amplified by quantitative reverse transcription-PCR in 20-µl reaction mixtures using a LightCycler FastStart DNA Master Sybr green 1 kit (catalog no. 03515869001; Roche) with forward primer TACACCCAGTCACAATAGGAGAGTG and reverse primer CCATGCATTCATTGTC ACACTTGTGG for the influenza A virus hemagglutinin (HA) gene using a Roche LightCycler 2.0 real-time PCR machine under the following conditions: 95°C for 10 min, followed by 50 cycles of 10 min at 95°C, 5 min at 69°C, and 22 min at 72°C. Samples of known viral titers were also analyzed to develop a standard curve of influenza A virus particles

released/ml, to which experimental samples were normalized. Relative influenza A virus particle release in each cell type was compared to that for mock-infected cells and presented as a ratio of infected/mock-infected cells.

**Assessment of the influenza A virus-induced interferon response.** To determine the effect of Bax and/or Bak KO on influenza A virus-induced interferon signaling, cells were infected as described above. This was followed by secondary infection in both mock- and influenza A virus-infected cells at 24 hpi with green fluorescent protein (GFP)-tagged Newcastle disease virus (NDV) (provided by Adolfo Garcia-Sastre, Mt. Sinai University and Medical Center, New York, NY) at an MOI of 1 or 2. NDV-GFP is suppressed by interferon activity. Following secondary infection, cells were incubated for an additional 24 h at 37°C under a humidified 5% CO<sub>2</sub> atmosphere to allow NDV-GFP expression. Cells were then collected and immediately analyzed by fluorescence-activated cell sorter (FACS) analysis. An increase in interferon activity caused by the initial influenza A virus infection causes a corresponding decrease in the level of NDV-GFP expression, which can be quantified using FACS analysis as an indirect measure of interferon activity. The experiment was run in triplicate, and 10,000 events per sample were analyzed. Mean fluorescence was expressed as the ratio of infected to mock mean fluorescence.

**Protein analysis and immunocytochemistry.** For determinations of cytochrome *c* release in MDCK cells, mitochondria from mock- and influenza A virus-infected cells were separated from cells at 8, 12, 18, and 24 hpi by homogenization and differential centrifugation, and total protein lysates from both the cellular and mitochondrial fractions were isolated and analyzed by western blotting. Bax and Bak expression profiles in MDCK cells were determined by collecting total protein lysates from mock- and influenza A virus-infected cells at 24 hpi and analyzing them by Western blotting using anti-Bax (catalog no. sc493; Santa Cruz Biotech) and anti-Bak (catalog

no. sc832; Santa Cruz Biotech) antibodies. For determinations of apoptosis protein expression levels during infection of WT, Bax KO, Bak KO, and Bax/Bak DKO cells, protein expression profiles of mock- and influenza A virus-infected cells were obtained at 48 hpi to allow the full expression of response proteins during influenza A virus-induced cell death and analyzed by western blotting. Cells were then scraped from the plates, and the cell pellet was resuspended in ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5 mM EDTA) containing a protease inhibitor cocktail (catalog no. 11836-170-001; Roche) and centrifuged for 10 min at 1,000 x g at 4°C. The supernatant was collected, transferred into a new ice-cold tube, and stored at -20°C. For determinations of influenza A virus nucleoprotein (NP) nuclear localization in WT, Bax KO, Bak KO, and Bax/Bak DKO cells, cells were infected at an MOI of 5 and collected by trypsin digestion at 24 hpi. Cells were then washed once with ice-cold 1x PBS, resuspended in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol) containing a protease inhibitor cocktail (catalog no. 11836-170-001; Roche), and incubated on ice for 10 min to cause cell swelling. Cells were then burst using a Dounce homogenizer and centrifuged to obtain a nuclear pellet. The supernatant was collected as the cytoplasmic fragment, mixed with 5x RIPA buffer, and incubated on ice for 20 min. The nuclear pellet was resuspended in 0.25 M sucrose containing 10 mM MgCl<sub>2</sub> and layered over a 0.88 M sucrose cushion containing 10 mM MgCl<sub>2</sub>, followed by centrifugation at 2,800 x g. The supernatant was aspirated, the pellet was resuspended in 1xRIPA buffer, and vortexed on ice to release nuclear proteins, followed by incubation on ice for 20 min. Both cytoplasmic and nuclear fractions were then cleared of debris by centrifugation. The total protein content was determined using the Bio-Rad protein assay (catalog no. 500-0006; Bio-Rad). For western blot analysis, equal quantities of

proteins (30  $\mu$ g) were loaded onto a polyacrylamide gel and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred onto a nitrocellulose membrane (catalog no. RPN-303E; Amersham) overnight at 4°C. The nonspecific proteins were blocked using 4% milk solution and subjected to different primary antibodies: anti-cleaved caspase-7 (catalog no. 9491s; BD Pharmingen), anti-active caspase-3 (catalog no. 559565; Sigma), anti-Bcl-2 (catalog no. sc492; Santa Cruz Biotech), anti-influenza A virus NP (provided by Adolfo Garcia-Sastre, Mt. Sinai University and Medical Center), and anti- $\beta$ -tubulin (catalog no. sc9104; Santa Cruz Biotech), which was used as a loading control. Positive signals were detected using ECF (catalog no. RPN5787; GE Healthcare) according to the manufacturer's protocol and visualized using a Storm 860 scanner and software (GMI, Inc., MN) or by ECL (catalog no. RPN2132; GE Healthcare) and visualized using Amersham Hyperfilm ECL photoradiographic film (catalog no. 28906835; GE Healthcare).

For immunocytochemical analysis,  $4 \times 10^5$  cells were seeded onto heat-sterilized glass coverslips. After overnight incubation at 37°C, attached cells were carefully rinsed with 1x PBS and infected as described above. At 24 or 48 hpi, cells were fixed with ice-cold 3.5% paraformaldehyde for 10 min and rinsed with ice-cold 1x PBS. Nonspecific binding sites were blocked using 1x PBS containing 18  $\mu$ g/ml bovine serum albumin before immunoblotting with the rabbit antibodies anti-Bax (catalog no. sc493; Santa Cruz Biotech), anti-active caspase-3 (catalog no. 559565; Sigma), anti-influenza A/WSN virus (provided by Adolfo Garcia-Sastre, Mt. Sinai University and Medical Center, New York, NY), or anti-influenza A virus NP. Cells were then rinsed three times in 1x PBS, followed by incubation with anti-rabbit immunoglobulin G-Alexa Fluor 488 secondary antibody (catalog no. A11008; Invitrogen) solution for 2 h. Cells were then rinsed twice with 1x PBS before staining polymerized actin with phalloidin-

tetramethyl rhodamine isocyanate (phalloidin-TRITC; catalog no. P1951; Sigma) and before staining nuclei with 4-,6-diamidino-2-phenylindole (DAPI) (catalog no. D8417; Sigma). Stained cells were then mounted using GelMount (catalog no. M-01; Biomed) and visualized using a confocal microscope (Leica, Germany).

**Determination of lysosomal localization and function.** Cells were infected and fixed on slides as described above. Lysosomal localization was determined by staining cells with 1  $\mu$ l/ml LysoTracker Red DND-99 (catalog no. L7528; Invitrogen) for 30 min immediately prior to fixation. Cells were then rinsed once with 1x PBS and fixed using ice-cold 3% paraformaldehyde in 1x PBS for 10 min. Nuclear localization was also determined by staining with 10  $\mu$ g/ml of DAPI (catalog no. D8417; Sigma) for 10 min at RT prior to mounting. Cells were then mounted with gel mount (catalog no. M-01; Biomed) and visualized by confocal microscopy (Leica, Germany). For measurements of lysosomal activity, cells were infected and collected as described above. The activity of the lysosomal enzyme acid phosphatase was assessed using an acid phosphatase assay kit (catalog no. CS0740; Sigma) according to the manufacturer's protocol. Briefly, cells were infected as described above, rinsed once, and collected by scraping. A total of  $10^6$  cells from each sample were then resuspended in acid phosphatase substrate solution (*p*-nitrophenyl phosphate) for 30 min at RT. The reaction was stopped by the addition of a 0.5 N NaOH solution to the mixture, and the absorbance of each sample at 405 nm was measured spectrophotometrically with an Ultraspec III spectrophotometer (Pharmacia LKB, Sweden). To determine the overall lysosomal volume of mock- and influenza A virus infected cells, cells were infected as described above and incubated with 1  $\mu$ l/ml LysoTracker Red DND-99 for 30 min immediately prior to collection. Cells were then rinsed once with 1x PBS and collected by trypsin digestion, followed by FACS analysis. The experiment was performed in

triplicate, and 10,000 events per sample were analyzed. The mean fluorescences of both mock-infected and influenza A virus-infected samples were compared as an indicator of lysosomal staining intensity, a marker of lysosomal volume.

**Determination of LC3 cleavage and translocation.** To confirm the induction of an autophagy following influenza A virus infection in the absence of Bax, we assessed the localization of LC3 at 24 hpi. A total of  $4 \times 10^5$  cells were plated onto heat-sterilized glass coverslips in 35-mm plates, and LC3-GFP was transiently expressed by transfection with a C1-LC3-GFP construct (provided by Guido Kroemer, Institut Gustave-Roussy, Villejuif, France) using Lipofectamine 2000 (catalog no. 52887; Invitrogen) and incubated for 16 h to allow LC3-GFP expression. Cells were then infected as described above. At 24 hpi, cells were fixed with ice-cold 3.5% paraformaldehyde (catalog no. 04042; Fisher) for 10 min and rinsed once with 1x PBS. Cells were then embedded by gel mount (catalog no. M-01; Biomed) and observed by confocal microscopy. The generation of a punctate GFP expression pattern is indicative of LC3 translocation and autophagosome formation, which occurs exclusively during autophagy. A transfection efficiency of at least 50% was confirmed using fluorescence microscopy (data not shown). Mock-infected cells were also analyzed to ensure that LC3-GFP expression alone did not cause an autophagic response.

## RESULTS

### **Influenza A virus infection induces apoptosis and disables mitochondrial function.**

Influenza A virus infection drives an apoptotic response in host cells (Mori, Komatsu et al. 1995; Takizawa, Fukuda et al. 1995; Schultz-Cherry, Krug et al. 1998; Takizawa, Tatematsu et al. 1999; Zhirnov, Konakova et al. 1999; Wang, Zhang et al. 2000; Nichols, Niles et al. 2001; Wei,

Zong et al. 2001; Lowy 2003; Ludwig, Planz et al. 2003; Wurzer 2003; Wurzer, Ehrhardt et al. 2004; Zamarin, Garcia-Sastre et al. 2005; Ludwig, Pleschka et al. 2006; Zamarin, Ortigoza et al. 2006; Uchide and Toyoda 2007; McLean, Ruck et al. 2008; A. Shirazian, D. Matassov, J. E. McLean, and Z. Zakeri, unpublished data). In an effort to understand if and how influenza A virus-induced apoptosis is affected by mitochondrion-driven apoptosis signaling, we initially used MDCK cells, which are both permissive to infection and highly susceptible to influenza A virus-induced killing. Cell death in response to influenza A virus infection was assessed initially by the Trypan blue exclusion assay described in Materials and Methods. Subsequently, influenza A virus-induced death was characterized by several markers of apoptosis at different times post infection. We concluded that the level of cell death measured by Trypan blue was similar to that obtained by other markers and furthermore that cell death was apoptotic, consistent with findings reported previously by several other groups. As shown in Fig. 1.1A, page 186, by 24 hpi, more than 50% of influenza A virus-infected cells died, and by 48 hpi, 90% of infected cells died. Hoechst analyses examining the level of condensed and fragmented nuclei in infected cell populations showed that nuclear alterations typical of apoptosis followed a pattern consistent with death as measured by Trypan blue (Fig. 1.1A, page 186). This indicates that influenza A virus induced death is most likely apoptotic, a fact that was confirmed by the measurement of activation of caspase-3 (data not shown; Shirazian et al., unpublished).

One milestone during mitochondrion-mediated apoptosis is cytochrome *c* release from the inner mitochondrial space to the cytosol, where it activates apoptosis. This release is typically modulated by the activity of Bcl-2 family proteins and usually occurs without an immediate decline in the function of mitochondrial enzymes (Wei, Zong et al. 2001; Hardwick and Bolster 2003; Feldstein, Wernenburg et al. 2006; Kroemer, Galluzzi et al. 2007; Chipuk and Green

2008). We assessed the level of cytochrome *c* release in response to influenza A virus infection by the evaluation of the presence of cytochrome *c* in different cell fractions (i.e., the cytoplasmic versus mitochondrial fraction obtained by differential centrifugation of mock infected and infected MDCK cells at various times post infection). The presence of cytochrome *c* was evaluated and analyzed by western blotting using anti-cytochrome *c* antibodies. The distribution of cytochrome *c* shifted dramatically from the pelleted, mitochondrial fraction to the soluble, cytoplasmic fraction as early as 18 hpi; this distribution was maintained through 24 hpi (Fig. 1.1B, page 186).

Bax and Bak form cytochrome *c* release channels in the outer mitochondrial membrane, allowing cytochrome *c* release to the cytosol. We therefore assessed the levels of Bax and Bak expression in mock-infected and infected MDCK cells at 24 hpi.  $\beta$ -Tubulin was used as a loading control. As shown in Fig. 1.1C, page 186, Bax expression was downregulated, while Bak expression almost totally disappeared after influenza A virus infection.

Bax and Bak are considered to be pro-apoptosis partners during intrinsic pathway signaling (Chipuk and Green 2008), acting in tandem to induce cytochrome *c* release and subsequent caspase activation. As Bax, but not Bak, expression is still evident in infected cell populations, we assessed the localization of Bax at 24 hpi by using a Bax-specific antibody and confocal microscopy. A diffuse cytoplasmic distribution of Bax, typical of inactive Bax in healthy cells, was found in the mock-infected cells (Fig. 1.1D, page 186). In contrast, infected cells showed a punctate Bax staining pattern, which is typical of Bax activation in cells undergoing an apoptotic response, as reported previously (Hardwick and Bolster 2003; Wurzer 2003) (Fig. 1.1E, page 186). (The appearance of nuclear localization is due to DAPI staining in these pictures.) These results suggest that the apoptotic response generated by influenza A virus

infection is due to Bax-mediated cytochrome *c* release and that Bak is actively downregulated by the virus during infection. The observed elimination of Bak and activation (movement to mitochondria) of Bax during infection suggest that Bax, but not Bak, is important for the efficient induction of death by influenza A virus during infection.

**Bax KO inhibits, while Bak KO potentiates, influenza A virus-induced mitochondrion-mediated apoptosis.** Our findings that Bax and Bak activities are differently affected by influenza A virus infection prompted us to further explore the activities of these two proteins during influenza A virus-induced cell killing. We used C57Black, simian virus 40-immortalized MEFs that were WT or that possessed a genetic KO of Bax, Bak, or both (Wei, Zong et al. 2000). We infected WT, Bax KO, Bak KO, and Bax/Bak DKO cells with influenza A virus and assessed the level of cell death after 24 and 48 hpi. As shown in Fig. 1.2A, page 187, while the death rates of infected Bax KO cells do not differ from those of the WT, the rate of death of Bak KO cells is markedly higher than that of WT cells at both 24 hpi ( $P < 0.0001$ ) and 48 hpi ( $P < 0.0002$ ). These results suggest that influenza virus requires neither Bax nor Bak to kill the cells, that the cells are more vulnerable when Bax is present in the absence of Bak, and that Bax is required for death to be apoptotic. The increase over the control in rates of death observed in Bax/Bak DKO cells at both 24 hpi ( $P < 0.03$ ) and 48 hpi ( $P < 0.000002$ ) suggests that the virus can trigger alternate death pathways that are otherwise not seen when both of these proapoptotic proteins are present.

To assess the amount of apoptosis in response to influenza A virus under each KO condition, we first quantified the numbers of condensed and fragmented nuclei at 24 hpi and 48 hpi using Hoechst staining. The increase in numbers of fragmented nuclei in WT, Bak KO, and Bax/Bak DKO correlates with the levels of cell death following infection (Fig. 1.2B, page 187).

However, Bax KO cells, which experience rates of death very similar to those of the WT after infection, develop almost no fragmented nuclei compared to the WT at both 24 hpi ( $P < 0.00003$ ) and 48 hpi ( $P < 0.0002$ ). Influenza A virus-induced changes in nuclear morphology are increased in Bak KO cells compared to those of WT cells at 24 hpi ( $P = 0.04$ ) and 48 hpi ( $P < 0.0002$ ). At 24 hpi, mock-infected cells of all types manifest primarily healthy nuclei (Fig. 1.2C to F, page 187). Influenza A virus-infected WT, Bak KO, and DKO cells manifest many condensed and fragmented nuclei (Fig. 1.2G, H, and J, page 187). However, Bax KO cells, shown in Fig. 1.2I, page 187, display almost no fragmented nuclei at 24 hpi. These results suggest that Bax activation is intimately involved in the induction of apoptosis by influenza A virus.

To confirm the identity of WT, Bax KO, Bak KO, and Bax/Bak DKO cells, total protein lysates were analyzed by western blotting for the presence of Bax and Bak. Confirmation of Bax and/or Bak KO is shown in Fig. 1.2K and L, page 187, where Bax is present in both WT and Bak KO cells but not in Bax KO and Bax/Bak DKO cells, while Bak is present in WT and Bax KO cells but not in Bak KO and Bax/Bak DKO cells. To confirm infection of each cell type, cells were infected at an MOI of 5 and fixed at 24 hpi. Immunocytochemistry using anti-influenza A/WSN virus was performed with infected cells and observed by confocal microscopy. As shown in Fig. 1.3, page 188, by 24 hpi, virus-induced vesicles were present in all cell types, confirming the ability of influenza A virus to infect and replicate regardless of the presence of Bax or Bak.

The observed differences in death rates in Bax and/or Bak KO cells following infection may arise from differences in subsequent perturbations of apoptotic signaling. To further characterize the involvement of Bax and Bak in influenza A virus-induced apoptosis, we examined cytochrome *c* localization at 24 hpi by immunocytochemistry. As shown in Fig. 1.4A

to D, page 189, cytochrome *c* is distributed in a punctate pattern consistent with mitochondrial localization, and there is minimal cytoplasmic localization of cytochrome *c* in mock-infected cells at 24 hpi. In influenza A virus-infected WT (Fig. 1.4E, page 189) and Bak KO (Fig. 1.4G, page 189) cells, strong cytoplasmic cytochrome *c* staining is evident at 24 hpi. This corresponds to the observed apoptotic death in these cells following infection. Bax KO (Fig. 1.4F, page 189) and Bax/Bak DKO (Fig. 1.4H, page 189) cells do not exhibit detectable cytoplasmic cytochrome *c* staining, indicating a block in cytochrome *c* release after infection in cells lacking Bax. We also looked for an activation of caspase-3 using an antibody specific to active caspase-3. As shown in Fig. 1.4I to L, page 189, caspase-3 is not activated by 48 hpi in mock-infected WT or KO cells, corresponding to the lack of death observed in these populations. However, in influenza A virus-infected WT (Fig. 1.4M, page 189), Bak KO (Fig. 1.4O, page 189), and Bax/Bak DKO (Fig. 1.4P, page 189) cells, strong staining indicative of activated caspase-3 was found, further demonstrating the induction of apoptosis in response to influenza A virus. On the other hand, in Bax KO MEFs (Fig. 1.4N, page 189), we found no such staining, indicating no activation of caspase-3 after infection in the absence of Bax.

Executioner caspase activation was confirmed by western blotting of extracted whole-protein lysate from mock- and influenza A virus-infected MEFs of WT and KO cells at 24 hpi. Antibodies specific to the activated forms of caspase-3 and caspase-7 were used to confirm the activation of caspase. Activated caspase-3, shown in Fig. 1.5A, page 190, was observed in both WT and Bak KO cells following infection. Caspase-3 activation was also apparent in Bax/Bak DKO cells albeit at reduced levels compared to those of the WT. Likewise, activated caspase-7, shown in Fig. 1.5B, page 190, was seen only in influenza A virus-infected WT and Bak KO cells and was absent in Bax KO cells following infection. In contrast to the caspase-3 activation

shown in Bax/Bak DKO cells following infection, caspase-7 activity in these cells was only slightly apparent at 24 hpi. To confirm that we had a robust level of infection in all the cells, an antibody specific to influenza A virus NP was used to detect the production of viral protein. Figure 1.5D, page 190, shows that high levels of influenza A virus NP are present in infected WT cells and each KO cell condition, confirming that each cell type is permissive to viral infection. Consistent with the viral RNA levels (discussed below) (Fig. 1.5C, page 190), all three KO conditions showed stronger NP staining than the WT. Bcl-2 expression was not affected by influenza A virus infection in any of these cells (Fig. 1.5C, page 190).  $\beta$ -Tubulin was used as a loading control (Fig. 1.5E, page 190). These results suggest that the observed differences between cell types arise downstream of Bcl-2 activity and not from indirect effects upstream of Bax and Bak.

We next quantified the combined activities of caspase-3 and caspase-7 following infection by assessing the cleavage of a fluorogenic caspase-3/7 substrate, rhodamine 110, bis(*N*-carbobenzyloxy-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide [ZDEVD-R110]), as described in Materials and Methods. A substantial increase in fluorescence was seen following infection in WT, Bak KO, and Bax/Bak DKO cells, closely corresponding to the observed patterns of apoptotic nuclei in these cells in response to the virus (Fig. 1.5F, page 190). Bax KO cells demonstrated no appreciable increase in fluorescence levels following influenza A virus infection, providing further evidence that caspase-3 and caspase-7 are not activated under this condition.

Overall, we demonstrate that mitochondrion-mediated apoptotic signaling through Bax is essential for an apoptotic response to influenza A virus. Cells lacking Bax still die in response to infection although not by apoptosis.

**Bax KO forces influenza A virus-infected cells into an increased autophagic state.** Although influenza A virus-induced signaling for apoptosis is inhibited in Bax KO cells, the number of cells that die in response to infection is approximately equal to that of the WT, suggesting that when Bax-mediated apoptosis is restricted during infection, an alternative death pathway is triggered. We have found that in influenza A virus infected MDCK cells, a pharmacological blockade of caspase activation yields an increase in rates of autophagosome production and cell death (Shirazian et al., unpublished). Therefore, we evaluated the role of autophagy or lysosomal activity during influenza A virus-induced death of cells lacking Bax or Bak. To this end, we determined the activity of lysosomal acid phosphatase as an indicator of lysosomal activity. As shown in Fig. 1.6A, page 191, influenza A virus-infected WT cells showed a decrease in levels of acid phosphatase activity compared to that of mock-infected cells, with a smaller decrease displayed by Bak KO cells after infection. In contrast, a marked increase in acid phosphatase activity levels was seen in Bax KO cells after infection, with Bax/Bak DKO cells exhibiting a lower level of increase in response to influenza A virus.

Using LysoTracker staining and FACS analysis, we also found an increase in lysosomal volume in WT, Bak KO, and Bax/Bak DKO cells, with a slight decrease in the lysosomal volume of Bax KO cells after infection (Fig. 1.6B, page 191). Bax has been shown to induce lysosomal permeabilization under conditions of cellular stress (Boya, Andreau et al. 2003), and pharmacological and genetic inhibition of Bax has been shown to inhibit lysosomal permeabilization as well as mitochondrion-induced apoptosis (Boya, Andreau et al. 2003; Fink and Cookson 2005, Jaatela, Cande et al. 2004). We speculate that the influenza A virus-induced decrease in lysosomal activity (as demonstrated by acid phosphatase activity) in WT and Bak KO cells reflects a Bax-dependent leakiness of the lysosomes (evident by the increase in

lysosomal volume after infection), resulting in a loss of acidity and, hence, a loss of lysosomal enzyme activity. In Bax KO cells, there is an increase in levels of acid phosphatase activity together with a slight decrease in lysosomal volume, which is likely due to a lack of lysosomal permeabilization after infection with influenza A virus in these cells. Together, these data suggest an increase in lysosomal activity following infection in cells lacking Bax. However, Bax/Bak DKO cells also show an increase in acid phosphatase activity after infection but without the corresponding lack of lysosomal swelling seen in Bax KO cells, suggesting the existence of a parallel pathway to lysosomal permeabilization following infection when both Bax and Bak are missing.

As stated above, blocking caspase activity increases the formation of autophagosomes following infection (Shirazian et al., unpublished). We therefore assessed the autophagic response to influenza A virus in cells lacking Bax, which do not activate caspase-3, by examining LC3 localization during infection. Inactive LC3 is cytoplasmic, resulting in a diffuse staining pattern unless cleaved and activated during autophagy. LC3 cleavage results in its translocation to autophagosomal membranes, where it accumulates and appears as a brightly punctate staining pattern. To examine LC3 localization, we expressed a C1-LC3-GFP construct in the different cell types, infected the cells, and assessed the expression and localization of the construct. As shown in Fig. 1.6C to F, page 191, in WT and all KO cells, mock-infected cells displayed the diffuse pattern of expression of LC3-GFP consistent with an inactive, cytoplasmic LC3-GFP distribution. Following infection, cells constitutively expressing Bax (WT and Bak KO) continued to display the diffuse LC3-GFP expression pattern at 24 hpi (Fig. 1.6G and I, page 191, respectively). This result indicates that autophagosome formation is not triggered following infection of cells expressing functional Bax and is consistent with an apoptotic

response. However, in cells lacking functional Bax (Bax KO and Bax/Bak DKO cells), LC3-GFP shifts to a punctate pattern of distribution by 24 hpi (Fig. 1.6H and J, page 191, respectively), consistent with LC3 translocation to autophagosomal membranes. These results indicate that when Bax-mediated caspase activation is blocked during influenza A virus infection, an alternative cell death path that employs an increase in the autophagic state is in play, which leads to cell death by this alternative route.

**Bax expression is required for efficient replication of influenza A virus, while Bak suppresses replication.** The observations reported here demonstrate that influenza A virus-induced apoptosis is driven primarily by the intrinsic pathway, most likely derived from Bax translocation and the subsequent cytochrome *c* release from mitochondria. The downregulation of Bak expression following infection indicates that Bak is suppressed by the virus, suggesting an adaptation of the virus to suppress an antiviral function. To evaluate this possibility, we examined the effect of the elimination of Bax and/or Bak on the virus replication cycle as determined by the titers of infectious virus. Cell supernatant samples from infected cells were collected every 24 h until 144 hpi. These samples were used to infect monolayers of MDCK cells for analysis of viral replication by plaque assay. The virus titer reached a maximum of  $10^7$  PFU/ml in WT cells at 48 hpi and decreased slightly thereafter (Fig. 1.7A, page 192). However, Bak KO cells exhibited a much faster production of virus and surpassed the WT maximum titer by 24 hpi. The titer in Bak KO cells then reached a maximum titer similar to that of the WT by 72 hpi of just over  $10^7$  PFU/ml, and these cells maintained this titer to 144 hpi ( $P < 0.000002$  at 24 hpi;  $P = 0.08$  at 48 hpi;  $P = 0.14$  at 72 hpi;  $P = 0.016$  at 96 hpi;  $P = 0.09$  at 120 hpi;  $P = 0.01$  at 144 hpi) (Fig. 1.7A, page 192). On the other hand, Bax/Bak DKO cells reached a maximum titer of  $10^8$  PFU/ml at 24 hpi, which is 10-fold higher than that of the WT, and the titer then

declined slightly until 144 hpi ( $P = 0.0004$  at 24 hpi;  $P = 0.002$  at 48 hpi;  $P = 0.14$  at 72 hpi;  $P = 0.016$  at 96 hpi;  $P = 0.09$  at 120 hpi;  $P = 0.01$  at 144 hpi) (Fig. 1.7A, page 192). In contrast, the production of infectious virus as indicated by plaque assay is substantially inhibited in Bax KO cells, reaching a maximal titer of only  $10^5$  PFU/ml by 48 hpi, much lower than that of the WT ( $P = 0.012$  at 24 hpi;  $P = 0.003$  at 48 hpi;  $P = 0.0004$  at 72 hpi;  $P = 0.01$  at 96 hpi;  $P = 0.00001$  at 120 hpi;  $P = 0.0005$  at 144 hpi) (Fig. 1.7A, page 192). By 48 to 60 hpi, virtually all of the cells were dead and viral replication had ceased, with viral replication in Bax KO cells never having reached the level seen in the WT; the results beyond 48 hpi represent survival of virus. These results suggest that the intrinsic signaling of apoptosis through Bax is an integral step in the influenza A virus replication cycle and that Bak is antiviral during the early stages of infection.

To confirm the involvement of Bax in the production of influenza A virus, we determined if Bax supplementation in Bax KO cells would rescue the Bax KO cells' deficiency in virus production. We expressed a plasmid C2-Bax-GFP construct in WT and Bax KO cells. Following influenza A virus infection for 48 hpi, the supernatant was collected for plaque assay. Empty C2-GFP plasmid was used as the control for each cell type. Under all conditions, plasmid expression was confirmed by microscopic examination of GFP expression (data not shown). As shown in Fig. 1.7B, page 192, the addition of Bax to Bax containing WT cells had almost no impact on viral titer, but the replacement of Bax in Bax KO cells increased the viral titer fivefold ( $P = 0.0007$ ), approaching normal titers. Therefore, we conclude that Bax-mediated signaling plays an integral role in influenza A virus replication.

The difference in virus titers among KO constructs may be derived from differences in viral genomic replication, assembly, or maturation. If these differences are due to genomic replication, fewer virions (and, therefore, less viral RNA) will be released from Bax KO cells

than from WT cells, resulting in a lower plaque assay titer. However, if these differences are due to the assembly or maturation of progeny virions, Bax KO cells may release numbers of virions similar to those of WT cells but will produce fewer infectious progeny. Therefore, even if Bax KO and WT cells released similar amounts of viral RNA (viral particles), a plaque assay will show lower viral titers from Bax KO cells. To determine whether the differences in viral replication between cell types is due to differences in overall viral release or in virion infectivity, we quantified the amount of viral RNA released after infection. Viral RNA was extracted from the supernatant of infected cells at 24 hpi and subjected to reverse transcription-PCR using primers for influenza A virus HA. The amounts of RNA detected in all three KO cell types (Bax KO, Bak KO, and Bax/Bak DKO cells) remained similar at approximately double the amount released from infected WT cells (Fig. 1.7C, page 192). It appears that although each KO condition allows an increase in the level of production of viral RNA similar to that of the WT, this increase is not coupled with an associated increase in viral infectivity. Thus, the differences in the clonogenicity of the virus cannot be explained entirely by differential viral genomic expression and may be derived in part from defects in virus assembly or maturation. We therefore searched for these defects.

**Decrease in titer by Bax KO is not directly related to interferon.** Interferon inhibits the replication of many viruses through the use of several mechanisms (Wang, Zhang et al. 2000; Munoz-Jordan, Sanchez-Burgos et al. 2003; Park, Shaw et al. 2003; Kochs, Garcia-Sastre et al. 2007; McLean, Ruck et al. 2008). Although the influenza A virus NS1 protein is a potent inhibitor of interferon (Garcia-Sastre, Egorov et al. 1998; Bergmann, Garcia-Sastre et al. 2000; Wang, Zhang et al. 2000; Brydon, Smith et al. 2003; Kochs, Garcia-Sastre et al. 2007), the differential induction of interferon in Bax and/or Bak KO cells compared to that of the WT could

also account for the differences in virus replication observed between cell types. We therefore assessed interferon activity in infected WT, Bax KO, Bak KO, and Bax/Bak DKO cells using GFP-tagged NDV, which is highly sensitive to interferon. Interferon activity after infection of cells with influenza A virus can be indirectly quantified by secondary infection with NDV-GFP, followed by the measurement of GFP fluorescence by FACS (Munoz-Jordan, Sanchez-Burgos et al. 2003; Park, Shaw et al. 2003; Munoz-Jordan, Laurent-Rolle et al. 2005). As shown in Fig. 1.7D, page 192, levels of interferon activity are elevated (NDV-GFP levels are decreased) by influenza A virus infection under WT and all KO conditions. Infected Bax KO cells showed a level of NDV-GFP replication similar to that of the WT, whereas infected Bak KO cells showed a significant decrease in levels of NDV-GFP replication, representing an approximately 30% increase in interferon activity compared to those observed for both WT ( $P = 0.002$ ) and Bax KO ( $P = 0.037$ ) cells. The Bax/Bak DKO cells show a response similar to those of Bax and WT cells. These results suggest that Bak potentiates the interferon response in influenza A virus-infected cells and are consistent with the effects of Bak KO on virus replication and a model in which Bak suppresses the replication of influenza A virus. As the interferon response to infection observed in Bax KO cells is very similar to those of WT and Bax/Bak DKO cells, these data also indicate that excess interferon signaling is not responsible for the observed decline in the titer of infectious virus in Bax KO cells.

**Absence of Bax leads to inappropriate nuclear retention of influenza A virus NP.** The lack of caspase-3 activity following infection with influenza A virus results in influenza A virus NP retention within the nucleus (Nencioni, DeChiara et al. 2009; Wurzer 2003), sequestering viral genomic segments within the nucleus away from the cytoplasmic viral assembly sites. As influenza A virus NP acts as a shuttle for viral genomic segments from the nucleus to

cytoplasmic assembly centers, its location during the virus replication cycle markedly influences virus titers. A significant retention of influenza virus NP as a result of Bax KO cells within the nucleus would impair the proper assembly of progeny virions, thus reducing their infectivity. Similarly, lysosomal sequestration of viral segments in these cells during an autophagic response may also lead to improper viral assembly. To determine if the lack of Bax and/or Bak signaling plays a role in the nuclear export of influenza A virus NP or if the autophagic response seen in cells lacking Bax causes the sequestration of virus segments in autophagosomes, the localization of influenza A virus NP was determined at 24 hpi by using an antibody specific to influenza A virus NP coupled with organelle identification by DAPI (nucleus) and LysoTracker Red (lysosomes) under WT and all KO conditions. As shown in Fig. 1.8D, H, L, and P, page 193, at 24 hpi, influenza A virus NP does not colocalize with lysosomes in either WT or KO cells. This suggests that the reduced rate of virus replication in Bax KO cells does not derive from the lysosomal sequestration of viral genomic segments or viral progeny. As shown in Fig. 1.8C, page 193, very small vesicles containing influenza A virus NP are apparent in WT cells throughout the cytoplasm and at the cell membrane, with no nuclear NP staining being evident at 24 hpi. Influenza A virus-containing vesicles are similarly small in Bak KO cells, with viral NP localizing near the cell membrane (Fig. 1.8L, page 193), suggesting an efficient endocytic release of viral progeny despite the slight nuclear NP staining evident in roughly 50% of these cells. Influenza A virus NP localizes in the cytoplasm and near the cell surface, with little apparent nuclear NP staining evident in Bax/Bak DKO cells (Fig. 1.8P, page 193). However, in Bax KO cells (Fig. 1.8H, page 193), nuclear NP staining is evident in nearly all infected cells. To confirm that excessive nuclear retention of influenza A virus NP occurs in Bax KO cells but not in WT, Bak KO, and Bax/Bak DKO cells, nuclear and cytoplasmic proteins of infected cells

were obtained as described in Materials and Methods. As shown in Fig. 1.8Q, page 193, strong staining for influenza A virus NP is located in the cytoplasmic fraction of all infected cells. In WT cells, very little NP staining was seen in the nuclear fraction. However, there was strong NP staining in Bax KO cells, confirming NP enrichment in the nuclear fraction of these cells. Bak KO and Bax/Bak DKO cells also showed some NP enrichment after infection although not as severe as that for infected Bax KO cells. While these results suggest that both Bax and Bak have an effect on viral NP trafficking between the nucleus and cytoplasm, they indicate that the abolition of Bax-mediated apoptosis, with concurrent Bak activity, yields a particularly effective restriction of NP export from the nucleus to cytoplasmic viral assembly centers.

## **DISCUSSION**

Several groups have linked an apoptotic response in influenza A virus-infected cells to the efficiency of virus replication. While purposeful cell killing may seem to be counterintuitive for maximizing virus replication, an activation of apoptosis is required by influenza A virus for proper viral RNP complex release from the nucleus to cytoplasmic viral assembly centers (Wurzer 2003) as well as for proper viral HA processing prior to progeny release (Olsen, Kehren et al. 1996). The specific importance of mitochondrion-mediated caspase activation in this event has not been clearly studied. Here we report that influenza A virus manipulates both Bax- and Bak-mediated apoptosis, affecting both the cell death phenotype and efficiency of virus replication and propagation.

Bcl-2 suppresses apoptosis by sequestering Bax and Bak (Chipuk and Green 2008), and Bcl-2 overexpression in MDCK cells causes a reduction in both apoptosis and virus replication (Olsen, Kehren et al. 1996). MDCK cells, which lack functional Bcl-2 (Hinshaw, Olsen et

al.1994; Li, Backer et al. 2003), permit influenza A virus infection. We will present evidence of high-virus-titer generation as apoptosis proceeds with cytochrome *c* release and the activation of the caspase cascade elsewhere (data not shown). Therefore, the activation of Bax and/or Bak, which Bcl-2 opposes, may provide an integral step in the formation of viral progeny. Consistent with these results, we find that while Bak expression is severely downregulated in MDCK cells following infection, Bax translocation from a diffuse cytoplasmic distribution to a punctate pattern indicates Bax activation in response to the virus despite a reduction in expression levels following infection. These results suggest a selection pressure for influenza A virus favoring Bax activity with a concurrent disruption of Bak signaling during infection.

To understand the opposing effects of influenza A virus on Bax and Bak activity, we used MEF cells that lack Bax and/or Bak but express functional Bcl-2. In cells lacking Bak (which is severely downregulated during infection), the rates of both apoptosis (demonstrated by nuclear morphology, Trypan blue exclusion, cytochrome *c* release, and the activation of caspases) and viral replication are increased compared to those of the WT. Thus, selection pressure for the virus favoring mitochondrion-mediated apoptosis activation may explain the observed reduction in Bak expression levels following infection in MDCK cells.

In contrast to Bak, Bax activation is essential for influenza A virus-induced caspase activation, and Bax is required for efficient virus replication. This is demonstrated by the near-complete lack of apoptosis markers present in Bax KO cells after infection, including nuclear condensation/fragmentation, cytochrome *c* release, and the activation of executioner caspase. In the absence of Bax activity, the virus titer is 1% of that seen in the WT. This effect is ameliorated by ectopic Bax expression, which increases virus replication in Bax KO cells. Overall, though, the rate of cell death in response to the virus is not reduced compared to that of

the WT, indicating the involvement of an alternative death pathway. The general inhibition of caspases by the pancaspase inhibitor ZVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[*o*-methyl]-fluoromethylketone) shifts infected MDCK cells from apoptosis to autophagy and slightly decreases the overall rate of cell death (Shirazian et al., unpublished). Consistent with these results, Bax KO leads to the death of infected cells, with an increase in the levels of lysosomal activity and autophagy, as demonstrated by a dramatic increase in lysosomal acid phosphatase enzyme activity and LC3 translocation.

Interestingly, both cell death in response to infection and maximum virus titers are potentiated in Bax/Bak DKO cells compared to the WT. Similar numbers of apoptotic nuclei are found by comparing Bax/Bak DKO to WT cells following infection, indicating the involvement of an alternative, non-mitochondrial apoptosis pathway when Bax and Bak are absent. Alternative mechanisms may include the activation of the extrinsic apoptosis pathway, lysosomal protease, or calpain (Ruiz-Vela, Gonzales de Buitrago et al. 1999). The TRAIL and Fas/FasL death receptor systems can activate executioner caspases through caspase-8 (Takizawa, Fukuda et al. 1995; Takizawa, Tatematsu et al. 1999). The caspase cascade may also be activated through lysosomal proteases, and there can be significant cross talk between caspase and calpain (Ramphal, Small et al. 1980; Neumar, Xu et al. 2003). Although influenza A virus can trigger many different routes to caspase activation, the rate at which each one proceeds following infection is likely to vary. Our data argue that Bax-mediated apoptosis is a primary pathway by which influenza A virus induces cell death. More Bax/Bak DKO cells die than do WT cells following infection, without a corresponding rise in numbers of apoptotic nuclei, indicating that an alternative, nonapoptotic death pathway is initiated in a subset of the population. Consistent with this model, both apoptotic and autophagic cells are found in infected Bax/Bak DKO cells.

Also, the dramatic increase in the rate of cell death observed for infected Bak KO cells is attenuated for Bax/Bak DKO cells, indicating that the interplay between Bax and Bak is an important component of influenza A virus-induced, mitochondrion-initiated death.

The decline in virus titers in Bax KO cells is not accompanied by a decrease in levels of viral RNA released from infected cells. This indicates that the reduction of viral titers seen in Bak KO cells results from a block in viral maturation or processing rather than a direct effect on viral genomic replication. Autophagosomal sequestration of viral genomic segments is an unlikely explanation for titer reductions in the absence of Bax, since influenza virus NP is not found in lysosomes. However, while WT cells show no nuclear NP staining at 24 hpi, nearly all Bax KO cells demonstrate a marked nuclear retention of viral NP along with a concurrent cytoplasmic localization. Western blotting of both the nuclear and the cytoplasmic fractions of infected cells also demonstrates nuclear NP enrichment in Bax KO cells compared to the WT. These results suggest that although many viral RNP complexes reach cytoplasmic assembly centers following infection in Bax KO cells, a large subset of viral RNP complexes and, therefore, their associated viral genomic segments are retained in the nucleus. This would lead directly to improper viral assembly and is a possible explanation for the observed reduction in overall viral infectivity. The data support a model in which infecting influenza A virus encounters proviral Bax and antiviral Bak. Bak may inhibit the Bax-mediated initiation of cell death. It is likely that viral PB1-F2, which is similar in structure and function to Bax (Chipuk and Green 2008), is involved in the interaction. We are currently exploring this possibility.

In summary, we identify Bax-mediated apoptosis as a driving force behind influenza A virus-induced cell death. In the presence of Bax, infected cells undergo apoptosis, and virus replication succeeds, while Bak suppresses apoptosis and viral replication. The biochemical

interactions and dynamics between Bax-mediated cytochrome *c* release and various other death pathways that drive this decision remain to be elucidated. However, it is clear that the success of viral replication, i.e., the titer achieved during infection, depends on the interplay of Bax and Bak and how and when they affect cell death.

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### 3.4 Results, part 2. Influenza A and the Mitochondria

Although the argument that viruses promote cell death to propagate may seem counterintuitive, activation of the caspase cascade may be involved in the processing and maturation of influenza virus (Wurzer 2003). During apoptosis initiation, Bax and Bak both regulate the release of cytochrome c from the mitochondria to cytoplasm, which then triggers the caspase cascade. We have shown that Bax knockout disrupts caspase activation following Influenza A infection, and leads to a subsequent 99% reduction in extracellular virus titer. In contrast Bak knockout leads to enhanced apoptosis following infection, which in turn leads to an increase in the rate of progeny virion release during the early stages of infection. These results suggest that Bax functions as a pro-viral apoptosis promoter, while Bak functions as an anti-viral apoptosis suppressor during Influenza A infection.

As Bax and Bak help regulate mitochondrial function that is central to an infected cell's fate, we examined mitochondrial respiration following Influenza A infection in wild-type, Bax KO, Bak KO and Bax/Bak DKO cells at 24 hpi using both the MTT assay and FACS analysis of Mitotracker Red CMXRos<sup>®</sup> stained cells. As seen in Figure 1.9, page 194, (A) in wild-type cells Influenza A infection induced a small reduction in mitochondrial respiration at both 24 and 48hpi. However, a significant reduction in mitochondrial respiration in Bax KO cells (~4 times that of wild-type cells) is seen at 24 and 48hpi. Bak KO cells displayed the greatest decrease in mitochondrial respiration following infection, ~10 times the reduction seen in wild-type cells. Infection in Bax/Bak DKO cells leads to a slight reduction in mitochondrial respiration similar to wild-type. (B) A similar pattern was seen following FACS analysis of Mitotracker Red

CMXRos<sup>®</sup> stained cells. Uptake of Mitotracker Red CMXRos<sup>®</sup> into the mitochondria is dependent upon mitochondrial membrane potential, yielding decreased staining intensity with decreased mitochondrial membrane potential. As seen after MTT assay, FACS analysis following infection of wild-type cells reveals a slight reduction in mitochondrial activity, while infection in Bax KO cells causes elevated mitochondrial failure. Bak KO cells are most susceptible to mitochondrial failure following infection, while Bax/Bak DKO cells show only a slight reduction in mitochondrial activity, similar to wild-type. Knockout of either Bak or Bax increases mitochondrial susceptibility to Influenza A virus, while the double knockout appears to compensate.

In addition to regulating cytochrome c release during apoptosis initiation, Bax and Bak also regulate mitochondrial size and shape by their involvement in mitochondrial fission and fusion in both healthy and dying cells (discussed in Background section). In healthy cells, Bax promotes the fusion of mitochondria by regulating Mfn2 complex assembly, which occurs at sites of mitochondrial fusion (Karbowski, Norris et al. 2006). In turn, mitochondrial fusion inhibits apoptosis (Perfettini, Roumier et al. 2005).

As Bax and Bak are both intimately involved with mitochondrial fission and fusion, we examined mitochondrial morphology following Influenza A infection in wild-type, Bax KO, Bak KO and Bax/Bak DKO cells at 24hpi by staining with Mitotracker Red CMXRos<sup>®</sup> as described in Materials and Methods and observation under confocal microscopy. Figure 1.10, page 195 illustrates that (A) Most mock-infected wild type cells (~98%) exhibit robust ovoid mitochondria, while (B) almost all Bax KO cells (92%) were found to contain intricate networks of small mitochondria end-to-end in a ‘beads on a string’ arrangement. (C) Most of the mock-infected Bak KO cells (88%) contained

robust ovoid mitochondria similar to wild-type, while (D) approximately half of the Bax/Bak DKO cells displayed robust ovoid mitochondria, with the other half displaying smaller mitochondria in a ‘beads on a string’ arrangement. Following Influenza A infection, (E) wild-type cells (88%) retained their robust ovoid mitochondria as seen in mock-infected samples. (F) Nearly all Bax KO cells showed robust ovoid mitochondria similar to wild-type cells following infection, displaying a very different morphology from mock-infected Bax KO cells. (G) Conversely, many Bak KO cells (60%) lost their robust ovoid mitochondrial morphology following infection, instead displaying smaller ‘beads on a string’ mitochondria. (H) Influenza A infection in Bax/Bak DKO cells results in approximately half of the cells displaying robust ovoid mitochondria, with the other half displaying smaller beads on a string mitochondria, similar to mock-infected cells.

Our results indicate that mitochondrial fusion is Bax-dependant in healthy cells, as Bax knockout disrupts mitochondrial fusion leading to a distinct ‘beads on a string’ mitochondrial morphology. However, Influenza A infection ameliorates this effect suggesting that an as-yet unidentified virus-host interaction in the absence of Bax promotes mitochondrial fusion. These data also indicate that Bak, in the absence of Bax, promotes mitochondrial fission during infection, further suggesting virus-host interactions at the mitochondria controlling these events.

### **3.5 Chapter 1 Discussion**

We have demonstrated that Influenza A infection induces Bax-dependent apoptosis that is required for efficient virus replication (McLean, Datan et al. 2009). As Bax and Bak activity are both important to mitochondrial morphology and function, we examined these attributes following Influenza A infection in Bax and/or Bak knockout

cells. In wild-type MEF cells, infection caused only a slight reduction in mitochondrial activity compared to mock-infected controls, as demonstrated by both MTT and MitoTracker Red CMXRos staining. Concurrently, mitochondrial morphology was not noticeably affected following Influenza A infection in wild-type cells.

In contrast (and in agreement with others (Karbowski, Norris et al. 2006)), mock-infected Bax KO cells exhibit interconnected networks of very small mitochondria, possibly due to the lack of Bax-dependent mitofusin-2 (mfn2) activation in these cells. In Bax KO cells, Influenza A infection leads to a decline in mitochondrial activity and a major shift in mitochondrial morphology from the interconnected small mitochondrial networks seen in mock-infected cells to larger, dissociated mitochondria similar to those found in wild-type cells. It is possible that an Influenza A protein (possibly PB1-F2, which is known to colocalize with mitochondrial membranes (Gibbs, Malide et al. 2003)) interacts with mfn2 or a related protein in the absence of Bax and stimulates mitochondrial fusion. An alternative explanation is that Bak, not Bax, regulates mitochondrial fusion during infection, possibly due to specific Bak-virus interactions. The loss of mitochondrial activity in Bax KO cells following infection indicates that Bax activity helps maintain mitochondrial function, perhaps by stimulating mitochondrial fusion.

Bak KO cells suffer a massive loss of mitochondrial activity and subsequently increased apoptosis following infection compared to wild-type. Prior to infection, Bak KO cells exhibit large mitochondria similar to wild-type cells. However, following infection the mitochondria within infected cells shift from their normal appearance seen in mock-infected Bak KO cells to interconnected networks of small mitochondria, similar

to that seen in mock-infected Bax KO cells. This is a surprising result as Bax activity, which is unfettered in these cells, is known to induce mitochondrial fusion. These results therefore suggest that Bak is required for mitochondrial fusion following infection. The loss of mitochondrial activity in Bak KO cells indicates that Bak participates with Bax in maintaining mitochondrial function during Influenza A infection. Examination of Bax/Bak DKO cells revealed that these cells do not suffer the extreme loss of mitochondrial function seen in Bax or Bak single knockout cells. Additionally, Bax/Bak DKO cells do not exhibit the changes in mitochondrial morphology seen in single knockout cells.

Our results indicate that specific virus-Bax-Bak interactions are required to maintain both mitochondrial morphology and function during Influenza A infection. Further, as the individual absence of Bax or Bak leads to severe effects on both mitochondrial morphology and function, while concurrent knockout of both proteins ameliorates these effects, our data suggest that Bax and Bak play opposing roles in the regulation of mitochondrial morphology and function during Influenza A infection. Similarly, we find that Bax and Bak play opposing roles in the regulation of apoptosis and viral replication during infection. Bax is pro-apoptosis and pro-viral during Influenza A infection while Bak is anti-apoptosis and anti-viral (McLean, Datan, et al., 2009). As Bax and Bak-dependent changes in mitochondrial morphology have been linked to changes in mitochondrial health and cellular apoptosis, which have themselves been linked to Influenza A replication, identification of the particular virus-host interactions governing these effects is likely to produce valuable insight into the biochemistry of

Influenza A replication and may provide valuable targets for therapeutic intervention during *in vivo* infection.

## 4 Chapter 2. Flaviviruses

Although specific information concerning flavivirus manipulations of cell death is lacking, the data to date indicate that these manipulations are primarily dependent upon cell type. Several systems exist in which flavivirus infections induce the death of infected cells by apoptosis (Catteau, Courageot et al. 2007). Dengue-1 induces apoptosis in the central nervous system of infected neonatal mice (Despres, Frenkiel et al. 1998), as well as in neuro 2a cells (Depres, Flamand et al. 1996) and hepatoma cells (Marianneau, Cardona et al. 1997). Increased apoptosis also contributes to the neuropathogenesis of Dengue-1 infection *in vivo* (Marianneau, Flamand et al. 1998). Similar findings have been presented for West Nile virus, implicating apoptosis via caspase-9 and caspase-3 activity (Yang, Ramanathan et al. 2002; Samuel, Morrey et al. 2007) in neuropathogenesis. Apoptosis is also associated with liver pathogenesis in yellow fever infections (Bray 2005; Quaresma, Barros et al. 2006), and the induced death of Japanese encephalitis virus-infected neurons (Ghoshal, Das et al. 2007). Although instances of flavivirus-induced apoptosis in certain cell types are irrefutable, several reports of flavivirus-induced autophagy have recently been presented that shed new light on the complexities of flavivirus regulation of cell death. In hepatoma cells and fibroblasts, both Japanese encephalitis virus and Dengue-2 virus infection lead to PI3K-mediated protection against apoptosis (Lee, Liao et al. 2005). A common consequence of PI3K signaling is autophagy (Gutierrez, Master et al. 2004; Baehrecke 2005; Codogno and Majer 2005; Hu, Hacham et al. 2008; Deng, Singh et al. 2010). Dengue-2 virus infection induces autophagy in hepatoma cells (Lee, Lei et al. 2008), and infection-induced autophagosomes are used as sites of Dengue-2 virus replication in fibroblasts

(Panyasrivanit, Khakpoor et al. 2009). Autophagosomes have similarly been demonstrated as the site of virus replication for the closely related viruses cytopathic bovine diarrhea virus (Meyers, Stoll et al. 1998; Birk, Dubovi et al. 2007) and hepatitis C virus (in Huh-7 cells) (Sir, Chen et al. 2008; Dreux, Gastiminza et al. 2009). Flaviviruses are discussed in further detail in Chapter Two.

It has been clearly demonstrated that both apoptosis and autophagy play a role during *in vivo* infection and that this effect is primarily dependent upon cell type. However the specific virus-host interactions governing the fate of infected cells and their effect on flavivirus replication and pathogenesis have not been elucidated. A major goal of the research presented in Chapter Two of this thesis is to identify both viral and cellular factors that control the decision of flavivirus infected cells to die or survive, and the effect of this decision on *in vitro* viral replication.

#### **4.1 The Global impact of Flaviviruses**

Around the world, more than 2.5 billion people live at constant risk of infection with flaviviruses, a group of over 80 related ssRNA viruses with several members that currently top lists for both infection frequency and severity (Harris, Holden et al. 2006). In humans, flavivirus infection typically occurs through arthropod vectors (mosquitoes and ticks), although several viruses in this group have no known vector and rely instead on horizontal transmission. Flavivirus infections, while often asymptomatic, many times progress to severe febrile, encephalitic, hemorrhagic and hepatic illnesses, causing morbidity and mortality around the world and putting great strain on the healthcare systems of regions afflicted with epidemics.

Flaviviruses are small (50-60nm diameter), lipid-enveloped, positive ssRNA viruses that infect a wide range of both vertebrate and invertebrate hosts (Brinton 1983). Flaviviruses belong to the family Flaviviridae, which consists of three closely related genera: pestiviruses, a group that includes bovine diarrhea virus (BVDV), a pathogen of considerable importance to the cattle industry; hepaciviruses, which includes the human pathogen Hepatitis C virus (HCV); and flaviviruses, which currently include some of the most common and devastating human viral pathogens, including:

Yellow Fever virus (YFV). Yellow fever was one of the most lethal and feared diseases before the development of an effective vaccine in the 1940's (Tauraso, Spector et al. 1972). Yellow fever is the original hemorrhagic fever. Transmitted by mosquitoes, it results in hepatic, renal and myocardial injury as well as hemorrhage and shock often leading to death. The earliest reliable description of yellow fever was written by the Spanish friar Lopez de Cogolludo describing the Yucatan epidemic of 1648 in central America (Guerra 1964), and it was a major scourge to the New World and west Africa during colonial the 18<sup>th</sup> and 19<sup>th</sup> centuries. Common clinical features of yellow fever include febrile illness, jaundice, nausea and vomiting blood that often progress to hemorrhage, severe hypotension, shock and death. The disease is caused by Yellow fever virus, and was the first human ailment proven transmissible by a 'filterable agent', or virus. Today, the virus infects over 200,000 people per year in the tropical regions of Africa and South America, resulting in 30,000 deaths annually around the world. The virus is maintained in a sylvatic cycle between non-human primates and tree-hole breeding *Aedes* mosquitoes. Infections among men working the jungle perimeter are

common, and can spread to urban populations through infection of the urban vector, *Aedes aegypti* (Monath 2001).

Japanese encephalitis virus (JEV). Japanese encephalitis is a mosquito-borne disease whose symptoms range from nonspecific febrile illness to severe meningoencephalomyelitis resulting in seizures or flaccid paralysis that often causes irreversible neurologic damage or death (Leyssen 2001). Over 3 billion people live in areas endemic for Japanese encephalitis virus, primarily in Southeastern Asia and the Pacific (Solomon, Ni et al. 2003), and it is currently the most important cause of viral encephalitis worldwide (Tsai 2000). Annually, 30,000-50,000 cases of Japanese encephalitis are reported globally, resulting in 10,000-15,000 deaths (Wang, Fu et al. 2007). Epidemics of Japanese encephalitis (or summer encephalitis) have been described in Japan since the 1870's. The principal vector of Japanese encephalitis virus is *Culex tritaeniorhynchus* which breeds in the calm waters of irrigated rice patties and is primarily zoophilic. The virus is maintained in wading birds, which act as reservoirs, but infection regularly crosses over to pigs, horses and humans. An effective vaccine was developed over 40 years ago, but a multiple dose requirement and logistical problems that plague its delivery to rural areas prevent many from receiving the vaccine (Libraty, Nisalek et al. 2002).

West Nile virus (WNV). West Nile virus is the causative agent of West Nile encephalitis, a febrile illness that leads to neuropathology and death in 4-15% of cases (Hayes and Gubler 2006). West Nile virus was first isolated in 1937 from the blood of a febrile patient in the West Nile region of Uganda. Until 1999, it was regarded as a relatively non-virulent flavivirus. Nearly 40% of the Egyptian population in the Nile

delta were seropositive for the virus in the 1950's, although it was implicated in only a handful of febrile illness and sporadic encephalitis outbreaks in Africa, the Mediterranean Basin, Europe, India, and Australia (Russel and Dwyer 2000; Hayes and Gubler 2006; Murgue, Murri et al. 2006; Gubler 2007) between 1937 and 1999. In 1999, West Nile virus was detected for the first time in the Western hemisphere, making a dramatic entrance into the New York City area. The outbreak resulted in severe neurological and encephalitic manifestations, causing 59 hospitalizations and 7 deaths (12% of hospitalized patients) (Asnis, Conetta et al. 2000). Genomic analysis suggested the epidemic had originated from a West Nile virus strain endemic to Israel (Lanciotti, Roehrig et al. 1999; Chowers, Lang et al. 2001; Nash, Montashari et al. 2001; Hayes 2006). Hastened by the migratory travel of infected birds and the high availability of its principal mosquito vector, *Culex spp.*, West Nile virus marched rapidly across North America, and by 2004 was endemic to all of the continental United States and Canada (Rappole, Derrickson et al. 2000; Lord and Day 2001; Petersen and Hayes 2004; Gubler 2007). Currently, ~80% of West Nile virus infections are asymptomatic, ~20% present as a dengue-like viral syndrome that may be severe but is usually self-limited, <1% lead to neuroinvasive disease including encephalitis, meningitis, and flaccid paralysis. Those that survive neurological manifestations of West Nile infection frequently develop life-long neurological sequelae (Hayes and Gubler 2006). The establishment of persistent infections with West Nile virus has been reported in both humans and mice, and hosts may shed virus in their urine for months to years following full recovery from acute illness (Tesh, Siirin et al. 2005).

St. Louis encephalitis virus (SLEV). St. Louis encephalitis is a disease that occurs in sporadic outbreaks throughout North and Central America. These outbreaks do not have an annual cycle, and typically appear without warning owing to the lack of monitoring and general data about the virus and its transmission cycles. Several environmental factors play into establishment of an outbreak, mainly by supporting the growth of mosquito vectors and bird reservoir populations. As with other flaviviruses, most cases (~80%) of St. Louis encephalitis are asymptomatic (Tesh, Siirin et al. 2005). Clinical manifestations of infection vary from mild febrile illness to severe neuroinvasive disease including aseptic meningitis or encephalitis. The case fatality rate of St. Louis encephalitis is ~3-20%, predominantly in the elderly and very young. Renal involvement is also common, and persistent infection and virus shedding from the kidney epithelium is common well after recovery from acute illness (Tesh, Siirin et al. 2005). Although major outbreaks of St. Louis encephalitis occur infrequently, once an outbreak occurs the virus typically becomes endemic to the affected area, and sporadic cases over the following years are common. St. Louis encephalitis is transmitted primarily by *Culex* spp. mosquitoes (Reisen, Fang et al. 2005), which transmit the virus from birds (which do not become ill after infection and act as a reservoir for the virus) to humans. The first recorded outbreak of St. Louis encephalitis occurred in St. Louis, Missouri in 1933 that caused 1095 cases of hospitalization and 201 deaths (Day 2001). Retrospective analysis suggests that 19 cases of encephalitis in the region the prior year were likely due to infection with the virus (Luby 1979). There are currently no vaccines or treatments for St. Louis encephalitis.

Tick-Borne Encephalitis virus (TBEV). Tick-borne encephalitis is the most dangerous neuroinfection of Europe and Asia. Primarily transmitted by the tick *Ixodes ricinus* (*I. persulcatus* in the eastern Baltic States) (Haglund and Günther 2003), tick-borne encephalitis affects ~3000 people annually. Previously known as Russian Spring and Summer Encephalitis (RSSE), the first records of tick-borne encephalitis-like disease are found in church records from the 18<sup>th</sup> Century (Lindquist and Vapalahti 2008). The disease was described as a clinical entity in Austria in 1931, and Tick-borne encephalitis virus was discovered in 1937 on a Far-East Russian expedition hunting for the agent responsible for acute encephalitis associated with tick bites (Gritsun, Lashkevich et al. 2003). Several grades of disease severity are recognized in association with Tick-borne encephalitis virus infection, ranging from febrile illness to severe meningoencephalitis, and permanent neurological sequelae are common in those that survive infection (Haglund and Günther 2003).

Dengue virus (DENV). Dengue virus is currently the world's most common mosquito-borne illness and is the leading cause of children's hospitalization in Southeast Asia (Panyasrivanit, Khakpoor et al. 2009). Dengue infection commonly leads to Dengue fever, an influenza-like illness with symptoms that range from mild to life threatening (discussed below) (Hencahl and Putnak 1990). Currently, 50-100 million people are infected with Dengue virus worldwide each year, including ~500,000 resulting in life-threatening Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS). With a case fatality rate of approximately 5% (25,000 deaths per year) (Luo, Xu et al. 2008), more people die from DHF/DSS each year than all other hemorrhagic fevers combined (Morens 2009). Dengue infection rates have climbed for over 50 years due to the

increasing range of its principal mosquito vectors *Aedes aegypti* and *Aedes albopictus* (Knudsen 1995; Hopp and Foley 2001; Rogers, Wilson et al. 2006). Currently there are no treatments or vaccines available for Dengue infection aside from supportive care, and prevention is primarily dependent upon mosquito vector control programs (Webster, Farrar et al. 2009).

Other flaviviruses. While the viruses and associated diseases described above cause substantial human morbidity and mortality, in total there are over 80 flaviviruses around the world that infect a wide range of vertebrate and invertebrate hosts. These include: Alfuy, Apoi, Aroa, Bagaza, Banzi, Bouboui, Bukalasa bat, Bussuquara, Cacipacore, Carey Island, Cowbone Ridge, Dakar bat, Edgehill, Entebbe bat, Gadgets Gully, Ilheus, Israel Turkey meningoencephalitis, Jugra, Jutiapa, Kadam, Karshi, Kokobera, Kountango, Kunjin, Kyassanur Forest disease, Langat, louping ill, Meaban, Montana myotis encephalitis, Modoc, Murray Valley encephalitis, Naranjal, Negishi, Ntaya, Omsk hemorrhagic fever, Phnom-Phen bat, Powassan, Rio Bravo, Rocio, Royal Farm, Saboya, Saumarez Reef, Sal Vieja, San Perlita, Sepik, Sokoluk, Spondweni, St. Louis encephalitis, Stratford, Tamana bat, Tembusu, Tyuleniy, Uganda S, Usutu, Wesselsbron and Zika viruses (Westaway, Brinton et al. 1985).

#### **4.2 Clinical Manifestations of Dengue Infection**

In 1801, Queen Maria Luisa of Spain wrote this account of her illness during an epidemic of Dengue-like illness at Aranjuez, Spain in a series of letters to her presumed lover Manuel Gordoy:

June 9: *“I’m not well at all, with a continual bitterness in my mouth, to the point that it distorts my whole visage, making me yellow as saffron, with pain at the pit of my stomach; the hips and womb hurt and make me very uncomfortable; the sadness is strong, with a general heaviness and weakness, my legs are swelling, they tell me I need to exercise on foot and on horseback. I don’t know yet what is my ailment. It worries me because I have what I’ve never felt before, and if this doesn’t stop, I believe this bothersome woman will last only a short time, and you will all be rid of her. In my handwriting you’ll recognize my unsteady hand; God’s will be done.”*

June 12: *“I’m feeling better, because it has been the cold in fashion, that they call dengue, and since yesterday I’ve had some blood, which is what is making me uncomfortable, and after talking some time the throat hurts.”*

June 13. *“Friend Manuel, I’m very sorry to have worried you but in truth the day I wrote to you I felt very ill, but it was that epidemic that we’re having, so general that few escape it. I got away without fever, but in others it causes quite good ones. But it puts one in, and then leaves, a weakness as from a disease, but I’m now well, and only my throat itches, besides having my period rather well, after some months without this relief, so that I’m back in my old self(?) and with appetite” (Rigau-Perez 1998).*

The first comprehensive clinical description of Dengue Fever was reported by Dr. Benjamin Rush during the Philadelphia outbreak of 1793, which he described as “breakbone fever”, “breakheart fever” or “bilious remitting fever” to describe the extreme pain of movement, extreme psychological depression and blackened vomit associated with the illness (Rush 1796). Clinical and experimental observations since the 1920’s

have since led to the description of a spectrum of clinical symptoms related to Dengue Fever (DF) and Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS).

Classical Dengue Fever (DF) is typically a disease of older children and adults, beginning with the sudden onset of fever (103 to 106° F, 39.4 to 41.1°C) 3 to 15 days after initial infection with the virus, usually presenting with a frontal or retro-orbital headache. Altered taste sensation and sore throat are also common. Flushing of the face and a generalized macular rash that blanches under pressure appear during the first 24 to 48 hours after fever onset. Anorexia, nausea and vomiting, generalized lymphadenopathy and cutaneous hyperplasia are all common during days 2-6. Defervescence (abatement of fever) usually occurs on days 2-6, and is typically associated with extreme sweating. Within 24 hours after the initial fever breaks, a secondary morbilliform or macropapular rash (characterized by raised, spotted lesions) often appears that lasts from 1-5 days, usually starting on the trunk and extending to the face and extremities. Upon appearance of this secondary rash, a second rise in temperature is common that lasts 2-3 days before recovery from illness, resulting in a saddle-back patient temperature profile during the course of infection. As the secondary rash fades, localized clusters of petechiae (red or purple spots on the body) appear on the feet, legs, hands, fingers and mucous membranes, indicating pinpoint hemorrhagic lesions across the body. Gastrointestinal bleeding, menorrhagia (abnormally prolonged and/or heavy menstrual bleeding), and bleeding from other organs are also common symptoms (Gubler 1998; Sabin 1952; Hayes and Gubler 1992, Rigau-Perez, Clark et al. 1998). Psychomotor depression is common during DF. Convalescence may take days to weeks, and weakness and depression often persist until full recovery (Gubler 1998).

Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) are more severe vascular manifestations of Dengue infection that are indistinguishable from classical Dengue Fever (DF) for the first 2-5 days after fever onset. In cases of DHF/DSS, the secondary rash and temperature spike associated with defervescence is followed not by recovery (as is the case for Dengue Fever), but instead by vascular permeabilization and plasma leakage. This leads to low blood volume, often resulting in hypovolemic shock, rapid deterioration of patient condition and death. Petechiae, bleeding at venipuncture sites, easy bruising and large ecchymoses (spontaneous bruises) are often observed in patients with DHF. The World Health Organization currently recognizes four grades of DHF/DSS severity. As mild hemorrhagic symptoms are common with DF, hemorrhage alone does not satisfy the diagnosis criteria for DHF/DSS. Grades I and II DHF are characterized by thrombocytopenia (insufficient blood platelets) and a concurrent drop in blood volume. Grade I DHF is declared when the patient has fever and non-specific constitutional symptoms of DHF (easy bruising, bleeding at venipuncture sites). A patient has Grade II DHF when the criteria for Grade I DHF are met *and* spontaneous bleeding occurs. Grade III and IV DHF are defined as Dengue Shock Syndrome (DSS) and are characterized by symptoms of hypovolemic shock. Grade III DHF/DSS is declared when the symptoms of Grade II DHF have been met *and* the patient displays hypovolemic shock with signs of circulatory failure, including weak rapid pulse and low blood pressure. This often rapidly deteriorates to Grade IV DHF/DSS, which is characterized by profound shock and undetectable pulse and blood pressure, often leading to death (Thomas, Strickman et al. 2003). As the dramatic drop in blood volume leading to shock is typically due to plasma leakage, proper diagnosis and

fluid replacement often prevents DHF from progressing to DSS. With appropriate supportive therapy, mortality rates can be reduced to less than 1% (World Health Organization website, accessed October 22, 2009). Patients that do progress to Grade III and IV DHF/DSS develop a weak and rapid pulse, persistent vomiting and intense, sustained abdominal pain. Rapid shifts from fever to hypothermia are common. Changes in the mental status of the patient are also common, shifting from bouts of restlessness to periods of sedation (Gubler 1998). Recovery from Grade III and IV DHF/DSS is often associated with pronounced bradycardia (low heart rate: under 65 beats/min.) and asthenia (overall lack of strength), as well as extreme psychological depression that often persists after recovery from acute illness (Gubler 1998; Lum, Suaya et al. 2008).

#### **4.3 Persistent infection with flaviviruses**

Persistent infections with flaviviruses are common (esp. kidney) well after full recovery from acute illness. Flaviviruses replicate to very high titer in macrophages, which are a primary site of virus replication during acute infection. As infected macrophages cross the epithelium, they transmit the virus to the surrounding epithelial cells. Infected epithelial cells then evade the host immune response and shed virus at low titer for weeks to years following full host recovery from illness (Tonry, Xiao et al. 2005; Siirin, Duan et al. 2007; Murray, Walker et al. 2010). West Nile virus (WNV) causes persistent infections in hamsters (Tesh, Siirin et al. 2005; Tonry, Xiao et al. 2005), rhesus monkeys (Pogodina, Frolova et al. 1983) and humans (Murray, Walker et al. 2010). Japanese encephalitis also commonly causes persistent infections in mice (Mathur, Arora

et al. 1983, 1986) and humans (Sharma, Mathur et al. 1991). St. Louis encephalitis virus causes persistent infection in hamsters (Siirin, Duan et al. 2007) and tick-borne encephalitis virus causes persistent infection in monkeys (Pogodina, Frolova et al. 1981) and humans (Gritsun, Frolova et al. 2003). Modoc virus also causes persistent infections in mice (Davis, Hardy et al. 1974; Johnson 1970) and hamsters (Davis and Hardy 1974). While little work on persistent infection with Dengue has been reported, clinical case reports have detailed the transmission of both Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) to immune-compromised transplant recipients from apparently healthy donors with a history of prior Dengue-related illness but no detectable blood virus or virus-related antibody (Chacko, John et al. 2004; Tan, Loh et al. 2005). Transmission by this manner implies persistent infection within donor tissues, and suggests that blood testing negative for Dengue may not necessarily equate to a non-infected host. More distantly related members of the Flaviviridae also induce persistent infections in host tissues. Bovine Diarrhea virus (Flaviviridae; *pestivirus*) can induce persistent infections in mousedeer (Uttenthal, Grøndahl et al. 2005), pigs (Terpstra and Wensvoort 1997) and cattle (Njaa, Clarke et al. 2000; Fray 2001; Wittum, Grotelueschen et al. 2001) while Hepatitis C virus (Flaviviridae; *hepacivirus*) can create persistent infections in chimpanzees (Weiner, Erickson et al. 1995) and humans (Brillanti, Foli et al. 1993; Ferrari, Valli et al. 1994; Gretch, Bacchi et al. 1995). Thus representative members of all known clades of the flaviviruses as well as more distantly related members of the Flaviviridae are known to generate persistent infections within a significant proportion of infected individuals. The number of flaviviruses and their

relatives that share this feature makes obvious the importance of viral persistence in the epidemiology and clinical evaluation of flavivirus-induced disease.

#### **4.4 The Challenge of Multiple Dengue Serotypes**

While vaccine development in single-serotype flaviviruses like Japanese encephalitis virus, Yellow Fever virus and Tick-borne encephalitis virus have been largely successful, a Dengue vaccine continues to confound researchers due to the strict requirement of simultaneously stimulating equal immunity to each of four distinct serotypes. The four serotypes of Dengue virus (DENV1-4) are actually four different, very closely related viruses. Infection with any of any one of these serotypes often leads to the production of subneutralizing antibodies to the other three serotypes (Beltramello, Williams et al. 2010; Zellweger, Prestwood et al. 2010). Severity of dengue disease has been correlated with viral titer in humans (Sabin 1952). Subneutralizing antibodies have been seen to increase viral titer in non-human primates (Anderson, Wang et al. 1997), suggesting that, as a result of an earlier infection by one serotype the host becomes more susceptible to severe illness (Dengue Hemorrhagic Fever and Dengue Shock Syndrome) following infection with another. Although the pathogenesis of Dengue DF/DHF/DSS is not well characterized, the increased severity of secondary Dengue infection has been linked to antibody-dependent enhancement of secondary Dengue infection (Mongkolsapaya, Dejnirattisai et al. 2003). Antibody-dependent enhancement increases viral entry into cells, increasing subsequent viral load in infected tissues (Mongkolsapaya, Dejnirattisai et al. 2003; Moi, Lim et al. 2010). As severity of Dengue disease has been correlated with viral titer in humans and passive transfer of sub-neutralizing antibodies increases viral

titer in non-human primates (Anderson, Wang et al. 1997), antibody-dependent enhancement has arisen as an attractive model to explain the increased severity of secondary Dengue infection, as it explains both the increased viral loads and the severe immunopathogenesis of secondary Dengue infection.

Following primary infection (no prior exposure to Dengue), antibodies are produced that grant the host immunity to that serotype. However, while these antibodies work well against the Dengue serotype for which they were made, they also cross-react inefficiently with other Dengue serotypes, leading to sub-neutralizing activity for other serotypes. Therefore, upon subsequent infection with a different Dengue serotype (secondary infection), these antibodies bind to the E protein of the invading viruses. Instead of neutralizing the secondary serotype, these sub-neutralizing antibodies mediate virus-antibody complex formation with Fc receptors on the surface of monocytic cells, including macrophages. The increased viral attachment mediated by this immune complex formation promotes viral entry and leads to increased infected cell mass, dramatically increasing viral load *in vivo* (Goncalvez, Engle et al. 2007). Further, the cross reaction of these antibody responses generates an upregulation of inflammatory cytokine production. This causes lymphocytes to migrate to the area, providing Dengue new hosts for antibody-dependent enhanced infection (further increasing viral load). Inflammatory cytokines also promote complement activation, which has been linked to apoptosis in the vascular endothelium resulting in vascular permeabilization and progression to DHF/DSS (Mongkolsapaya, Dejnirattisai et al. 2003).

#### **4.5 History of the Dengue viruses**

The earliest records of Dengue-related illness were written in China during the Chin Dynasty (265 to 420 A.D.) as part of an encyclopedia of disease symptoms and remedies. Called ‘water poison’ by the people of the time, it was believed to be associated with flying insects near water (Thu 2009). Outbreaks of Dengue-like illness were also recorded in the French West Indies in 1635 and in Panama in 1699, although the first well-documented cases of Dengue-like illness did not occur until 1779 when epidemics of Dengue-like ‘joint fever’ swept Jakarta, Indonesia and Cairo, Egypt. This was followed by an outbreak in Philadelphia, USA the following year (Carrey 1971). Over the next 160 years, epidemics of Dengue-like illness were infrequent (10-40 years between outbreaks) but large. Major Dengue epidemics erupted in Zanzibar in 1823 and 1870. The West Indies experienced their first Dengue epidemic in 1827. Calcutta had several waves of epidemic Dengue in 1824, 1853, 1871 and 1905, and Hong Kong had an outbreak in 1901 (Schlesinger 1977).

As epidemics of Dengue-like illness swept through ports around the globe it was described by many names, including break-bone fever, quebranta huesos, dengüero, dengue fever, dandy fever, bouquet fever, giraffe fever, polka fever and the 7 day fever (Sabin 1955). The word ‘Dengue’ is derived from the Swahili “Ka dinga pepo”, which translates to “a kind of sudden cramp-like seizure from a spirit or plague”. The term was popularly identified with the Spanish word “Dengue”, which translates in Spanish to “affectation; false pretense” (Megenny 1983). In the British West Indies, “Ka dinga pepo” was popularly identified with the English word “dandy”, meaning foppish; pretentious (Ariza 2005). In both cases, the terms were used to describe the stiff body

movements of those suffering from Dengue-related joint pain. Other common names for Dengue fever including ‘bouquet fever’, ‘giraffe fever’, and ‘polka fever’ refer to the petechiae and skin lesions that occur on most patients infected with Dengue virus.

Between 1780 and 1940, Dengue-affected regions would typically have only one of the four Dengue serotypes endemic to an area, allowing local populations to develop immunity to Dengue-related illness. Although transmission of Dengue to locals was limited, travelers new to afflicted regions would frequently develop Dengue-like symptoms, indicating active circulation of the virus. Worldwide spread of the virus between Asia, Africa and the Americas was facilitated by the expansion of the global economy by naval shipping at the time. As infected mosquito vectors could use the stored water on a ship to breed, they subsequently maintained the Dengue transmission cycle with people aboard the ship, allowing the virus to survive long distances aboard infected vessels. When an infected ship delivered goods to a new port, both the mosquitoes and the virus were delivered as well (Gubler 1998). If the serotype of Dengue delivered in this fashion matched the Dengue serotype already circulating in the area, little would happen due to the pre-existing immunity of the local population. However, many ships travelling between Asia, Africa and the Americas, regions with historically different Dengue serotypes, would occasionally transmit differing serotypes to each continent. Presumably, it was when a new serotype was introduced to an area that epidemics struck. Since the local population had no immunity to the introduced serotype, and because secondary Dengue infections are typically more severe than initial infections (discussed above), these outbreaks were often large.

Although Dengue fever epidemics worldwide have been increasingly common since 1779, epidemics of Dengue-like illness with severe hemorrhagic symptoms did not appear until near the beginning of the twentieth century. The first was at Queensland, Australia in 1897. Due to the sudden increase in severity of this epidemic, Dengue research was spurred in earnest and in 1907 Dengue fever became the second human disease (after Yellow fever, another flavivirus disease) whose etiology was confirmed as caused by a 'filterable agent', or virus (Ashburn 1907). In 1922, an outbreak of Dengue with hemorrhagic symptoms in the southern United States affected 1-2 million people. Durban, South Africa followed with a large hemorrhagic Dengue epidemic in 1927, followed by another in Greece the following year, and another in Formosa, China in 1931 (Halstead 1965).

The early 1940's saw a global shift in the ecology of the Dengue viruses. The massive globalization of troops and supplies across all continents provided the perfect opportunity for the global spread of hyperendemic Dengue (more than one endemic serotype), particularly to areas of conflict in Southeast Asia and the Pacific islands. Similarly to their travel aboard naval vessels in the centuries before, mosquito vectors were transported from all points of the globe to these regions of conflict aboard military ships and aircraft. As a result, most countries in Southeast Asia were hyperendemic for Dengue by the end of WWII (Hammon 1960). The first confirmed cases of Dengue Hemorrhagic Fever were reported in Manila, Philippines in 1953-1954 (Hammon, Schrack et al. 1958). The initial DHF outbreak in the Philippines was followed by major DHF epidemics in Bangkok, Thailand in 1958 (Halstead 1965) and Vietnam in 1963 (Halstead, Voulgaropoulos et al. 1965). By the 1970's, all of Southeast Asia was

afflicted with endemic DF/DHF (Gibbons and Vaughn 2002). During this time, the trend of increasing Dengue severity was avoided in the Americas due to the success of an *Aedes aegypti* eradication program implemented by Dengue-affected countries (Istúriz, Gubler et al. 2000). However, this program was dissolved during the 1970's and *Aedes aegypti* re-invaded the continent. By 1990 the mosquito vector had regained most of the territory it had lost during the vector eradication campaign. In areas across South and Central America, many that had been Dengue-free for 35-130 years, DF/DHF epidemics became increasingly more frequent as the mosquitoes returned. In 1977, Cuba experienced a large epidemic affecting over 500,000 people that infected nearly 45% of the urban Cuban population (Guzman, Kouri et al. 2000). Another Cuban Dengue epidemic in 1981 spurred the introduction of a mosquito control program that all but eliminated both the mosquito and the virus from the country by 1983. However, during the 1990's the Cuban vector eradication program was disbanded, and *Aedes aegypti* returned, bringing hyperendemic Dengue with it. A Dengue outbreak in Havana during 1997 resulted in 38.5 times more DHF and case fatality than the previous epidemic in 1981, owing largely to the establishment of hyperendemic Dengue across Cuba in the years between outbreaks (Guzman, Kouri et al. 2002). By 1997, 24 American countries were reporting laboratory-confirmed endemic dengue (Pinheiro and Corber 1997). During 2002, 30 American countries reported over 1 million cases of DF/DHF (Guzman and Kouri 2003). Currently over 100 countries are hyperendemic for Dengue virus, and its mosquito vectors continue their expansion across the planet (Gubler, Reiter et al. 2001). This expansion has brought *Aedes aegypti* to the United States, and the mosquito is currently endemic in Florida and Texas (Moore and Mitchell 1997). Sustained

transmission of Dengue virus in Florida was reported for the first time in 2009 (Franco, Hynes, et al., 2010)

Continued global expansion of Dengue and the increase in Dengue disease severity over the past 50 years have created a particularly devastating threat to global human health. The sheer number of Dengue infections and subsequent fatalities worldwide necessitates the development of effective anti-viral treatments and vaccines. Animal models of flaviviral pathology are being developed (Charlier, Leyssen et al. 2004). Although important strides in the understanding of the molecular biology behind Dengue infection have been made, the existing lack of knowledge regarding the basic characteristics of *in vivo* and *in vitro* infections restricts the development of refined animal models and anti-viral treatments. The development of specific knowledge regarding the biochemical activity of each flavivirus protein during viral replication is an important step towards the development of anti-viral therapeutics for use during acute and persistent infection.

#### **4.6 Flavivirus Genome and Replication Cycle**

The flavivirus genome is ~11kB long, and contains a single open reading frame that encodes ten viral genes. The genome contains a 5' UTR cap, but lacks a 3' poly(A) tail. Within a few hours, an infected cell can generate tens of thousands of newly synthesized viral genomes. The 5' portion of the coding region encodes three structural proteins that form the physical structure of the virion (C, prM and E, discussed below). The remaining seven non-structural proteins are encoded by the 3' coding portion of the genome (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, discussed below), and they

dictate the effectiveness of viral replication, viral assembly and host immune evasion. Flaviviruses initially attach to heparin sulfate on the cell surface through interactions with the envelope protein. Upon attachment, the virus enters the cell through endocytosis. Once the virus is exposed to the low pH environment of cellular endosomes, viral fusion is triggered and the nucleocapsid is released into the cytosol. The ssRNA viral genome is then uncoated and translocates to the endoplasmic reticulum due to a targeting sequence in the 5'UTR, where its m<sup>7</sup>GpppN<sub>1</sub>mpN<sub>2</sub> cap structure aids in initiation of translation. Ribosomes translate the viral genome into a ~370 kDa polyprotein containing all ten viral proteins in the order C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5 (Wu, Bera et al. 2005). The viral polyprotein is then cleaved by both viral and cellular proteases to release its individual constituents (Leyssen 2000) (discussed below). Intracellular membrane rearrangements are a common feature of flavivirus infection, and three main structures accumulate within infected cells: Convoluting membranes, paracrystalline arrays and vesicle packets/smooth membrane structures (Mackenzie, Jones et al. 1999).

#### **4.6.1 Flavivirus 3' and 5' Untranslated Regions (UTR's)**

The coding region of the flavivirus genome is flanked by 5' and 3' UnTranslated Regions (UTR's) that contain cis-acting RNA domains controlling viral protein translation, RNA synthesis and encapsidation. Translation of the viral polyprotein is achieved through cap-dependent initiation at the 5'UTR (Chiu, Kinney et al. 2005). Complementarity of RNA between the 3' and 5' UTR's allows cyclization of the flavivirus genome, which is essential for efficient NS5 RdRp activity and flavivirus replication (Charlier, Leyssen et al. 2002; Alvarez, De Lella Ezcurra et al. 2005; Gritsun

and Gould 2007). Severe deficiencies in virus RNA synthesis occur with deletion of specific domains at the 5' and 3' UTR, indicating their essential role in flavivirus replication.

#### **4.6.1.1 Flavivirus 5' UTR**

The flavivirus 5' UTR is ~100 nucleotides long and is highly conserved among the different Dengue serotypes (Brinton and Disposito 1988; Markoff 2003). Upon decapsidation, its type 1  $m^7GpppN_1mpN_2$  RNA cap structure is recognized by cytoplasmic cap binding protein eIF4E, which associates with initiation factor eIF4G. This complex serves as a scaffold for attachment to eIF3, which is itself attached to the small ribosomal subunit, promoting ribosomal attachment at the 5'UTR and translation of the viral polyprotein at the 5'-most AUG codon (Chiu, Kinney et al. 2005).

The 5' UTR also contains two RNA domains with essential functions in flavivirus RNA synthesis. The first domain of the 5' UTR is 70 nucleotides long and folds into a large stem-loop that serves as the promoter for viral NS5 viral RNA dependent RNA polymerase (RdRp) activity and amplification of the viral genome. Direct binding of NS5 to the SLA is required for viral replication *in vitro* (Filomatori, Lodeiro et al. 2006), allowing NS5 to discriminate between cellular and viral RNAs and ensuring that NS5 RdRp activity is focused on viral genome synthesis. The second domain in the 5'UTR, called the 5'UAR, is a 16 nucleotide sequence that folds into a short stem loop structure (SLB). The SLB is complementary to a portion of the 3' UTR of the flavivirus genome (3'UAR), allowing cyclization of the genome. This brings the regions of each UTR that are essential for NS5 activity together, allowing viral RNA synthesis (Markoff 2003).

#### **4.6.1.2 Flavivirus 3' UTR**

The flavivirus 3' UTR is ~450 nucleotides long, and lacks a poly(A)-tail due to NS3 virus-RNA-stimulated nucleoside tri-phosphatase (NTPase) activity during replication (discussed below) (Li, Clum et al. 1999). The 3' UTR ends in a highly conserved stem-loop structure (3'SL) that is an absolute requirement for flavivirus replication (Alvarez, De Lella Ezcurra et al. 2005). The 3'SL contains the 3'UAR region that is complementary to 5'UAR, allowing cyclization of the flavivirus genome during replication ensuring the generation of full length replicants by the viral replication complex (Hahn, Hahn et al. 1987). Additionally, a second site of 3' complementarity to the 5' region of the genome exists upstream of the 3'UAR. This site, called the 3'CS, is 11 nucleotides long contains complementary to a region within the C gene (the most 5-prime gene) of the coding region. Hybridization of both the 3'-5'UAR and 3'-5'CS domains is essential for flavivirus replication (Khromykh and Westaway 1997; Alvarez, De Lella Ezcurra et al. 2005).

#### **4.6.2 Flavivirus Proteins**

##### **4.6.2.1 Flavivirus Structural Proteins (C, prM, E)**

The flavivirus virion is composed of three viral structural proteins: the envelope protein, the capsid protein and the membrane protein. These proteins are arranged in a strict geometric formation, forming a 50-70nm icosahedral shell that surrounds the viral genome. Initial cleavage of these proteins from the viral polyprotein occurs by host

signalase activity, and further processing of each protein occurs by viral protease NS3/2B as well as by the activity of host cellular proteases signalase and furin.

#### **4.6.2.1.1 Capsid Protein (C)**

The viral Capsid (C) protein is an ~11kDa protein that shares less homology between flaviviruses than the other two structural proteins (E and prM, discussed below) (Mandl, Ecker et al. 1997), and can withstand large internal deletions without affecting virus replication (Kofler, Heniz et al. 2002). It is the first protein cleaved from the viral polyprotein and forms homodimers that assemble into the nucleocapsid to drive genomic packaging into budding virions. Multiple copies of the C protein surround a single copy of the viral genomic RNA to form the viral nucleocapsid (Jones, Ma et al. 2003), which is then incorporated into the ER membrane. The C protein is very rich in lysine and arginine, giving it an overall positive charge that facilitates interaction with negatively charged viral genomic RNA (Khromykh and Westaway 1996). C protein association with the ssRNA viral genome is required for infectious virion production (Khromykh, Varnavski et al. 1998), and relies on cellular and viral protease processing of the Capsid protein to facilitate genome association with budding virions. The lack of pre-formed nucleocapsids in infected cells (Mukhopadhyay, Kuhn et al. 2005) indicates that nucleocapsid formation occurs in tandem with virus budding and assembly. Three main processing events on the Capsid protein facilitate the coordination of nucleocapsid formation with virion budding through the ER. Host signalase first cleaves the C-prM complex from the viral polyprotein, and an ER targeting domain on the C protein causes association with the ER membrane. The viral protease NS3/2B (discussed below) then cleaves the capsid protein from prM, exposing a host protease signalase cleavage site.

Cleavage at this site completes processing of the C protein which allows the simultaneous interaction of both the viral genome and the ER membrane with the protein. The mature C protein has an internal hydrophobic sequence that anchors it to the ER membrane via a hairpin loop (Kofler, Heniz et al. 2002), facilitating assembly of complete virions by associating the C-associated genome with the E-prM rich ER membrane. prM-E heterodimers (discussed below) then facilitate nucleocapsid budding through the ER membrane, surrounding the nucleocapsid and completing the assembly of an immature virion.

#### **4.6.2.1.1.1 Pre-Membrane Protein (prM)**

The pre-Membrane protein ensures that newly synthesized progeny virions can escape the host cell by inhibiting E protein binding to host cell membranes before virion release. prM is initially cleaved from the viral polyprotein by host proteases along with the Capsid protein (discussed above). An ER targeting domain on the Capsid protein localizes the C-prM complex to the ER membrane. The viral protease NS3/2B (discussed below) cleaves C from this complex (Yamshchikov and Compans 1994). The viral pre-membrane (prM) protein then assembles at the ER membrane, heterodimerizing with the E protein and enriching the ER with flavivirus E-prM heterodimers. The (+)-sense flavivirus genome, in complex with the capsid protein, buds through this membrane (Heniz, Stiasny et al. 1994) to facilitate viral assembly. The prM-E complexes embedded in these membranes promote budding from the ER (Lorenz, Kartenbeck et al. 2003) and surround the genome with exactly 180 copies of each protein. E-prM heterodimers are capable of generating non-infectious virus-like particles (VLP's) without the association

of the virus genome or the capsid protein (Lobigs and Lee 2004), indicating that genome association is not required for structural synthesis. During budding, the prM-E heterodimers arrange into 60 asymmetric spikes along each virion surface. At the ends of these spikes, prM caps the fusion domain of the E protein, preventing premature fusion with host cell membranes before release (Zhang, Corver et al. 2003; Zhang, Kaufmann et al. 2007; Li, Lok et al. 2008). During passage through the Golgi, cellular protease furin (Elshuber, Allison et al. 2003) and viral protease NS3/2B (Lobigs 1993) cleave prM from E. This causes E dimer formation which exposes the membrane fusion domains on E, maturing the virus and allowing it to be infectious once released (Elshuber, Allison et al. 2003; Yu 2008). The mature M protein remains associated with the virion membrane and provides some structural stability to the newly synthesized viral membrane (Heinz and Allison 2001).

#### **4.6.2.1.1.2 Envelope Protein (E)**

The flavivirus envelope glycoprotein (E) is the most antigenically active flavivirus protein in mammals, eliciting neutralizing and protective antibodies from infected hosts. The E protein coats the surface of the flavivirus virion, and serves as both the viral attachment protein and the viral fusion protein, mediating virus binding and entry as well as decapsidation of the viral genome. The E protein drives fusion to the prospective host cell through interactions with receptors on the cell surface (Hanna, Pierson et al. 2005). Initially, the E protein interacts with heparin sulfate on the cellular membrane, concentrating the virus to facilitate interaction with cell-type specific receptors for viral entry (Lee and Lobigs 2000; Belting 2003). The presence of N-linked aminoglycans on the E protein facilitate interactions with heparin sulfate (Hanna, Pierson

et al. 2005), and variations in glycosylation of the E protein have been linked to neurovirulence and disease severity (Post, Santos et al. 1992). Once concentrated at the cell surface by heparin sulfate, the virus interacts with cell-type specific receptors and triggers clatherin-mediated endocytosis (Chu and Ng 2004; Heniz, Stiasny et al. 2004; Lecot, Belouzard et al. 2005). Several cell membrane proteins have been implicated as putative receptors for flavivirus entry, including Heat Shock Protein (HSP) 90 and HSP 70 (Reyes-del Valle, Chavez-Salinas et al. 2005), and the cellular C-type lectin receptor DC-SIGN (Tassaneetrithep, Burgess et al. 2003; Pokidysheva, Zhang et al. 2006). Several other putative receptors exist that have not been fully characterized. The cell-type specific expression of these proteins may explain the tissue tropism seen during flavivirus infection. Once endocytosis is complete, the low pH of the early endosomes triggers E protein trimerization, exposing a membrane targeting sequence on the protein that causes it to insert into the endosomal membrane (Allison, Schlich et al. 2001; Stiasny and Heniz 2006). This, in turn, results in the fusion of the viral membrane to the endosomal membrane, creating a pore through which the viral genome escapes to the cytosol (Yamshchikov 1997; Kuhn, Zhang et al. 2002; Bressanelli, Stiasny et al. 2004).

#### **4.6.2.2 Flavivirus non-structural proteins**

The seven non-structural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These viral proteins are not incorporated into progeny virions, and instead are involved in the manipulation of specific aspects of host cellular biochemistry to promote virus replication and evasion of the host immune response. Although the particular mechanisms of viral replication and assembly are not fully elucidated, effective

replication requires the presence of most of the non-structural proteins assembled at the viral replication complex (Guyatt, Westaway et al. 2001; Malet, Egloff et al. 2007).

#### **4.6.2.2.1 NS1**

Flavivirus non-structural protein 1 (NS1) is a highly conserved 40-50 kDa glycoprotein found in both soluble (sNS1) and membrane-bound (mNS1) forms during infection. NS1 activity is vital to flavivirus replication, although the mechanisms remain elusive (Kumarasamy, Wahab et al. 2007). NS1 interaction with NS4A (the putative docking site for the flavivirus replication complex, discussed below) (Lindenbach and Rice 1999), and co-localization with dsRNA viral intermediates (Mackenzie, Jones et al. 1996; Westaway, Mackenzie et al. 1997) have been demonstrated. Also, NS1-deficient viruses do not replicate efficiently (Lindenbach and Rice 1997; Muylaert, Galler et al. 1997). Together, these data indicate that NS1 is a functional component of the viral replication complex, although its role has not yet been elucidated.

NS1 is initially cleaved from the viral polyprotein by the host cellular protease signalase, in complex with NS2A (discussed below). A protease associated with the ER membrane then cleaves the link between NS1 and NS2A, releasing soluble NS1 monomers (Falgout and Markoff 1995). NS1 homodimerization quickly occurs, exposing a membrane targeting domain that drives association of NS1 dimers with the host ER membrane (Winkler, Maxwell et al. 1989). NS1 is then secreted through the Golgi and expressed on the surface of infected cells (Chung, Thompson et al. 2007). NS1 expression on the cellular surface induces an immune response, and NS1-specific antibodies are produced in mammalian hosts that provide protection against flavivirus-

associated pathology during infection with Dengue-2 virus (Shu, Chen et al. 2000), Japanese encephalitis virus (Lin, Chen et al. 1998), Yellow Fever virus (Despres, Dietrich et al. 1991), West Nile virus (Chung, Nybakken et al. 2006), and Tick-borne encephalitis virus (Jacobs, Stephenson et al. 1992). As antibodies to structural flavivirus proteins (*i.e.* the E protein) may contribute to antibody-dependent enhancement of secondary dengue infection, the generation of NS1-specific antibodies has been developed as an alternative target for vaccine design (Schlesinger, Brandriss et al. 1987; Jacobs, Stephenson et al. 1994; Shu, Chen et al. 2000; Puttikhunt, Kasinrerak et al. 2003; Chung, Nybakken et al. 2006; Webster, Farrar et al. 2009). However, human NS1-specific antibodies are complement-fixing, and may contribute to the pathogenesis of secondary dengue infection (Falconer 1997). That said, recent studies have shown that West Nile virus NS1 actually inhibits complement fixation by binding to regulatory factor H and sequestering it, thereby minimizing activation of the complement pathway and allowing immune evasion (Chung, Liszewski et al. 2006). Dengue sNS1 is also secreted as a hexamer from infected cells, and is found in the blood sera of those experiencing a secondary, but not primary, dengue infection (Young, Hilditch et al. 2000). Due to its high level of secretion during both Dengue Fever and Dengue Hemorrhagic Fever, NS1-specific assays are being developed that are superior to viral RNA analysis as a rapid predictive tool for severity of Dengue infection (Kumarasamy, Wahab et al. 2007).

#### **4.6.2.2.1.1 NS2A**

NS2A is a small 25 kDa hydrophobic protein with several transmembrane domains that embed into the ER membrane, assisting in virus membrane formation

(Mackenzie, Khromykh et al. 1998; Leung, Pijlman et al. 2008) and packaging of virus RNA into the nucleocapsid (Kummerer and Rice 2002). It also acts as an interferon inhibitor, partially blocking STAT1 phosphorylation and resulting in the attenuation of downstream interferon signaling (Liu, Chen et al. 2004; Munoz-Jordan, Laurent-Rolle et al. 2005). NS2A binds with high specificity to the 3' UTR of viral ssRNA and other components of the viral replication complex, including NS3, NS4A and NS5 (Mackenzie, Khromykh et al. 1998). Presumably, this interaction facilitates newly synthesized viral nucleocapsid incorporation into progeny virions during assembly. In addition, NS2A deficient flavivirus replicons could not induce membrane rearrangements typical of wild-type flavivirus infection. However, this limitation could be rescued during NS2A-deficient flavivirus infection by supplying NS2A expression *in trans* (Leung, Pijlman et al. 2008) suggesting that virus-induced membrane rearrangements require NS2A activity. The mechanism by which NS2A binds to the virus replication complex or how it facilitates membrane rearrangements in host cells is not yet fully understood.

#### **4.6.2.2.1.2 NS2B**

NS2B is small ~14kDa membrane-bound viral protein that serves as a required cofactor for proper proteolytic processing of the viral polyprotein by viral protease NS3 (discussed below) (Falgout, Pethel et al. 1991; Chambers, Nestorowicz et al 1993; Falgout, Miller et al. 1993). NS2B contains an N-terminal hydrophilic domain that interacts with the N-terminal portion of soluble NS3 (Droll, Krishna-Murthy et al. 2000). These interacting domains stabilize the NS3 protein and the hydrophobic domains of NS2B anchor the NS2B-NS3 complex to the ER membrane. This promotes association with the viral polyprotein embedded in the ER membrane and facilitates its proteolytic

processing (Chambers, Nestorowicz et al. 1993; Katzenmeier 2004). In addition, NS2B association with NS3 completes the NS3 substrate binding domain, allowing full activation of the NS3 proteolytic domain (Erbel, Schiering et al. 2006). No other function of NS2B has been described.

#### **4.6.2.2.1.3 NS3**

NS3 is a soluble multifunctional ~69kDa viral protein (Yon, Teramoto et al. 2005) that is currently under scrutiny as a target for anti-viral drug development due to its essential role in viral polyprotein processing. Its N-terminal domain, in complex with NS2B (discussed above), functions as a viral trypsin-like serine protease (Bazan and Fletterick 1989; Weir et al. 1990; Bartenschlager, Ahlborn-Laake et al. 1993; Lobigs 1993). Together with intracellular proteases signalase and furin, NS3/2B releases individual proteins from the viral polyprotein and processes some into their mature forms (Bessaud, Pastorino et al. 2006). Specifically, NS3/2B drives polyprotein cleavage at the NS2A-NS2B (Preugschat, Yao et al. 1990), NS2B-NS3 (Falgout, Pethel et al. 1991), NS3-NS4A and NS4B-NS5 (Cahour, Falgout et al. 1992) junctions. NS3/2B is also involved in proteolytic maturation of C (Lobigs and Lee 2004), NS2A and NS4A (Miller, Kastner et al. 2007) following their release from the viral polyprotein. The NS3 C-terminal domains acts as a viral helicase, unwinding viral dsRNA intermediates during replication (Utama, Shimizu et al. 2000; Wu, Bera et al. 2005). As with many RNA helicases, NS3 is also a virus RNA-stimulated nucleoside tri-phosphatase (NTPase), which provides the energy required for helicase activity (Li, Clum et al. 1999). NTPase activity of NS3 results in cleavage and the loss of the flavivirus ssRNA poly(A) tail from

newly synthesized virions (Li, Clum et al. 1999). NS3 is also a 5'-RNA triphosphatase and mediates the first of three sequential enzymatic reactions involved in generation of the 5' RNA cap structure of the flavivirus genome, allowing initiation of viral translation (Benarroch, Selisko et al. 2004; Yon, Teramoto et al. 2005).

#### **4.6.2.2.1.4 NS4A**

Flavivirus NS4A is a ~16 kDa (127 amino acid) membrane-associated protein whose role is the most poorly characterized among flavivirus proteins (Speight and Westaway 1989; Miller, Kastner et al. 2007). Mature NS4A is the least conserved of the flavivirus proteins and consists of a cytosolic N-terminal tail (amino acids 1-49) and three membrane-bound helices (amino acids 49-74, 74-100 and 100-127) (Speight and Westaway 1989, Miller, Kastner et al. 2007) Once the viral genome is translated in the ER, NS4A is initially cleaved from the viral polyprotein in complex with NS4B and the 2K region between them (NS4A-2K-NS4B) by the viral protease NS3/2B (Roosendaal, Westaway et al. 2006). The C-terminal end of NS4A is cleaved by host signalase in the ER lumen at the N-terminus of the 2K region, removing the link with the neighboring protein, NS4B (Lin, Amberg et al. 1993) and allowing full NS4A activity. Collectively the data suggest that NS4A induces ER membrane rearrangements, the viral replication complex assembles on ER membrane-bound NS4A to form the virus replication complex, which then colocalizes with autophagosomal membranes (Lee, Lei et al. 2008; Panyasvrit, Khakpoor et al. 2009). NS4A also inhibits Interferon  $\alpha/\beta$  signaling by downregulating STAT1 activation (Jones, Davidson et al. 2005; Munoz-Jordan, Laurent-Rolle et al. 2005), effectively downregulating the immune response to infection.

NS4A is the determinant viral factor for infection-induced intracellular ER membrane curvatures that result in massive ER membrane rearrangements (Mackenzie, Khromykh et al. 1998; Roosendaal, Westaway et al. 2006; Miller, Kastner et al. 2007). Proteolytic cleavage of the 2K fragment of NS4A from certain flaviviruses, including Dengue virus, is necessary for NS4A induced ER membrane rearrangements (Roosendaal, Westaway et al. 2006; Miller, Kastner et al. 2007), possibly by allowing the folding of ER-membrane bound NS4A. The first membrane domain of NS4A spans the ER membrane, the second embeds in the luminal leaflet of the membrane and the third spans the ER membrane again, forming a U-shaped structure within the membrane (Miller, Kastner et al. 2007). The precise virus-host interactions that govern NS4A-mediated ER membrane rearrangements remain unclear. Steric hindrance between these membrane-bound domains and the ER membrane may cause membrane curvature, ER rearrangements and ER stress directly by bending the membrane, although this has not been confirmed. Alternatively, NS4A may interact with cell factors to trigger host-mediated membrane rearrangements.

The virus replication complex assembles on ER membranes, the same membranes that NS4A rearranges. NS4A participation in the formation of the flavivirus replication complex has been indicated during both Dengue-2 virus and Kunjin virus infection (Mackenzie, Khromykh et al. 1998; Lindenbach and Rice 1999). NS4A co-localizes with dsRNA, and the interaction between NS4A and NS1 is required for efficient viral RNA synthesis, indicating that NS4A acts as the docking site for the replication complex by tethering other nonstructural proteins to intracellular membranes (Bartenschlager, Lohmann et al. 1995; Miller, Kastner et al. 2007). In addition, NS4A indirectly supports

dsRNA stability by binding with host cell polypyrimidine tract-binding protein (PTBP), which binds to viral dsRNA intermediates and stabilizes their association with the viral replication complex (Jiang, Yao et al. 2009).

#### **4.6.2.2.1.5 NS4B**

NS4B is a small membrane-bound protein that acts as the primary flavivirus-encoded interferon inhibitor (Munoz-Jordan 2003, 2005; Evans and Seeger 2007). NS4B is initially cleaved from the viral polyprotein in complex with NS4A and the 2K region between them (NS4A-2K-NS4B) by the viral protease NS3/2B (Roosendaal, Westaway et al. 2006). These membrane bound viral proteins then begin to induce host membrane rearrangements. Release of NS4B from this complex by host signalase mediated cleavage allows its functionality as an interferon inhibitor (Roosendaal, Westaway et al. 2006). NS4B expression, and to a lesser extent NS2A and NS4A, inhibits STAT1 phosphorylation, effectively blocking its translocation to the nucleus and diminishing the upregulation of host interferon-stimulated anti-viral genes (Munoz-Jordan, Sanchez-Burgos et al. 2003). A single amino acid substitution in the central portion of NS4B of West Nile virus confers a highly attenuated phenotype in mice (Wicker, Whiteman et al. 2006), further demonstrating NS4B's essential role in host immune evasion. NS4B also interacts with NS3 in the viral replication complex and increases the activity of its helicase domain by dissociating viral ssRNA from NS3 (Umareddy, Chao et al. 2006).

#### 4.6.2.3 NS5

NS5 is a ~100 kDa protein that is the main viral protein involved in flavivirus replication (Malet, Eglhoff et al. 2007), and inhibitors of NS5 activity are attractive candidates for development of anti-viral chemotherapy (Ray and Shi 2006). NS5 contains two main active domains: an N-terminal ~30kDa methyltransferase domain involved in 5' RNA cap formation and a C-terminal ~70kDa RNA-dependent RNA polymerase domain that makes copies of the viral ssRNA genome for incorporation into newly synthesized virions (Zhou, Ray et al. 2007).

Synthesis of viral genomes is accomplished by the RNA-dependent RNA polymerase (RdRp) domain of NS5. vRNA association with NS5 is facilitated by RNA binding activity possessed by several of the proteins, both cellular and viral, that associate with the viral replication complex. The NS5 RdRp domain enters the viral genome at the 3' end and replicates the entire genome in a primer-independent manner (Selisko, Dutarte et al. 2006). These new copies of the viral genome are then processed by NS3 and NS5 (discussed below), and then associate with the capsid protein to form nucleocapsids which are packaged into viral membranes during maturation through the Golgi apparatus (Leysen 2000).

Proper 5' capping of viral genomes before assembly is essential for proper translation initiation of the viral polyprotein following infection (Bollati, Milani et al. 2009). However, flavivirus replication occurs in the ER, and the cellular capping machinery is located in the nucleus. Therefore, flaviviruses evolved their own 5' capping machinery to ensure proper viral translation. The flavivirus 5' cap is formed by a sequential series of enzymatic reactions that modify the structure. This process is initiated by NS3 RNA

triphosphatase activity (discussed above). A second step, the addition of GMP to the 5' end of the genome, is carried out by an as yet uncharacterized RNA guanylyl transferase (Furuichi and Shatkin 2000; Zhou, Ray et al. 2007). The final step in the formation of the 5' cap is the methylation at the N-7 position of guanine and at ribose 2'-OH position by the NS5 2'-O nucleoside methyltransferase domain (Koonin 1993; Eglhoff, Benarroch et al. 2002; Gu and Lima 2005). This yields a 5' cap 1 (m<sup>7</sup>GpppNm) structure that facilitates translation initiation of the newly synthesized genome following infection (Ray, Shah et al. 2006; Geiss, Thompson et al. 2009). Finally, NS5 also inhibits anti-viral interferon signaling during infection by preventing STAT1 activation in the interferon-stimulated JAK-STAT pathway (Best 2005).

#### **4.7 Modoc virus As a Model for Flavivirus Infections**

The need for effective research into flavivirus pathogenesis and vaccine development spurred initial research into finding animal models of these diseases. Research into viral chemoprophylaxis and vaccine design using the major pathogenic flaviviruses like Dengue virus or Yellow fever virus is hindered by the need for special equipment to work with these Biosafety Level 3 and 4 organisms. Leyssen et al., have described a mouse model for flavivirus-induced encephalitis using Modoc virus, a Biosafety level 2 virus. They have shown that infecting SCID (Severe Combined Immune Deficiency) mice with Modoc virus intranasally or intracerebrally results in encephalopathy very similar to that experienced by humans infected with other flaviviruses (Leyssen 2001). Modoc virus is a highly virulent murine flavivirus of the “No Known Vector” group that causes murine encephalitis. It was first isolated from a deer mouse in 1957 (Davis 1974), after the first

recorded human case of Modoc encephalitis occurred in a young boy after he played with a sick mouse in the foothills of the Sierra Mountains (Davis 1973). Very high viral titers are generated in the kidneys of infected animals, which then likely spread the virus effectively to others by territory marking and dominance battles involving urine spray. Modoc virus is easily administered through the intranasal route, and is 100% fatal in mice lacking protective genetic immunity. Modoc virus has the same conserved enzymatic motifs and genomic organization as those flaviviruses that are pathogenic to humans (Leysen 2002). The encephalitis it induces in immunocompromised mice also mimics human flavivirus encephalitis very closely, causing flaccid paralysis and life-long neurological sequelae in those animals that recover (Leysen 2001, 2003). SCID mice intranasally infected with  $10^4$  pfu show signs of morbidity including ruffled fur, hind leg paralysis and wasting syndrome within 6 days post-infection and mortality within 9 days. In cultured Vero cells, Modoc virus infection results in proliferation and dilation of the rough endoplasmic reticulum, and viral particles are released 2-3 days post-infection. Modoc virus was shown by Leysen et al. to be equally as sensitive as Yellow fever virus or Dengue virus for studying chemoprophylaxis in cell culture (Leysen 2001, 2003).

Modoc virus is an ideal candidate for the study of flaviviral infections and treatment, because of its similarity to other known flaviviral pathologies in mouse models. Its ability to replicate in cell culture equally as well as other flaviviruses, and the lack of need for special equipment in working with Modoc virus allow research to be conducted safely and inexpensively.

Flaviviruses present a unique and deadly challenge to the health care systems of the world. Many flaviviruses are pathogenic in humans, and the associated disease is often

life-threatening. Treatment of flavivirus infections is largely limited due to the lack of specific details about the molecular biology behind host-virus interactions during infection. Recent advances in the understanding of flavivirus-host interactions have identified manipulation of autophagy signaling for appropriate viral replication in some cell types. In this chapter, we present evidence that a consequence of upregulation of autophagy by flaviviruses in some cell types is protection from cell death, and identify a single flavivirus gene, NS4A, that is responsible for this effect.

## **4.8 Chapter 2 Results: Flavivirus NS4A-Induced Autophagy Protects Cells from Death and Enhances Viral Replication**

### **4.8.1 Modoc virus and Dengue virus infect and replicate in MDCK renal epithelial cells**

Flavivirus manipulations of cell fate are cell-type specific: while macrophages and neurons die following infection, hepatocytes and fibroblasts survive. Several reports of flavivirus-induced persistent infection of the renal epithelium indicate that renal epithelial cells also survive infection. To understand the particular virus-host interactions involved in renal flavivirus infection, we chose MDCK renal epithelial cells as our main model for *in vitro* study. To ensure that MDCK cells support Dengue-2 and Modoc virus infection and replication in our hands, we evaluated the infection and replication capability of each virus in MDCK cells by immunocytochemistry and qRT-PCR. For immunocytochemistry, cells were seeded onto glass coverslips, infected and fixed at 48hpi as described in Materials and Methods. Fixed cells were stained for flavivirus E protein with antibodies Di-4G2-4-15 and AlexaFluor 488 and observed by confocal

microscope (Leica, Germany). As shown in Figure 2.1A, page 196, mock-infected cells show no positive staining for the flavivirus E protein. At 48hpi both Dengue-2 and Modoc virus are abundant in vesicles primarily within the perinuclear region of infected cells (Figure 2.1B, 2.1C, respectively, page 196), a staining pattern consistent with previous findings of localization in the endoplasmic reticulum and Golgi bodies of infected cells (Cardiff, Russ et al. 1973). Many cells are positive for flavivirus E protein in both Dengue-2 and Modoc infected cells (60% and 54%, respectively - data not shown), confirming that these viruses are capable of infecting and replicating in MDCK cells.

To quantify flavivirus replication in MDCK cells, supernatant samples from Modoc and Dengue-2 infected cells were collected at 48 hour intervals until 144hpi. Viral RNA was extracted from each sample and subjected to qRT-PCR. For both Dengue-2 and Modoc viruses, primers for the NS4A gene were used and qRT-PCR was performed as described in Materials and Methods. As shown in Figure 2.1D, page 196, robust replication of both Dengue-2 and Modoc virus is evident by 48 hpi. A maximum virus titer of just over  $8 \times 10^7$  pfu/mL is reached by Dengue-2 virus at 144 hpi, while the maximum titer generated by Modoc at 144hpi is  $10^7$  pfu/mL. These data confirm that MDCK cells support *in vitro* infection and replication of both Dengue-2 and Modoc virus, and provide a basis for the use of MDCK cells as a model for our study.

#### 4.8.2 Modoc virus does not kill infected epithelial or fibroblast cells

As Modoc virus has been proposed as a research model for flavivirus infection *in vivo*, we first characterized Modoc-induced death of MDCK cells. Cells were seeded and infected at MOI=10, 100 and 1190 as described in Materials and Methods, and collected at 48, 144, and 288hpi for trypan blue exclusion analysis. The intact cell membranes of viable cells exclude trypan blue, while the permeabilized cell membranes of dead cells allow the dye to enter the cell and stain the cytoplasm. Therefore live cells remain clear while dead cells become blue. Living and dead cells were counted on a hemocytometer and percent cell death for each population was calculated. Data are expressed as percent cell death above mock. Each assay was run in triplicate, and error bars indicate one standard deviation from the mean. As shown in Figure 2.2A, page 197, Modoc virus does not lead to appreciable cell death in MDCK cells (<10%), even in the extreme conditions of 288hpi using undiluted stock virus (MOI=1190).

We next characterized Modoc cell killing in several other cell types and assayed for death at 24, 48, 96 and 144hpi to establish that this effect was not specific to MDCK cells. Swiss Webster primary mouse embryonic fibroblasts were chosen for their known knockout mutation in the mammalian flavivirus resistance gene OAS1b (Lucas, Mahimo et al. 2003); C57Black primary mouse embryonic fibroblasts were analyzed because OAS1b functions normally in these cells (Lucas, Mashimo et al. 2003); Vero cells were used because they have been used in several previously published *in vitro* studies with other flaviviruses (Davis and Hardy 1973; Ng, Pedersen et al. 1983; Westaway and Goodman 1987) MDCK cells were used as a control. As shown in Figure 2.2B, page 197, no significant levels of death are observed in any of the cell types tested through

144hpi (<10%), demonstrating that Modoc virus does not kill epithelial or fibroblast cells *in vitro*.

#### **4.8.3 Dengue-2 and Modoc virus kills macrophages while protecting epithelial and fibroblast cells from death.**

The fact that Modoc virus does not kill epithelial cells or fibroblasts from several mammalian species, even at high MOI and late hpi, suggests that pro-survival signaling is activated in these cells following infection. We therefore characterized the protection conferred by Dengue-2 and Modoc virus when cells responded to external cell death stimuli. We analyzed the protective capacity of Dengue-2 and Modoc virus infection in MDCK epithelial cells against a variety of toxins including camptothecin (CPT, [75 $\mu$ M], a topoisomerase inhibitor that leads to cell death by apoptosis through the p53 DNA damage response pathway (Hsiang, Hertzberg et al. 1985)); staurosporine (STS, [20  $\mu$ M], a broad spectrum kinase inhibitor that leads to cell death by apoptosis (Tamaoki, Nomoto et al. 1986; Bertrand, Solary et al. 1994)); cycloheximide (CHX, [150 $\mu$ M], a protein synthesis inhibitor that leads to cell death at high concentrations (Ennis and Lubin 1964)); and Influenza A WSN/33 virus infection (MOI=5, triggers apoptotic cell death (McLean, Datan et al. 2009)) as described in Materials and Methods. As shown in Figure 2.3, page 198, both Dengue-2 and Modoc virus infection confer substantial protection against death by all insults examined to MDCK renal epithelial cells. Pre-infection with Dengue-2 virus leads to a 39% decline in CPT induced cell death ( $p=0.0004$ ), a 40% decline in STS induced cell death ( $p=0.0017$ ), a 46% decline in CHX induced cell death ( $p<0.0001$ ), and a 31% decline in Influenza A induced cell death

( $p=0.002$ ) at 48hpi when compared to mock-infected, death stimulus-treated cells. Pre-infection with Modoc virus results in a 83% decline in CPT induced cell death ( $p=0.0016$ ), a 72% decline in STS induced death ( $p=0.0006$ ), a 51% decline in CHX induced cell death ( $p=0.004$ ), and a 36% decline in Influenza A induced cell death ( $p=0.002$ ) at 48hpi, when compared to mock-infected, death stimulus-treated cells. These results demonstrate that infection with either Dengue-2 or Modoc virus is sufficient to induce protection against death induced by several insults in MDCK cells. The generalized protective effect of infection with either virus against such a wide range of insults suggests a powerful and broad process at play.

As macrophage cell killing by flavivirus infection has been previously reported (King 2006), we replicated the cell killing or protecting capability of infection in macrophages in our laboratory. We obtained primary macrophages by peritoneal perfusion of adult Swiss Webster mice as described in Materials and Methods. As shown in Figure 2.4A, page 199, infection of macrophages with either Dengue-2 or Modoc virus results in ~22% cell killing ( $p<0.001$  for both), roughly equal to that caused by CPT exposure. Conversely in primary Swiss Webster mouse embryonic fibroblasts (obtained from ED10.5 embryos), infection with Dengue-2 or Modoc virus does not lead to cell killing (Fig. 2.4B, page 199). In fact, Dengue-2 infection resulted in a 59% decline in cell death by CPT, while Modoc infection leads to a 70% decline in death ( $p<0.0001$ ,  $p=0.0005$  respectively), demonstrating that infection with either virus confers protection to these cells. These results demonstrate that cell fate following flavivirus infection is cell-type specific, and indicate that pro-survival signaling is upregulated following infection in fibroblasts.

#### **4.8.4 Dengue-2 infected MDCK epithelial cells are protected from death while uninfected cells are not**

To confirm that infected cells within the population are those being protected against cell death, we assessed infection and caspase-3 activation following Dengue-2 infection and CPT treatment by immunocytochemistry and confocal microscopy as described in Materials and Methods. As expected in mock-infected cells, no staining for virus particles is apparent (Figure 2.5A, page 200), and active caspase-3 is observed following CPT treatment (Figure 2.5B, C, page 200). Following Dengue-2 virus infection, cells exhibit positive staining for the envelope protein (Figure 2.5D, page 200). Although caspase-3 activation is observed in infected cell populations following CPT treatment (Figure 2.5E, page 200), it is restricted to non-infected cells (Figure 2.5F, page 200). A similar exclusive staining pattern was observed for nearly all cells examined. These results demonstrate that Dengue-2 infection protects infected cells, but not uninfected bystander cells. Further, these data indicate that flavivirus-induced protection is due to intracellular signaling, and not to extracellular (cell to cell) communication.

#### **4.8.5 Flavivirus infection induces PI3K-dependent autophagy**

Our data demonstrate that Modoc virus and Dengue-2 virus infection have similar effects on cell survival in MDCK epithelial cells and several other cell lines, suggesting similarities in the biochemistry of protection. It is known that flavivirus infection triggers upregulation of autophagy (Gil, Martinez et al. 2004) and autophagy often stimulates pro-survival signaling. We therefore hypothesized that the protection observed following infection with either virus may be due to activation of autophagy-related signaling within infected cells.

We first confirmed that both Modoc and Dengue-2 viruses induce autophagy during infection. To this end, we examined LC3-GFP translocation following infection with Dengue-2 and Modoc virus in both HeLa and MDCK cells. LC3 remains in a cytosolic, inactive distribution until activated during autophagy upregulation. This cytosolic distribution results in a diffuse LC3-GFP expression pattern. Upon activation, LC3 is cleaved into LC3-I and LC3-II. LC3-II then translocates to and accumulates at autophagosomal membranes, resulting in a punctate GFP distribution.

We first infected HeLa cells stably expressing LC3-GFP with either virus and fixed them at 24 hpi for observation by confocal microscopy. As shown in Figure 2.6A, page 201, mock-infected HeLa cells show little LC3-GFP punctation, indicating low levels of autophagy typical of healthy cells. Infection with either Dengue-2 (Fig. 2.6B, page 201) or Modoc (Fig. 2.6C, page 201) virus induces a substantial increase in the number of cells containing punctate LC3-GFP, indicating an upregulation of autophagy following infection with either virus.

To confirm these results in our MDCK system, we next transfected MDCK cells with a C2-LC3-GFP plasmid and infected with either virus at MOI=10. At 48hpi, cells were fixed and mounted for observation by confocal microscopy. As shown in Figure 2.6D, page 201, at 48hpi mock-infected cells display only limited LC3-GFP punctation, indicating little activation of autophagy, while both Modoc (Figure 2.6E, page 201) and Dengue-2 (Figure 2.6F, page 201) infection produces pronounced LC3-GFP punctuation, indicating upregulation of autophagosome formation. As shown in Figure 2.6J, page 201, while only 17% of mock-infected cells display punctate LC3-GFP (indicating normal housekeeping activity), nearly 70% of both Modoc and Dengue-2 virus-infected cells

display LC3-GFP punctation, demonstrating that both viruses induce autophagy. Autophagosome formation is completely eliminated following treatment with the PI3K inhibitor Wortmannin in both Modoc (Fig. 2.6H, page 201) and Dengue-2 (Fig. 2.6I, page 201) infected cells. PI3K inhibition by this method reduces the number of infected cells that display punctate LC3-GFP to 17%, similar to mock levels of GFP punctation (Fig. 2.6J, page 201). These results support a model by which PI3K-dependent autophagy results from infection in both Modoc and Dengue-2 viruses, and are consistent with previous findings using Dengue-2 virus (Lee, Lei et al. 2008).

To confirm our results above we collected whole protein lysates from Dengue-2 infected cells at 0, 3, 6, 12, 24, 36 and 48hpi, and probed these lysates for cleaved LC3 by western blotting as described in Materials and Methods. As described above, LC3 cleavage is a commonly used indicator for autophagy upregulation. As shown in Figure 2.6K, page 201, minimal LC3 cleavage is seen in mock-infected cells at 0 and 48hpi, reflecting normal housekeeping functions of autophagy at work. However, in Dengue-2 infected cells, LC3 cleavage is seen to increase steadily until 36hpi, diminishing thereafter to 48hpi.  $\beta$ -tubulin was used as a loading control (Fig. 2.6L, page 201). These results demonstrate that LC3 cleavage is induced by Dengue-2 infection, suggesting that autophagy is upregulated following infection in MDCK cells. Furthermore, the decline in cleaved LC3 at 48hpi suggests the turnover of LC3 in infected cells, indicating the complete formation of autolysosomes following infection.

#### 4.8.6 Flavivirus-induced protection is dependent on autophagy related signaling

Flaviviruses induce autophagy in hepatocytes and fibroblasts (Lee, Liao et al. 2005; Lee, Lei et al. 2008; Panyasavritnit, Khakpoor 2009). Upregulation of autophagy often leads to PI3K-dependent pro-survival signaling. We therefore sought to identify the effect of key autophagy-related proteins on flavivirus-induced protection in epithelial cells. To analyze the role of PI3K signaling and autophagy in flavivirus-induced protection, we infected control and Wortmannin-treated MDCK cells with Modoc or Dengue-2 virus. At 24hpi, cells were treated with CPT, and analyzed by trypan blue exclusion at 48 hpi as described above. As shown in Figure 2.7A, page 202, Wortmannin treatment does little to cell survival in infected or un-infected cells, whereas CPT treatment kills 49% of mock-infected cells above control within 24 hours of exposure. However, infection with either Dengue-2 or Modoc virus provides protection against death, with Dengue-2 infection causing a 76% decline ( $p=0.0004$ ) and Modoc infection causing a 98% decline ( $p=0.0016$ ) in CPT-induced death. Inhibition of PI3K by Wortmannin treatment eliminates the protective response invoked by Modoc and Dengue-2 virus infection, allowing CPT-induced death to reach 50% above control in infected populations, killing these cells as effectively as uninfected cells.

As Wortmannin inhibits both Class I and Class III PI3K proteins, we next inhibited specifically Class III PI3K proteins during infection using the Class III PI3K inhibitor 3-methyl adenine (3MA), and repeated the assay. As shown in Figure 2.7B, page 202, Modoc and Dengue-2 infection and 3MA treatment alone do not kill cells, and levels of death for each are comparable to mock-infected cells at ~10%. As in previous experiments, CPT treatment induces ~50% death in mock-infected cells, while pre-

infection with either Dengue-2 or Modoc virus leads to a 70% reduction in death induced by CPT when compared to mock-infected, CPT-treated cells ( $p=0.0004$ ,  $p=0.0007$ , respectively). However, treatment with 3MA leads to the total abolition of both Dengue-2 and Modoc virus-induced protection against CPT-induced death. The data indicate that Class III PI3K activity is a regulatory factor for protection against cell death and suggest more specifically that autophagy signaling is responsible for the ability of the virus to trigger a response leading to protection. We therefore characterized Modoc and Dengue-2 induced protection in two different autophagy deficient cell lines: Beclin +/- and ATG5 -/- cells.

Beclin is a protein that directly contributes to autophagosome formation downstream of PI3K during autophagy induction (He and Levine 2010). We examined CPT-induced death after flavivirus infection in Beclin knockdown cells, which are heterozygous for the Beclin gene and have a reduced capability for autophagosome formation. WT 4-1 and Beclin +/- cells were infected with Modoc or Dengue-2 virus at MOI=10 and subsequently treated at 24hpi with CPT. At 48hpi, cells were collected and percent cell death was analyzed by trypan blue exclusion assay as previously described. As shown in Figure 2.7C, page 202, CPT kills 34% above control of treated wild-type cells, while both viruses were able to protect against death with Dengue-2 and Modoc viruses generating a 50% ( $p<0.0001$ ) and 24% ( $p=0.0002$ ) decline in cell death when compared to controls, respectively. However, in mock-infected Beclin +/- cells, CPT kills more effectively, resulting in 52% cell death above control. Moreover, in these cells virus-induced protection was completely eliminated, with Dengue-2 and Modoc infected cells actually exhibiting more cell death ( $p<0.0001$ ;  $p=0.0001$ ) when compared to infected CPT-

treated wild-type cells. These results suggest that Beclin activation, a key event in the autophagy response, is essential to Modoc and Dengue-2 induced protection.

We also analyzed the effect of ATG5 knockout in mouse embryonic fibroblasts. ATG5 is an autophagy related protein that acts downstream of Beclin-1, and is essential for formation of isolation membranes. Wild-type and ATG5 KO MEFs were infected with either Dengue-2 or Modoc virus at MOI = 10, incubated for 24 hours, treated with CPT and collected for trypan blue exclusion analysis as described above. As shown in Figure 2.7D, page 202, CPT kills 32% above control of wild-type cells, while infection with either virus protects against death. Dengue-2 and Modoc infection totally block CPT-induced cell death in wild-type cells ( $p < 0.001$ ,  $p = 0.001$ , respectively). In mock-infected ATG5 KO cells, CPT induces 37% cell death above control. Both Dengue-2 and Modoc infection fail completely to protect ATG5 KO cells against CPT, with CPT-induced death reaching levels similar to those seen in mock infections. These results demonstrate that flavivirus-induced protection is dependent upon ATG5 activity. Taken with the results above, our data suggest a role for autophagy signaling during flavivirus-induced protection against death.

#### **4.8.7 Upregulation of autophagy with or without flavivirus infection induces a protective response**

We next sought to determine the effect of upregulating autophagy signaling prior to infection on flavivirus-induced protection. As described above, autophagy is known to confer a protective effect against apoptotic triggers. Prior to infection, autophagy was upregulated by starvation prior to infection and treatment. As shown in Figure 2.8A, page 203, starvation alone does little to cell survival after 48 hours. Likewise, starvation

has no effect on survival of Dengue-2 or Modoc virus infected cells by 48 hpi. However, after CPT challenge marked differences were seen between control and starved cells. In control groups that were fed normally, Dengue-2 or Modoc virus infection both lead to an 44% reduction in cell death by CPT killing ( $p=0.0005$ ;  $p=0.0001$ , respectively), similar to our results reported above. However, starved cells (whose autophagy machinery has been upregulated to deal with nutrient deprivation) show an even more effective protection against CPT with or without infection. In starved mock-infected cells, CPT-induced death declined 72% when compared to their fed counterparts ( $p<0.0002$ ). CPT exposure with Modoc and Dengue pre-infection in starved cells yielded death levels similar to mock after CPT exposure. Infection therefore does not further protect starved cells, suggesting that autophagy, not viral infection itself, is responsible for Modoc and Dengue-2 induced protection against death.

To assess whether protection against death can also be triggered by alternate autophagy-inducing mechanisms, we treated MDCK cells prior to and during infection with 50nM rapamycin, a bacterial macrolide that upregulates autophagy by inhibiting mTOR (Ravikumar, Vacher et al. 2004), as described in Materials and Methods. For this experiment, we increased the concentration of CPT to analyze the effect of increased stress on the protection induced by infection. As shown in Figure 2.8B, page 203, mock infected cells without rapamycin treatment exhibited 74% cell death after 24 hours of 100 $\mu$ M CPT treatment. Dengue-2 and Modoc pre-infected cells each showed an ~85% decline in death ( $p<0.0001$  for both) when compared to mock-infected controls in response to 100 $\mu$ M CPT. In the presence of rapamycin, mock-infected, Modoc and Dengue-2 pre-infected cells were all equally protected against CPT-induced death, all

yielding no significant death (<10%).after CPT treatment. Since Modoc and Dengue-2 infection, rapamycin and starvation can each elicit autophagy and protection against CPT induced death, the data suggest that the mechanism by which autophagy is upregulated is not critical to the level of protection. Taken together, the data suggests that the protection elicited by these two viruses is dependent upon the host cell's ability to induce autophagy and/or autophagy-related signaling.

#### **4.8.8 Autophagy enhances Dengue-2 and Modoc virus replication**

Others have demonstrated that upregulation of autophagy is important to Dengue-2 replication in hepatocytes (Lee, Lei et al. 2008), and that the virus replication complex co-localizes with autophagosomal membranes (Panyasavrit, Khakpoor et al. 2009). As reported above a similar autophagy response is activated in epithelial cells during flavivirus infection. We therefore evaluated flavivirus replication by plaque assay in epithelial cells while inhibiting autophagy with Wortmannin and 3-methyladenine (3MA) as described in Materials and Methods. As shown in Figure 2.9A, page 204, Dengue-2 replication is apparent in control cells, generating  $8.16 \times 10^4$  pfu/mL by 96hpi. Inhibition of autophagy with 3MA or Wortmannin treatment each reduces Dengue-2 viral titer to  $6 \times 10^4$  pfu/mL at 96hpi, a 27% reduction in viral replication ( $p=0.001$ ,  $p = 0.002$ , respectively).

To quantify Modoc virus replication when autophagy is inhibited, we used both plaque assay and RT-PCR. In both cases, autophagy was inhibited by 3MA, and virus samples were collected at 96hpi. As shown in Figure 2.9B, page 204, Modoc virus replication is apparent in control cells at 96hpi, reaching  $2.26 \times 10^6$  pfu/mL. When autophagy is inhibited with 3MA, Modoc replication falls to  $6.67 \times 10^5$  pfu/mL, a 70%

reduction in virus replication ( $p < 0.0001$ ). To confirm our plaque assay data, we analyzed viral RNA in the supernatant of both control and 3MA treated cells. Virus RNA was extracted from supernatant samples at 96hpi and subjected to qRT-PCR analysis as described in Materials and Methods. For qRT-PCR results, CP values (possessing an inverse relationship to total virus RNA released) are shown. Each assay was run in triplicate, and error bars indicated one standard deviation from the mean. As shown in Figure 2.9C, page 204, total Modoc virus RNA in the supernatant of autophagy-inhibited cells is significantly reduced, at 3% of control at 96hpi ( $p = 0.02$ ), indicating that replication of Modoc virus is significantly enhanced by autophagy signaling, a result similar to that seen for Dengue-2 virus.

These results provide substantial evidence that autophagy is required for both Dengue-2 and Modoc virus replication in renal epithelial cells. The distant relation of these two viruses also suggests that upregulation of autophagy may be a general characteristic of flavivirus infection in epithelial cells.

#### **4.8.9 Protection against cell death is mediated by flavivirus NS4A**

Having established a link between flavivirus-induced protection, autophagy and flavivirus replication during infection with both Dengue-2 and Modoc viruses, we sought to identify key viral proteins involved in triggering these effects. We transfected MDCK cells with one of each of the ten viral genes of Dengue-2 virus, and the NS2A, NS4A, and NS4B genes (the small hydrophobic proteins) of Modoc virus, and allowed 20 hours for protein expression. We then assayed for protection against CPT-induced cell death as described in Materials and Methods. Live virus infection was done in tandem as a control. CPT kills 35% above control of mock-infected cells while Dengue-2 and Modoc

viruses both protect against CPT-induced death (Figure 2.10A, B, respectively, page 205), causing a 40% and 45% reduction in death by CPT, respectively ( $p=0.002$ ;  $p=0.003$ ). This is consistent with our previous results reported above. Empty TOPO plasmid has no effect on CPT-induced death, allowing 47% of cells to die from CPT treatment, similar to mock infection. Individual expression of C, prM, E, NS1, NS2B, NS3, NS4B and NS5 genes from Dengue-2 and NS4B from Modoc virus has no effect on cell survival in response to CPT, each allowing ~48% CPT-induced death, similar to mock infection. Expression of either Modoc or Dengue-2 NS2A kills a small number of cells. Dengue-2 NS2A expression increases death 10% over control and expression of Modoc NS2A increases death 4% over control ( $p=0.0002$ ;  $p=0.006$ ). A similar increase in death was seen in CPT-treated NS2A expressing cells (10% increase in death for NS2A from both viruses, *Dengue-2*:  $p=0.02$ ; *Modoc*:  $p=0.0001$ ). However, expression of NS4A from either Modoc or Dengue-2 is sufficient to protect against cell death similar to the whole virus. Dengue-2 NS4A expression leads to a 35% decrease in death by CPT exposure ( $p=0.002$ ), while Modoc NS4A expression leads to a 56% decrease in death by CPT exposure ( $p=0.004$ ) when compared to empty plasmid controls. These results demonstrate that expression of NS4A from either Dengue-2 or Modoc virus is sufficient to protect against death similar to live virus infection.

To ensure that NS4A-induced protection is not specific to CPT-induced death, we next tested whether Modoc and Dengue-2 NS4A protects against both CPT and STS-induced cell killing. It is important to note that although Staurosporine (STS) kills cells via broad-spectrum kinase inhibition, it does not affect ATM activity (Sarkira, Busby et al. 1999; Viniestra 2004). As shown in Figure 2.10C, page 205, NS4A expression does

not cause a significant increase in cell death over mock-transfected cells, consistent with previous results. In mock-infected cells, CPT exposure kills 57% of cell, while STS exposure kills 52%. Expression of empty plasmid control had no effect on either CPT or STS induced death, allowing 55% and 53% cell death, respectively. Dengue-2 NS4A expression decreases CPT-induced cell death by 48% in ( $p=0.0007$ ) and STS-induced death by 56% ( $p=0.002$ ) compared to empty plasmid controls. Expression of Modoc NS4A leads to a 48% decrease in CPT-induced death ( $p=0.0004$ ) and a 60% ( $p=0.002$ ) decrease in STS-induced death. These results demonstrate that expression of either Modoc or Dengue-2 NS4A is sufficient to protect against death. As with live virus infection, Modoc and Dengue-2 NS4A expression-induced protection is not specific to particular inducers, indicating that similar biochemistry is at play in live virus and NS4A-induced protection.

#### **4.8.10 NS4A expression induces LC3 cleavage**

As reported above, live virus infection with either Modoc or Dengue-2 virus stimulates LC3 cleavage and autophagy, which confers a protective effect to infected cells against several stressors. We therefore sought to determine whether NS4A expression also induces LC3 cleavage and autophagy. We examined by western blot LC3 cleavage in NS4A expressing cells. Whole-cell lysates of both live virus-infected and NS4A-transfected cells were obtained and blotted using a cleaved LC3II-specific primary antibody as described in Materials and Methods. As shown in Figure 2.10D, page 205, mock-infected cells exhibit low levels of LC3 cleavage at 48hpi. In contrast, both live Modoc and Dengue-2 infected cells, as well as Dengue-2 and Modoc NS4A-

expressing cells show upregulation of LC3 cleavage by western blot.  $\beta$ -tubulin was used as a loading control (Figure 2.10E, page 205). These results indicate that NS4A expression from either virus is sufficient to induce autophagy, and suggest that NS4A-induced autophagy is responsible for conferring protection against death.

To confirm that NS4A alone is responsible for LC3 cleavage and autophagy in these cells, we collected whole-protein lysates from cells transiently individually expressing NS4A or control proteins NS1, NS2A, NS4B or empty plasmid backbone. As shown in Figure 2.11A, page 206, each condition caused a small amount of LC3 cleavage, likely due to cellular stress induced by our transfection protocol as indicated by the LC3 cleavage seen in cells treated with the transfection agent (Lipofectamine 2000) but lacking plasmid (+L2000). However, LC3 cleavage is markedly increased in NS4A expressing cells, indicating that among the proteins examined NS4A activity alone holds the ability to induce LC3 cleavage in MDCK cells.  $\beta$ -tubulin was used as a loading control (Fig. 2.11B, page 206). Together with the results above, our data indicate that NS4A activity alone allows flaviviruses to induce autophagy and cell protection during infection.

#### **4.8.11 Inhibition of PI3K activity removes NS4A-induced protection**

To confirm that NS4A-induced autophagy is responsible for infection-induced protection, we manipulated the autophagy machinery and characterized NS4A-induced protection against CPT and STS-induced death. After transfection, we either induced autophagy with rapamycin or blocked autophagy using Wortmannin or 3MA as described in Materials and Methods. Cells were then treated with 75 $\mu$ M CPT or 10 $\mu$ M STS for 24

hours before collection and trypan blue exclusion assay as described above. As shown in Figure 2.12, page 207, approximately half of CPT and STS-treated mock cells die after 24 hours. Consistent with our previous results, NS4A from either virus induces a 70% decline in death by CPT (*Dengue-2*  $p=0.0006$ ; *Modoc*  $p=0.0004$ ) and a 30% decline in cell death by STS (*Dengue-2*  $p=0.0012$ ; *Modoc*  $p=0.0012$ ). Inducing autophagy with rapamycin treatment protects cells regardless of the presence or absence of NS4A from either virus, allowing almost no CPT-induced death. As with live Modoc and Dengue-2 virus infections, inhibiting autophagy using either Wortmannin or 3MA eliminated NS4A-induced protection against death by CPT and STS, allowing death similar to mock, killing approximately half of the cells in each case. These results demonstrate that NS4A from either Modoc or Dengue-2 virus is sufficient to induce autophagy, and indicate that NS4A-induced autophagy is responsible for NS4A-induced protection against death.

#### **4.8.12 Flavivirus Infection Induces ATM-dependent Autophagy**

Dengue-2 infection induces ER stress. We and others have shown that flavivirus infection induces autophagy upregulation. Recently, ER stress induced autophagy via UPR-independent ATM activation has been reported (Alexander, Cai et al. 2010). We therefore hypothesized that flavivirus-induced autophagy is initiated by ER stress-mediated ATM activation. As shown in Figure 2.13A, page 208, minimal LC3-GFP punctation is observed in mock-infected cells, while both Modoc (Fig. 2.13B, page 208) and Dengue-2 virus (Fig. 2.13C, page 208) both induce high levels of autophagy in infected cell populations (~70%; Fig. 2.13G, page 208). Inhibition of ATM activity by caffeine has no observable effect on mock-infected cells (Fig. 2.13D, page 208).

However, ATM inhibition eliminates autophagosome formation following infection with Modoc (Fig. 2.13E, page 208) or Dengue-2 (Fig. 2.13F, page 208) virus, reducing the number of GFP-positive cells displaying punctate LC3-GFP distribution to ~17% (Fig. 2.13G, page 208), similar to mock infection. These results indicate that flavivirus-induced autophagy is ATM-dependent, and suggest that flavivirus-induced autophagy is a consequence of ER stress.

#### **4.8.13 Flavivirus infection Leads to ATM-dependent Protection Against Cell Death**

As presented above, Dengue-2 and Modoc virus-induced protection from death is dependent upon upregulation of autophagy. We have also shown that flavivirus-induced autophagy is ATM-dependent. We therefore hypothesized that flavivirus-induced protection is similarly ATM-dependent. To assess the role of ATM in flavivirus-induced protection against death, we inhibited ATM with 20 $\mu$ M caffeine, infected MDCK renal epithelial cells with either Dengue-2 or Modoc virus, treated with 75 $\mu$ M CPT at 24hpi and evaluated CPT-induced death at 48hpi by trypan blue exclusion assay. As shown in Figure 2.14, page 209, while Dengue-2 and Modoc infection result in a 35% and 64% decline in CPT-induced cell death, respectively ( $p=0.0031$ ;  $p<0.0001$ , respectively), ATM inhibition results in a complete loss of Dengue-2 and Modoc-induced protection ( $p<0.0001$  for both). These results demonstrate that ATM-related signaling is critical for flavivirus-induced protection against death, and provide further evidence linking flavivirus-induced protection against death to flavivirus induced ATM activation and autophagy.

#### **4.8.14 Inhibition of ATM Activity Eliminates NS4A-induced Protection**

We have demonstrated that flavivirus-induced protection is ATM-dependent, and that NS4A expression induces autophagy and PI3K-dependent protection against death. To confirm that NS4A-induced protection against death is similarly ATM-dependent, we inhibited ATM with 20 $\mu$ M caffeine during expression of NS4A from either Dengue-2 or Modoc virus, treated with 75 $\mu$ M CPT at 24 hours post-transfection (hpt) and evaluated CPT-induced death at 48hpt by trypan blue exclusion assay. As shown in Figure 2.15, page 210, while expression of NS4A from Dengue-2 and Modoc infection both result in a 43% decline in CPT-induced cell death ( $p < 0.0001$  for both), ATM inhibition results in a complete loss of flavivirus induced protection from either Dengue-2 or Modoc virus ( $p < 0.0001$ ;  $p = 0.002$ , respectively). These results demonstrate that ATM-related signaling is critical for NS4A-induced protection against death, and provide further evidence linking flavivirus-induced protection against death to flavivirus-induced ATM activation and autophagy.

#### **4.9 Chapter 2 Discussion**

Both Dengue-2 and Modoc virus naturally infect the kidneys, liver, spleen, lungs, salivary glands, several types of immune cells and the central nervous system (Davis, Hardy et al. 1974; Lum, Lam et al. 1996; Jessie, Fong et al. 2004). These tissues represent a variety of cell types, including neurons, macrophages, monocytes and lymphocytes endothelial and epithelial cells, hepatocytes and fibroblasts (Leysen, Van Lommel et al. 2001, Jessie, Fong et al. 2004; Solomon 2004). The host innate immune

response incorporates many biochemical defenses designed to minimize both tissue damage and virus replication during infection. Among these defenses is the recruitment of programmed cell death pathways, including autophagy and apoptosis, that degrade viral components and/or quickly dispatch of infected cells (Everett and McFadden 1999; Tallóczy, Jiang et al. 2002; Chawla-Sarkar, Lindner et al. 2003). Viruses have had to evolve the means to evade or manipulate these responses to avoid immune detection and to increase their own replication.

Flaviviruses cause apoptosis in neurons and macrophages (Gulati, Chaturvedi et al. 1982; Halstead 1989; Despres, Falmand et al. 1996; Despres, Frenkiel et al. 1998; Jan, Chen et al. 2000; Espina, Valero et al. 2003), resulting in much of the pathogenesis associated with *in vivo* infection. Dengue-2 replicates to very high titer in these cells (which are the primary sites of virus replication during acute infection) before they die. As infected macrophages cross epithelia within the body (including the hepatic and renal tubular epithelium), they transmit the virus to surrounding epithelial cells and/or fibroblasts. In contrast to neurons and macrophages, these cells do not die following infection. Instead they survive and often become persistently infected (Tonry, Xiao et al. 2005; Siirin, Duan et al. 2007; Murray, Walker et al. 2010). Consistent with these results, we show that *in vitro* infection of macrophages with either Dengue-2 or Modoc virus results in cell killing similar to that of 70 $\mu$ M CPT (a well-characterized toxin) at 48hpi, while infection of renal epithelial cells and fibroblasts (MDCK, 293T, Vero, SW primary MEF, C57/Bl primary MEF) with either virus does induce cell killing even when infected at extremely high titer (MOI~1200) and collected after 12 days (288hpi; the duration of the experiment).

One possible explanation for the lack of death in these cells is that our experimental *in vitro* protocol did not allow sufficient virus uptake and/or replication. We therefore assessed the infectivity and replication of Dengue-2 and Modoc virus in MDCK cells using immunocytochemistry and RT-PCR, and found that both viruses infect and replicate to high titer in MDCK cells. Together, these results demonstrate that while renal epithelial cells and fibroblasts are susceptible to flavivirus infection and allow efficient virus replication, infection does not lead to cell killing. Thus this report provides further evidence that cell fate following flavivirus infection is cell-type specific, killing macrophages while sparing renal epithelial cells and fibroblasts.

Establishment of persistent infection is dependent upon the ability of the infected tissues to evade host immune detection and cell killing. As the kidney is a commonly persistently infected tissue during natural flavivirus infection, we evaluated the protective capacity of infection with Dengue-2 or Modoc virus in several kidney and fibroblast cell lines (MDCK, 293T, Vero, HeLa, SW and C57/Bl MEF's) against CPT, a well characterized cell-lethal toxin. We found that infection with either Dengue-2 or Modoc virus protects against death caused by CPT. Further, this protection is not specific against CPT, as infection also protects against STS, CHX and Influenza A infection-induced death. We also analyzed caspase-3 activation following infection and exposure to CPT by immunocytochemistry and found caspase-3 activation specifically in the subpopulation of cells that remained uninfected in the presence of virus. These results indicate that it is only those cells infected with either Dengue-2 or Modoc virus that are protected against the toxicity of CPT, while uninfected bystander cells are not. Considering also the number of signaling pathways affected by each of the stressors we

used, our results imply that flavivirus-induced protection is due to broad-spectrum intracellular upregulation of pro-survival signaling. The data also support a model in which persistent flavivirus infection of the kidney is due in part to flavivirus-induced protection against cell killing by immune or other mediators. We are currently exploring this possibility.

Dengue-2 infection induces autophagy (Lee, Lei et al. 2008) and the autophagy is essential for flavivirus replication in infected hepatocytes (Panyasavrinit, Khakpoor et al. 2009). Consistent with these results, we find that infection of renal epithelial and fibroblast cells with either Dengue-2 or Modoc virus induces PI3K-dependent autophagy, and chemical inhibition of autophagy by 3MA leads to a reduction in extracellular virus titer. These results demonstrate that autophagosome formation is important for flavivirus replication, and suggest that autophagosomes are the sites of virus replication in epithelial cells, similar to previously published results using hepatocytes.

A common consequence of autophagy signaling is the upregulation of PI3K-dependent pro-survival signaling and protection from death (Bröker, Kruyt et al. 2005; Kroemer and Jäätelä 2005; Ogata, Hino et al. 2006). To evaluate the link between flavivirus-induced autophagy and protection against death following infection, we assessed the protection conferred by flavivirus infection following both chemical and genetic inhibition of autophagy. Chemical inhibition of autophagy by Wortmannin eliminates any protective effect that Dengue-2 or Modoc virus confer on infected cells. Further, genetic inhibition of autophagosome formation by Beclin-1 knockdown or ATG5 knockout also removes the protection against death conferred by infection with either virus. These results demonstrate that flavivirus-induced protection is dependent

upon autophagy upregulation following infection. When autophagy is upregulated prior to infection by either starvation or rapamycin treatment, cells are protected with or without flavivirus infection, providing evidence that it is specifically autophagy signaling, and not other virus-mediated events, that provides protection against death following infection.

The level of protection that either virus exhibits with the presence of rapamycin or starvation is higher than that of Modoc or Dengue-2 infection alone. This most likely results from the fact that not all cells are infected so not every cell generates an autophagy response. In contrast, the entire population of cells is exposed to serum-free media or rapamycin and therefore a higher percentage of cells have upregulated autophagy signaling. Together, our data demonstrate that autophagy signaling is required for flavivirus-induced protection against death.

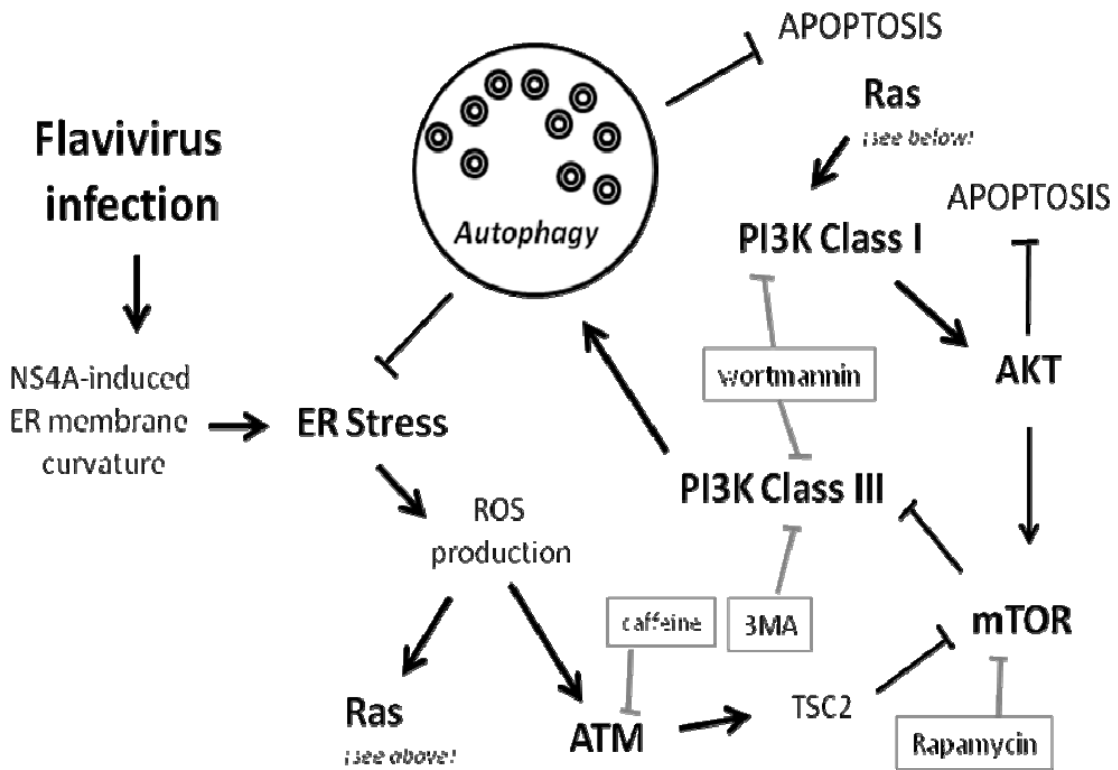
Once the cellular events leading to autophagy upregulation following flavivirus infection in epithelial cells were established, we turned our focus to the specific virus components leading to this effect. By expressing each virus gene individually, we determined that expression of only NS4A (from either Dengue-2 or Modoc virus) was sufficient to induce autophagy and protect against death by CPT. As with live virus infection, NS4A expression upregulates autophagy and induces broad-spectrum protection, shielding cells against both CPT- and STS-induced death. Manipulating autophagy signaling during NS4A expression nearly mimicks the effect on cell survival following CPT treatment seen following whole virus infection. Inhibition of PI3K signaling by Wortmannin and 3MA eliminates autophagy induced protection, while inducing autophagy by rapamycin confers protection regardless of NS4A expression.

Also, similar to whole virus infection, NS4A expression induces PI3K-dependent autophagy and subsequent protection against death. Taken together, our results indicate that flavivirus NS4A expression during whole virus infection leads to upregulation of autophagy. NS4A-induced autophagy subsequently leads to protection against death during infection.

Once we established the link from flavivirus induced autophagy upregulation and protection against death, we next investigated the causal mechanisms. As flaviviruses have previously been shown to induce ER stress, and as one consequence of ER stress is ATM-dependent upregulation of autophagy and pro-survival signaling, we hypothesized that ER stress may induce autophagy, subsequently leading to protection from death. We found that ATM inhibition prior to and during infection completely eliminates virus-induced upregulation of autophagy, indicating that ATM activation is essential to flavivirus-induced upregulation of autophagy. In addition, inhibition of ATM prior to infection eliminates the protection conferred by infection with either virus, providing further evidence that flavivirus-induced protection against death is dependent upon the ability of the cell to upregulate autophagy. As flaviviruses including Dengue-2 induce ER stress following infection, and as ATM activation follows ER stress, these results suggest that flavivirus-induced autophagy and protection against death are a consequence of ATM activation following infection-induced ER stress.

In conclusion, we demonstrate that infection with either Dengue-2 or Modoc virus induces protection in renal epithelial cells and fibroblasts against a wide range of apoptosis-inducing stimuli. The protection observed during flavivirus infection in MDCK renal epithelial cells is due to PI3K- and ATM-dependent upregulation of

autophagy. We also demonstrate that autophagy is essential to virus replication in MDCK cells, a result congruent to recent publications using hepatocytes (Lee, Lei et al. 2008, Panyasavrit, Khakpoor et al. 2009). Of the flavivirus proteins, only NS4A is sufficient to induce autophagy and subsequent protection from death in epithelial cells. As autophagosomes are important to enhancing flavivirus replication in these cells, flavivirus NS4A is thus identified as a potential target for anti-viral drug development.



**Flavivirus NS4A induces autophagy through a Class 3 PI3K- and ATM-dependent pathway.** Flavivirus-induced autophagy and protection against death are dependent upon the activity of both Class 3 PI3K and ATM proteins. Flavivirus infection and more specifically NS4A expression are known to cause ER stress. We hypothesize that increased ROS production in response to NS4A-induced ER stress triggers ATM activation. ATM activity inhibits mTOR, a major inhibitor of Class 3 PI3K-induced autophagy. The resultant upregulation of Class 3 PI3K activity then induces autophagosome production and subsequent protection against death.

## **General Summary and Discussion**

As we have presented, many viruses target the molecular machinery of apoptosis and autophagy to either induce or inhibit cell death, in both instances (but with different circumstances or timing) maximizing virus replication. A detailed understanding of the important virus-host interactions necessary for viruses to regulate cell death may therefore lead to the development of novel anti-viral therapeutics. In this work, we have examined the manipulation of cell death following infection of mammalian cells with viruses from either of two unrelated RNA virus families, the Orthomyxoviridae and the Flaviviridae. We have found that while both of these groups manipulate cell death to ensure maximum replication, their method of manipulation and their effects on cell survival are very different.

Influenza A (family Orthomyxoviridae) rapidly induces apoptosis following infection, an effect we have found to be primarily Bax-dependent. Bax-dependent apoptosis is important to replication of Influenza A virus, as Bax-deficient cells produce 99% fewer extracellular infectious virus than wild-type cells. We have linked the lack of infectious titer in Bax-deficient cells to failure to activate caspase-3 activation, resulting in retention of Influenza A NP in the nuclei. Nuclear retention of NP (an important regulator of translocation of the virus genome) restricts virus genome segments from reaching cytoplasmic virus assembly centers, presumably resulting in the production of incomplete progeny and reducing titer of infectious virus. As expression of Bax is required for efficient Influenza A virus replication, we have therefore identified Bax as a potential target for Influenza A anti-viral drug development.

In contrast to the rapid cell killing induced by Influenza A, both Dengue-2 and Modoc virus (family Flaviviridae) induce strong cell-type specific protection against cell death following infection. We have found that infection with either Dengue-2 or Modoc virus kills macrophages, while infection protects against death in epithelial cells and fibroblasts. Flavivirus-induced protection against death in epithelial cells is a consequence of ATM- and PI3K-dependent autophagy upregulation following infection. As ATM activity is required for flavivirus-induced autophagy and protection against cell death, it is likely that ER stress plays a major role in the upregulation of autophagy following infection. Further, we have shown that autophagy signaling enhances flavivirus replication, as inhibition of autophagy during infection results in a reduction of extracellular virus titer. In addition, we have traced flavivirus-induced autophagy and protection of these cells to the activity of NS4A, a poorly characterized small hydrophobic flavivirus protein. Expression of NS4A from either Dengue-2 or Modoc virus in epithelial cells results in ATM- and PI3K- dependent upregulation of autophagy and leads to protection similar to live virus infection. The distant relationship of these two viruses within the flaviviruses suggests that upregulation of autophagy in these cells may be a general feature of the group. As autophagy enhances flavivirus replication, we have therefore identified NS4A as a potential target for flavivirus anti-viral drug development.

The specific mechanisms of cell death manipulation by these two virus families are in near opposition to each other, with one killing cells following infection and the other protecting against death. However, both use the cell death machinery to maximize virus replication. We have identified several key proteins, both viral and cellular, that

participate in the virus-host interactions required for manipulation of cell death and replication by Influenza A, Dengue-2 and Modoc viruses. Future study into the mechanisms behind these interactions will yield important clues as to the biochemistry of infection, leading to the development of novel therapeutics and an expansion of our anti-viral arsenal.

# Figures

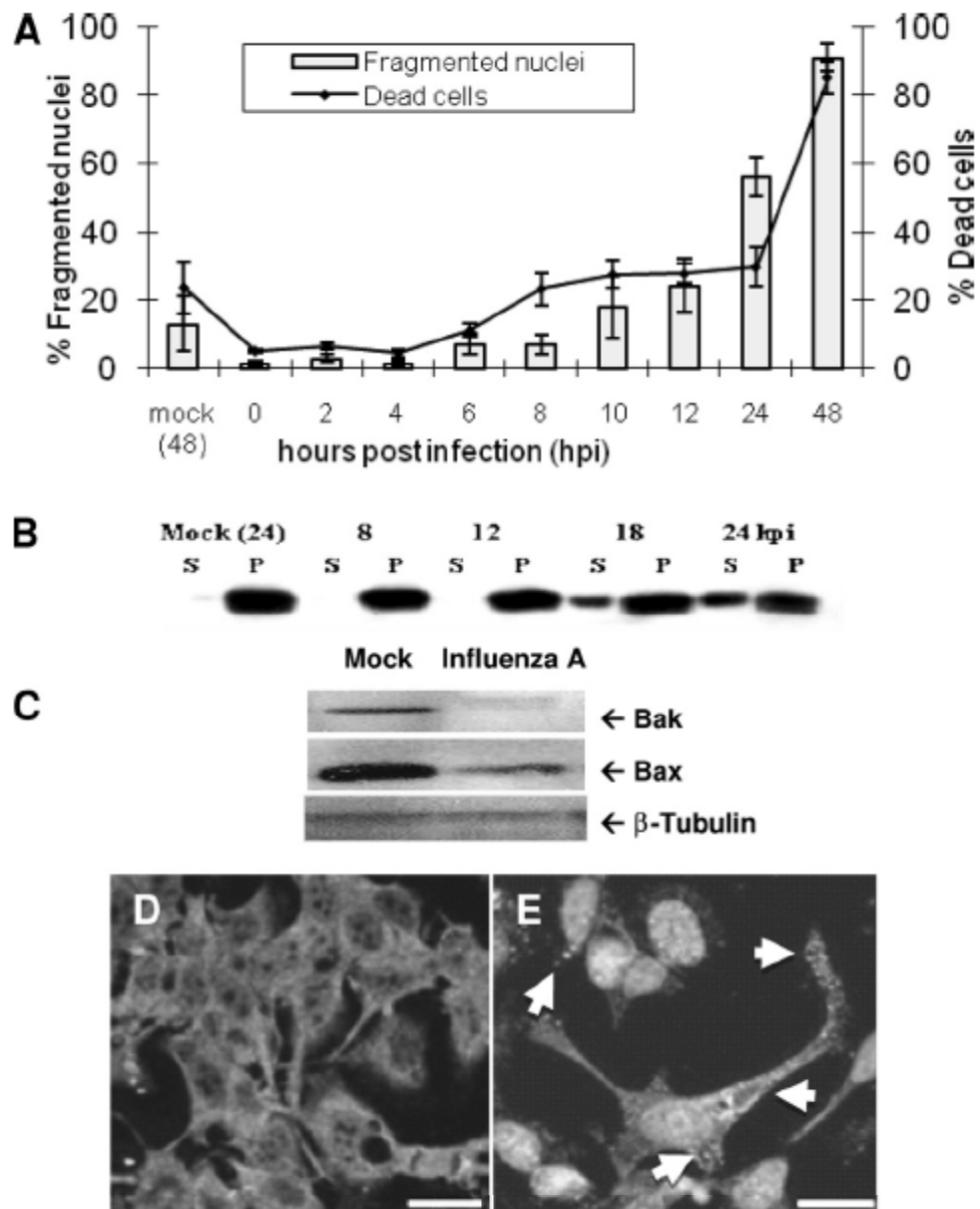


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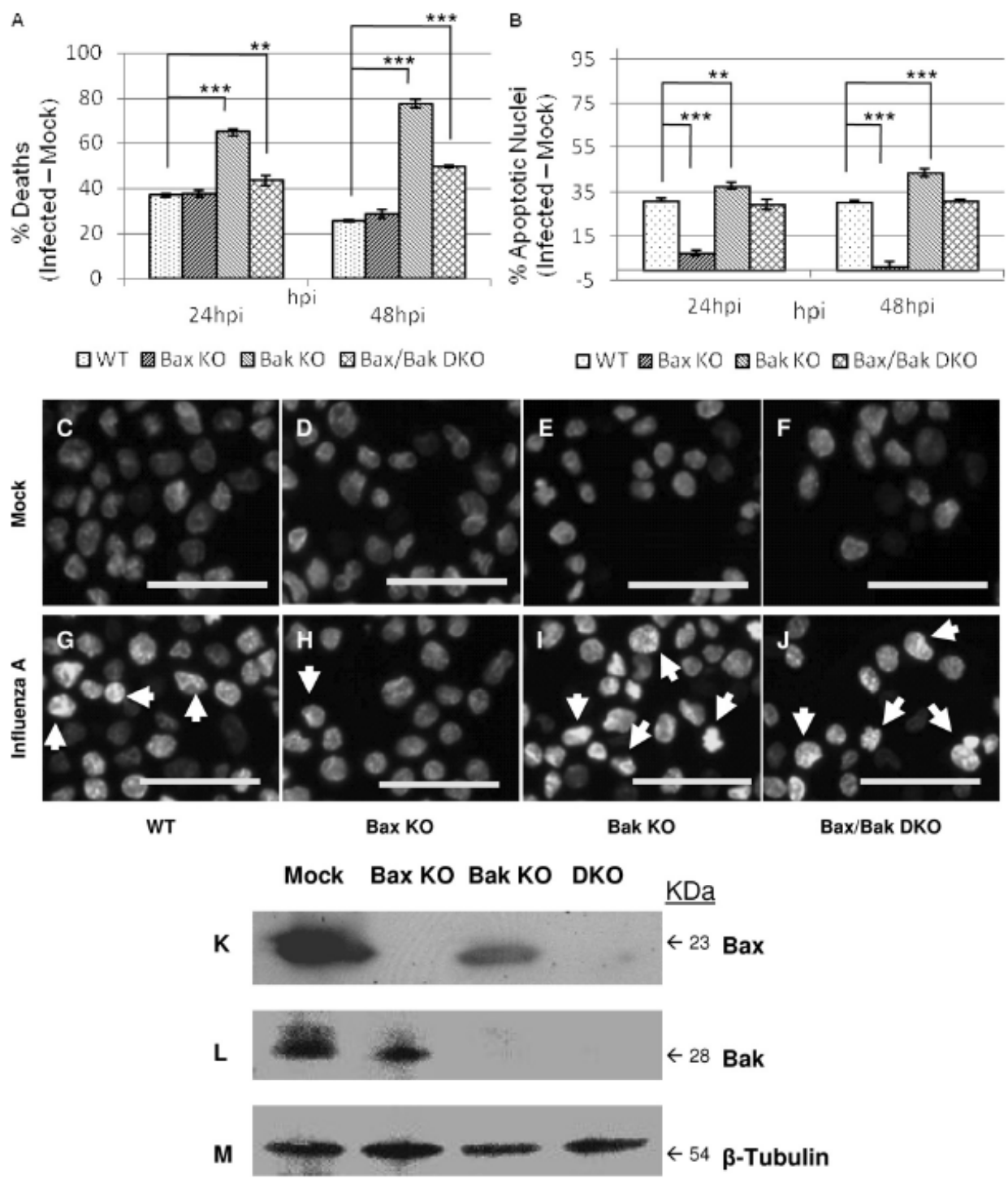


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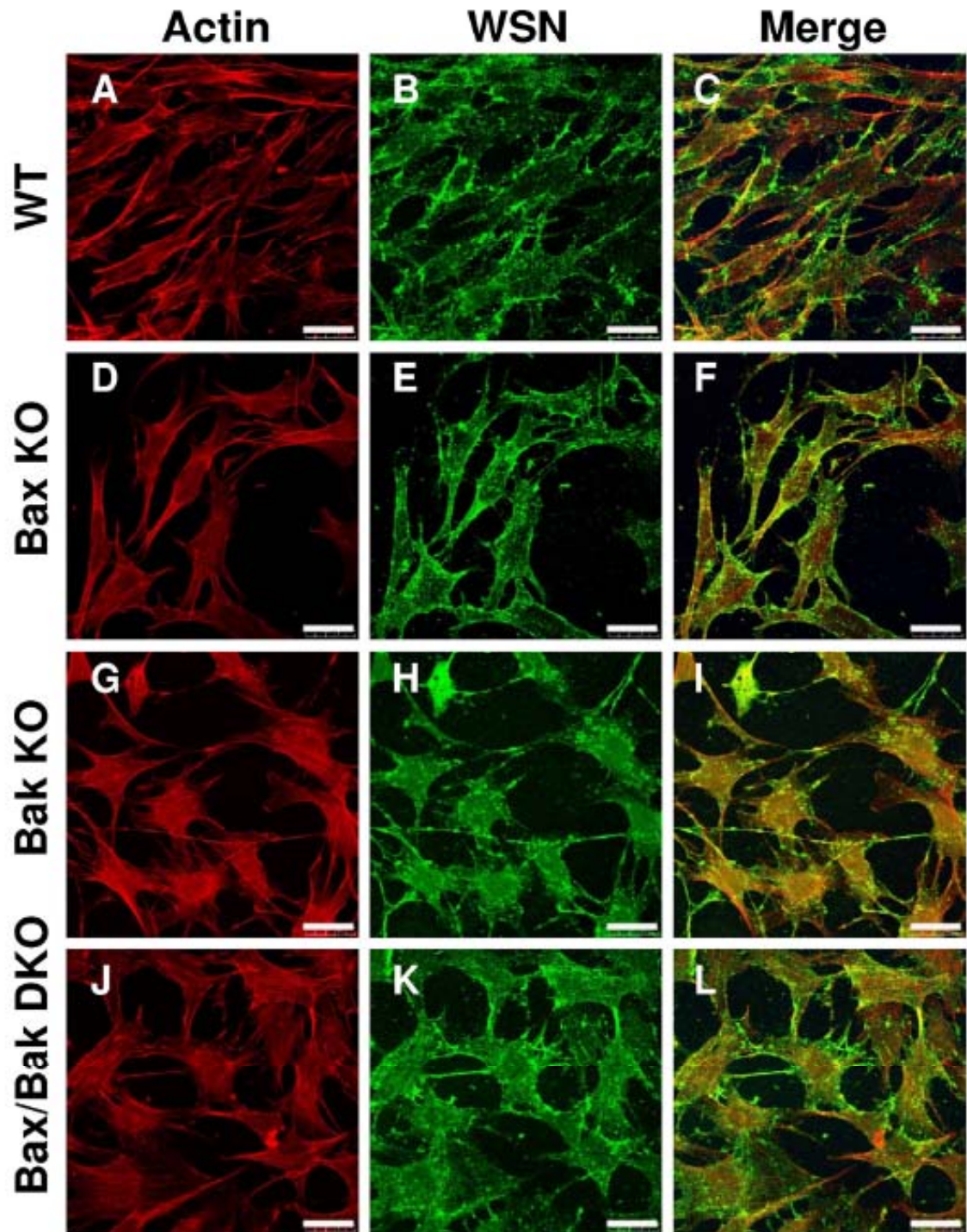


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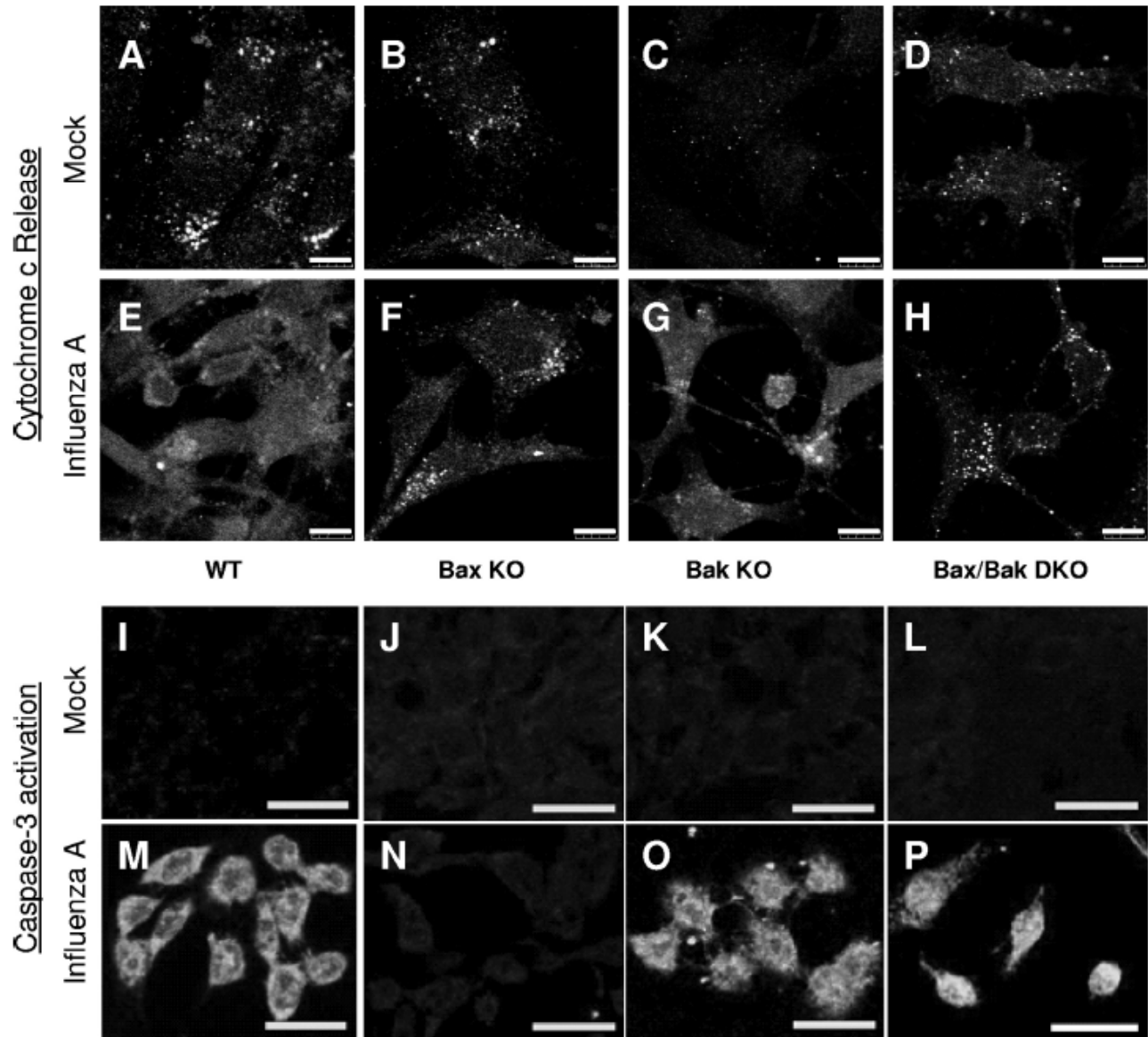


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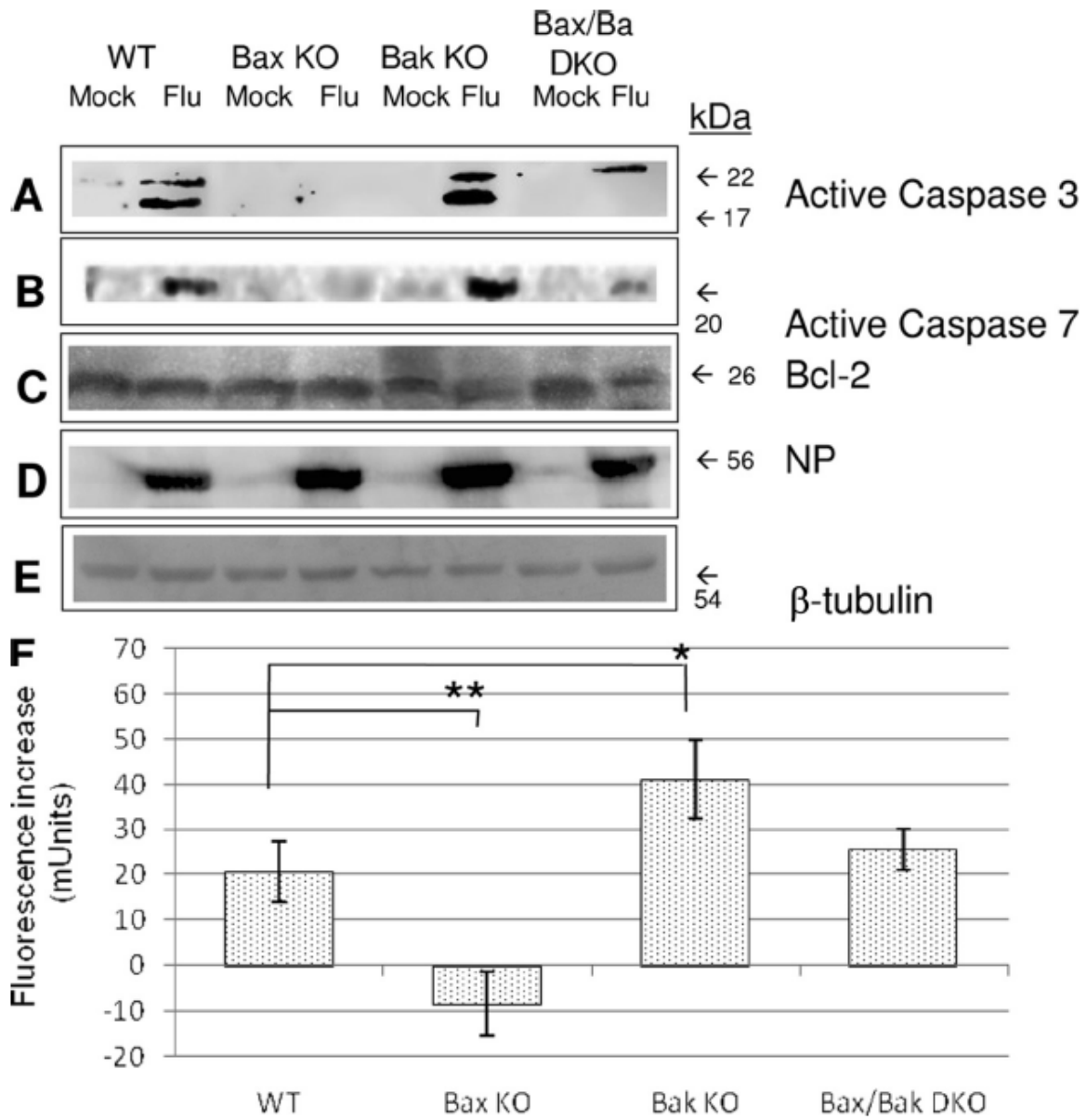


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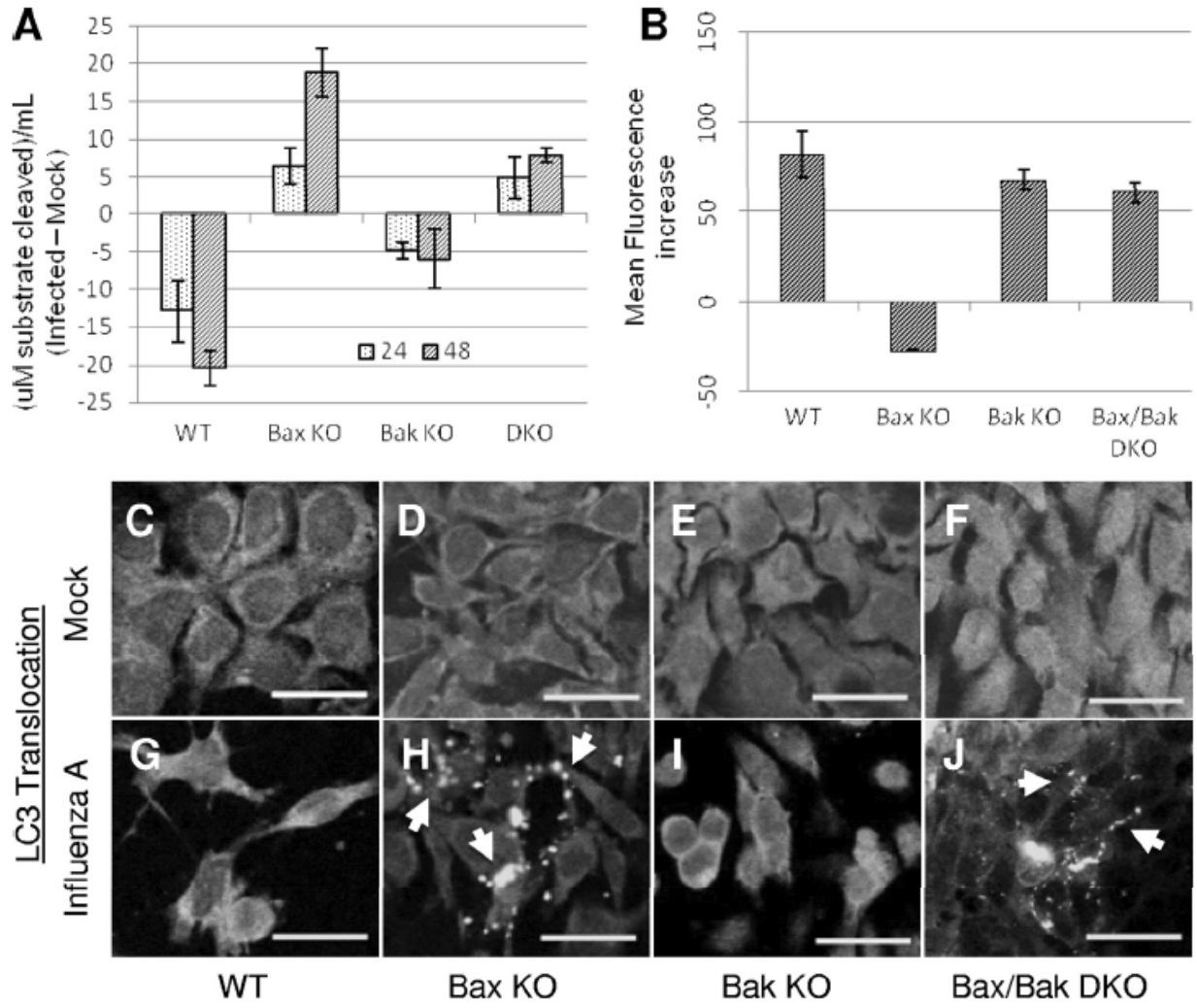


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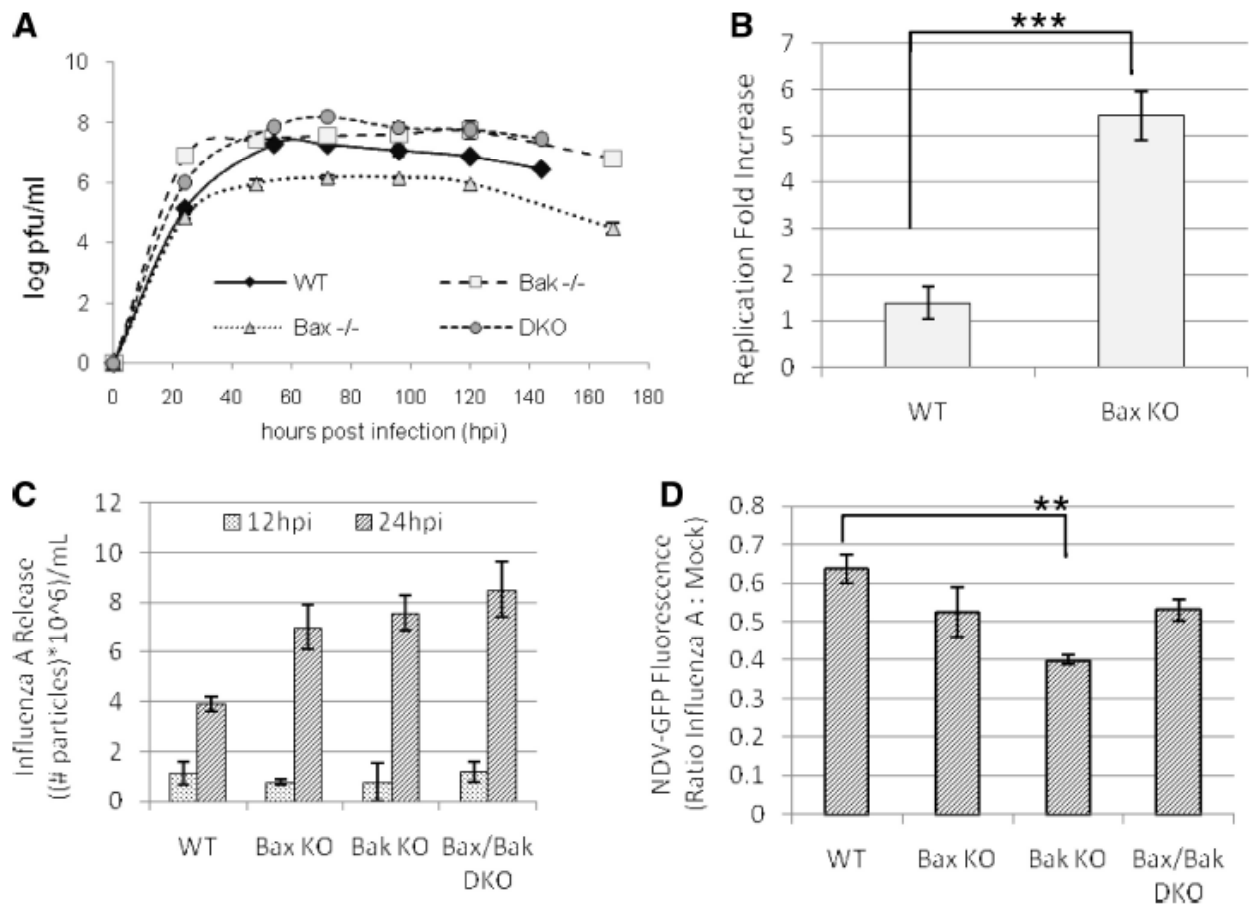


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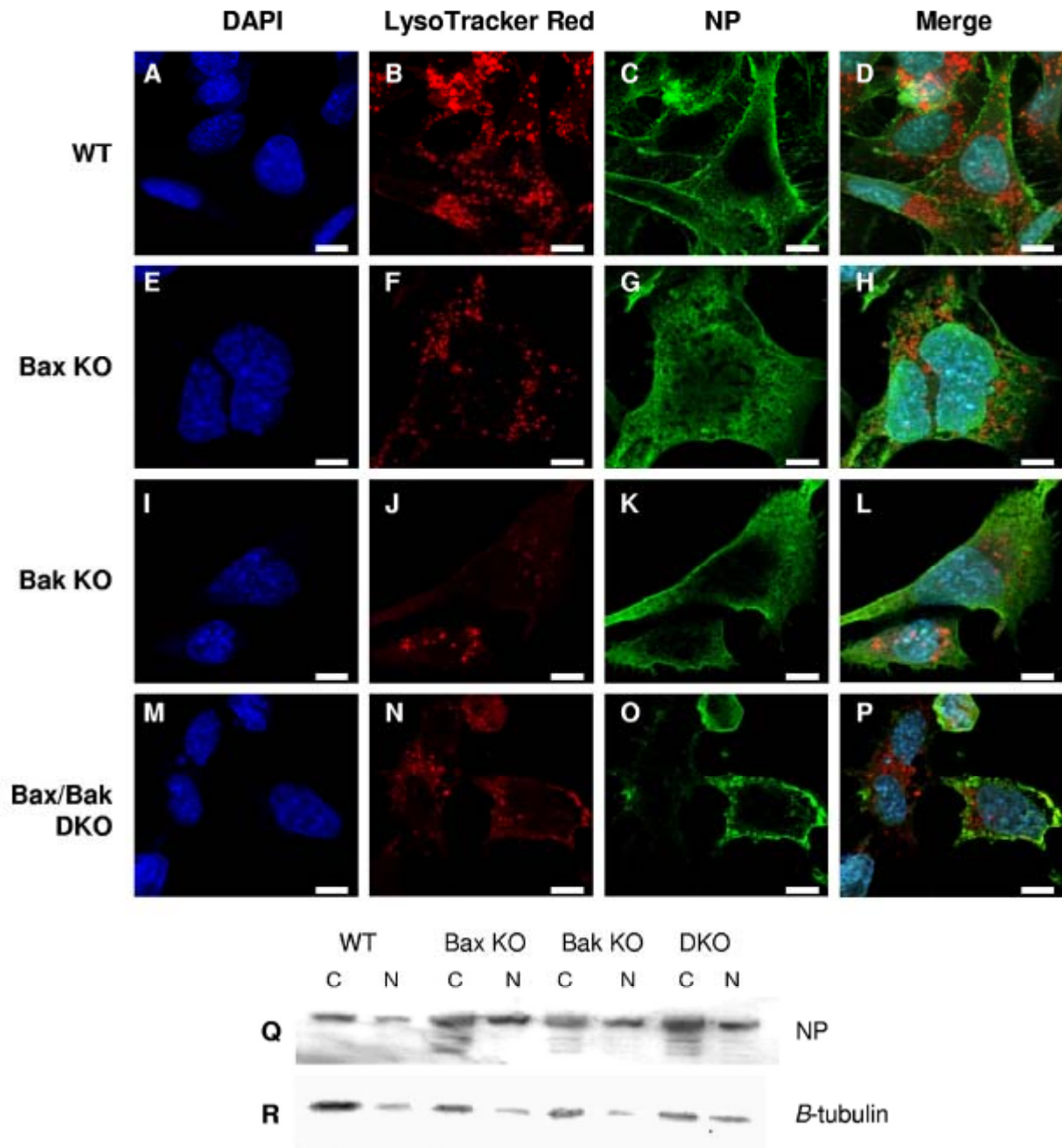


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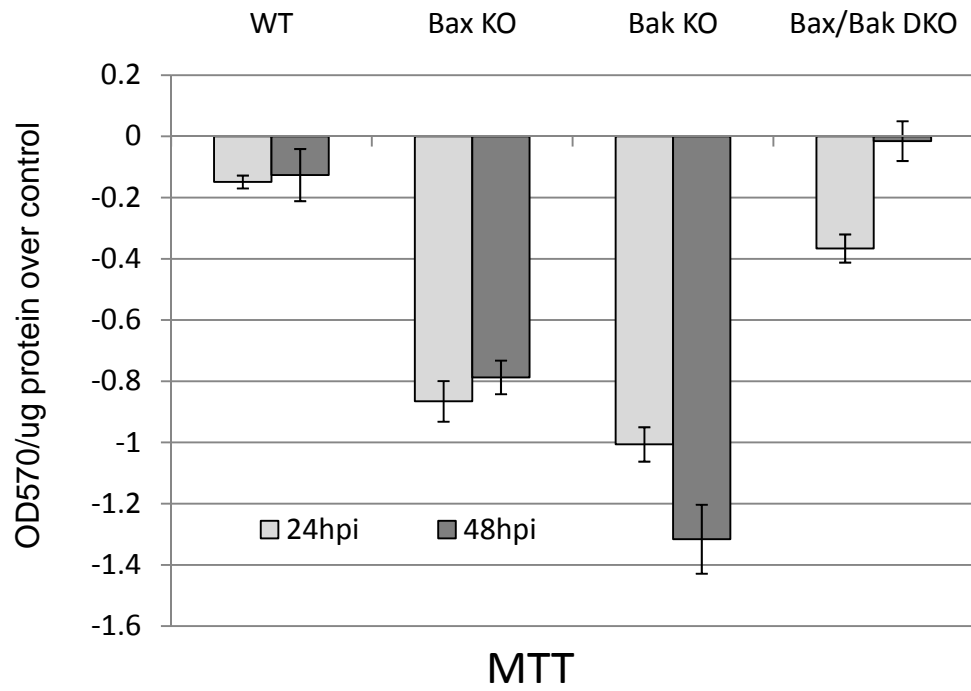
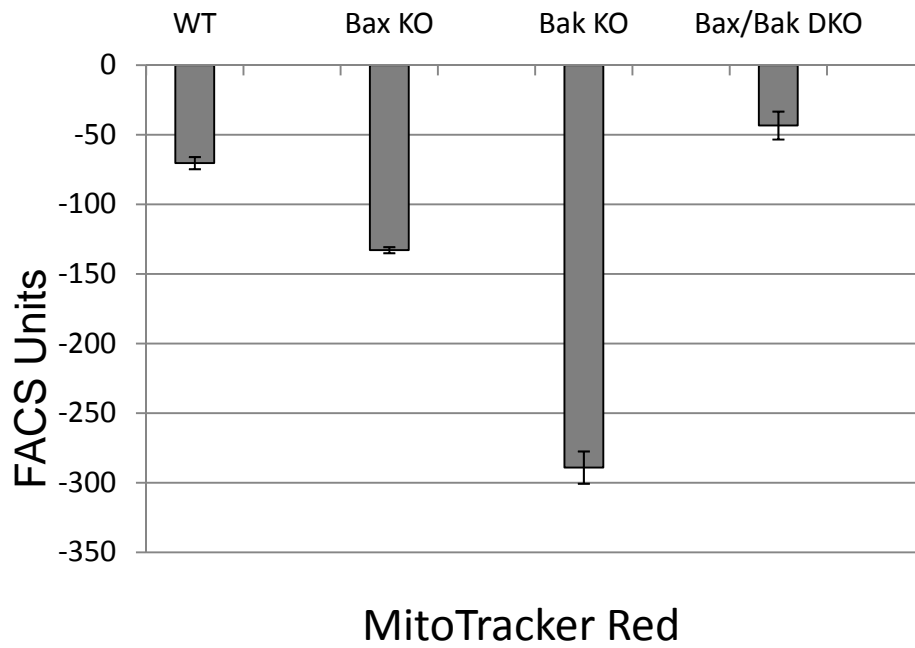
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Figure 1.9

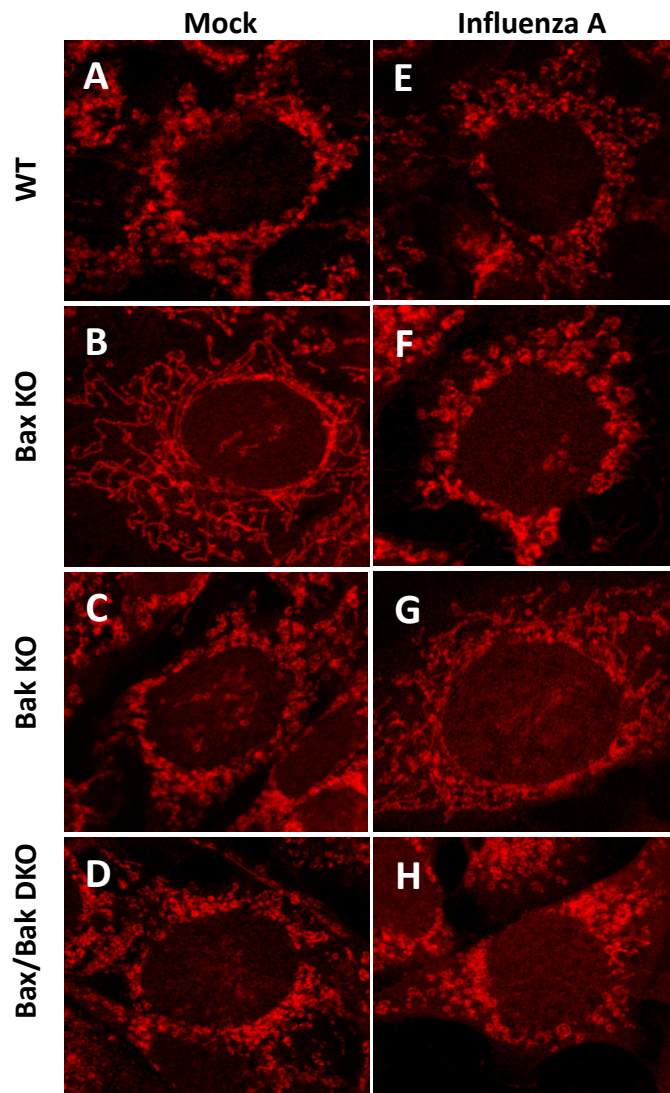


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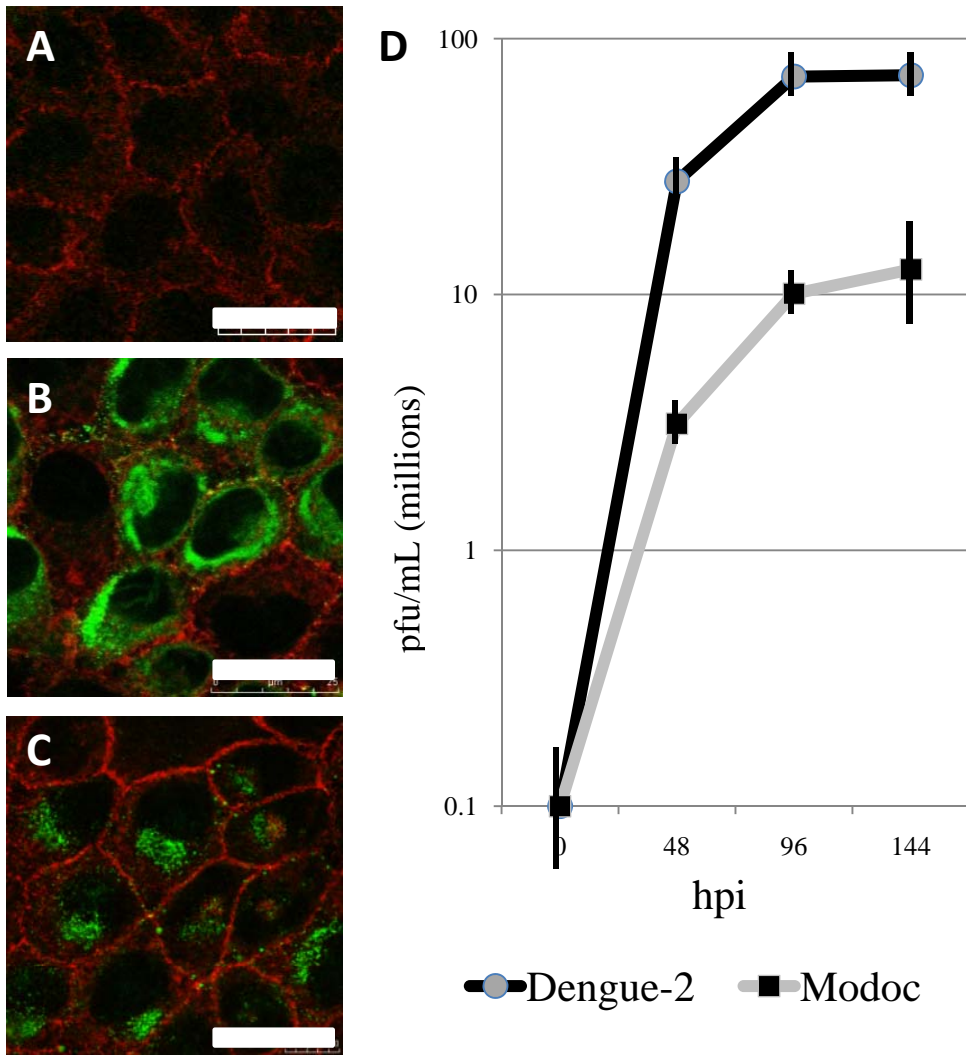


Figure 2.1

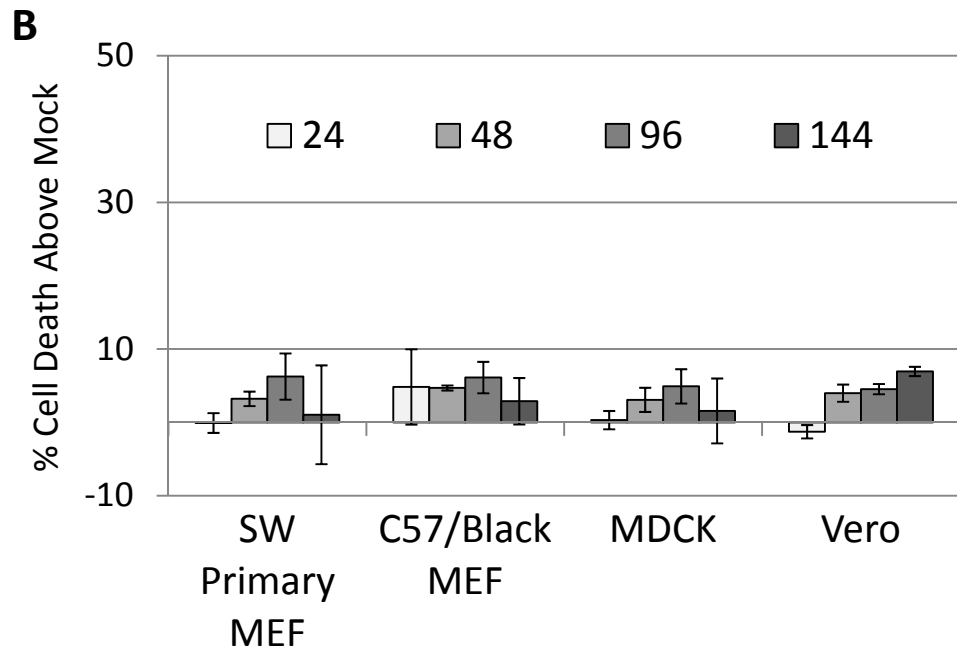
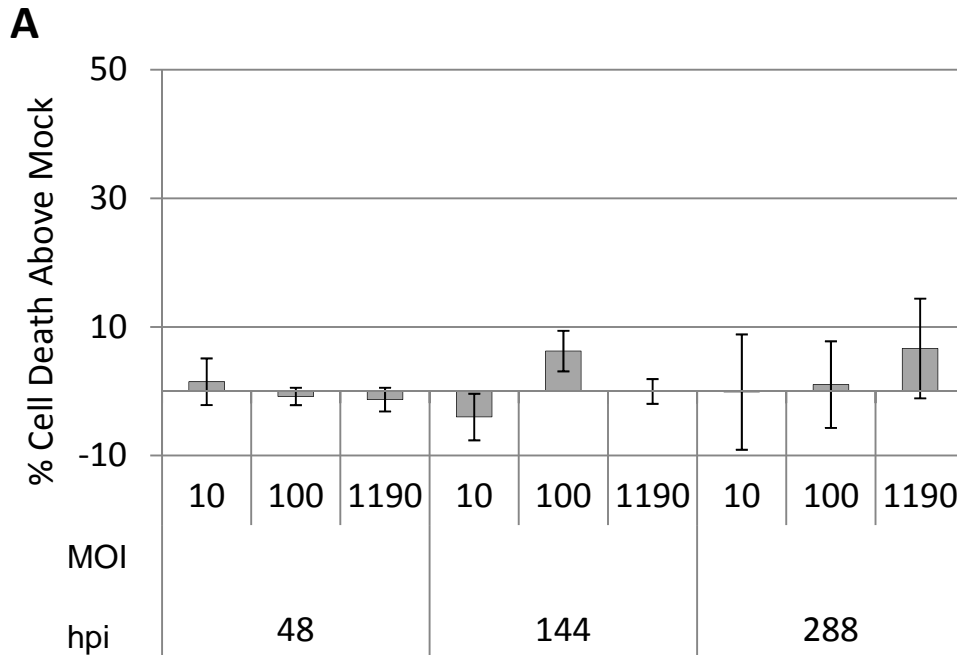


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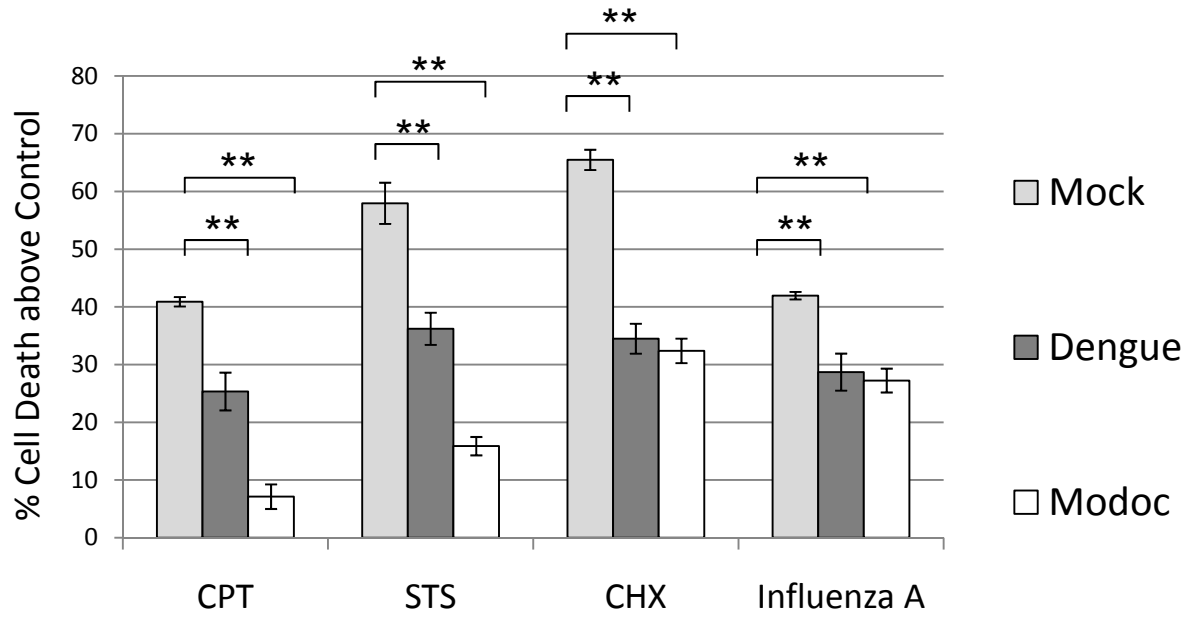


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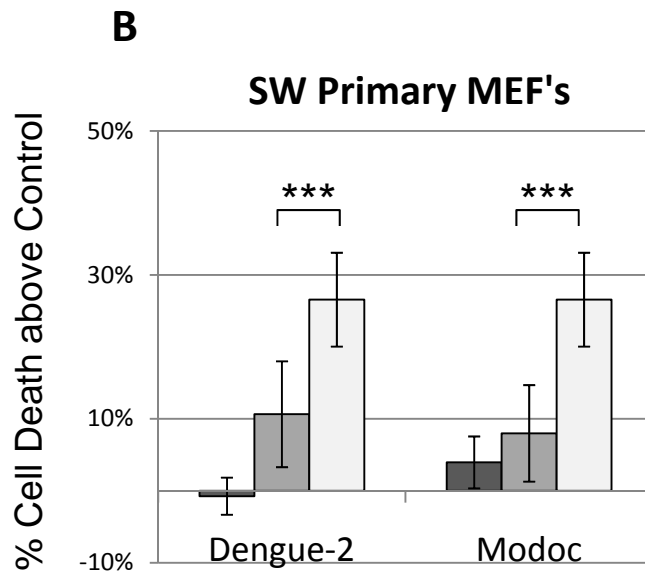
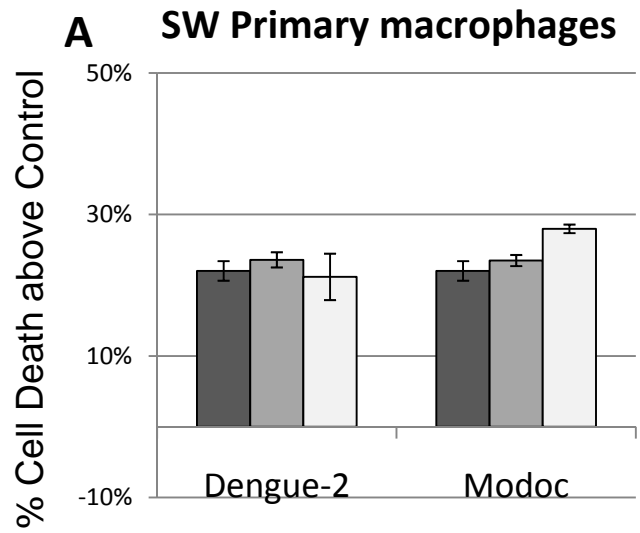


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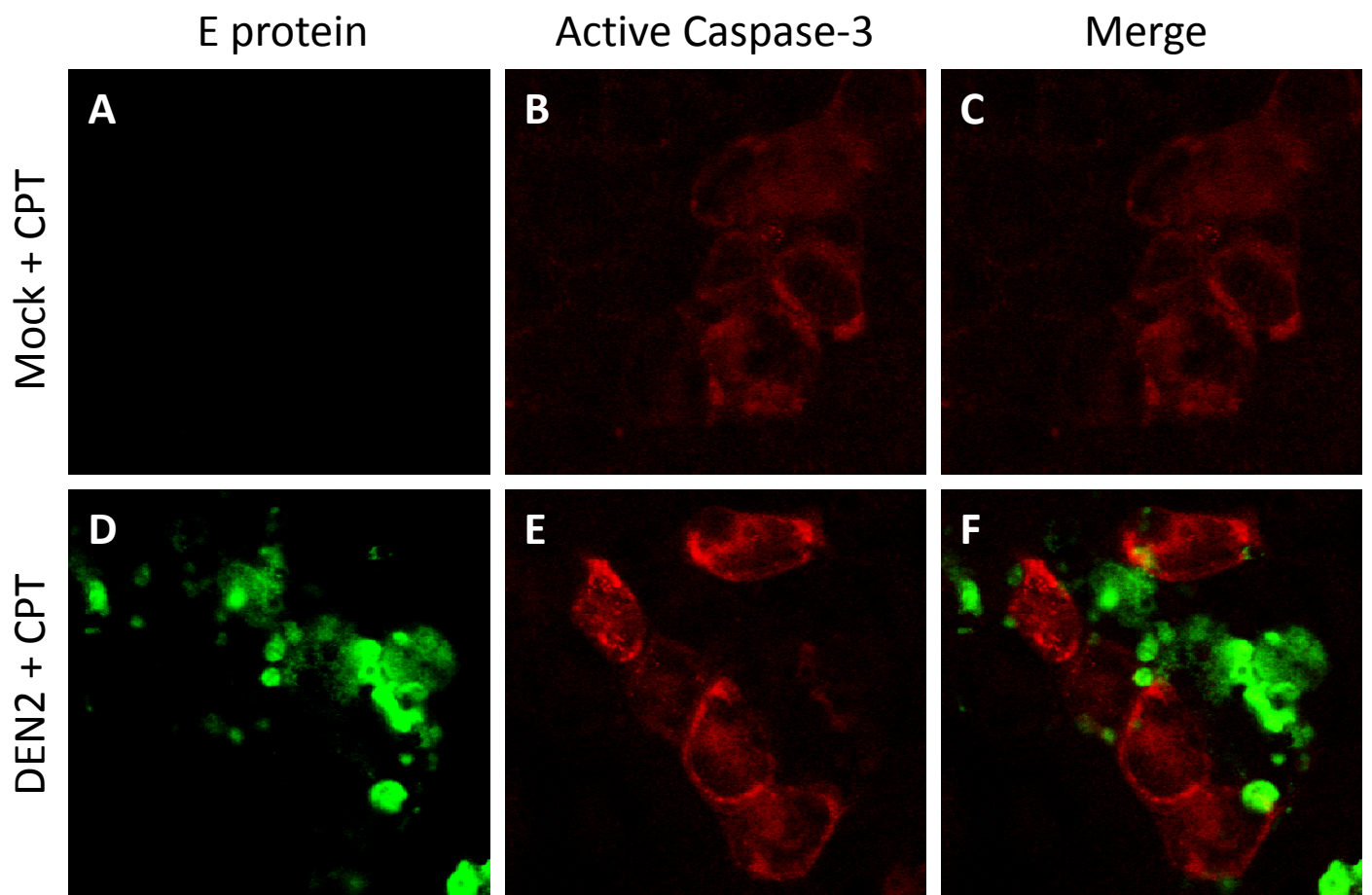
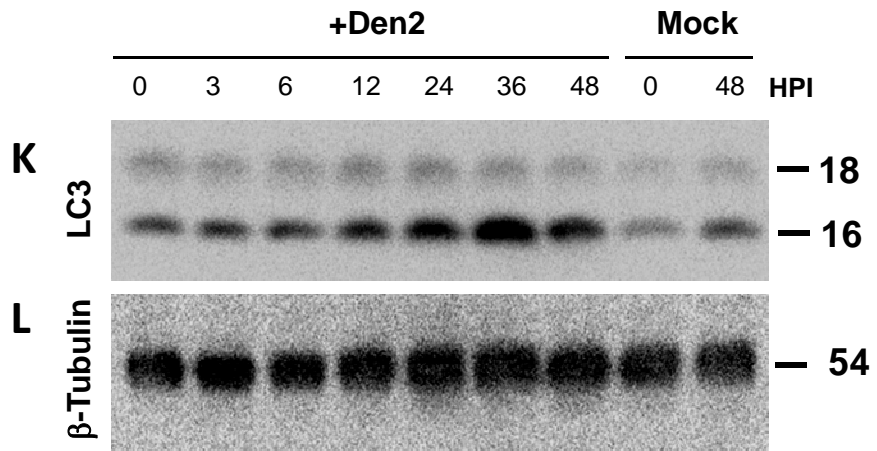
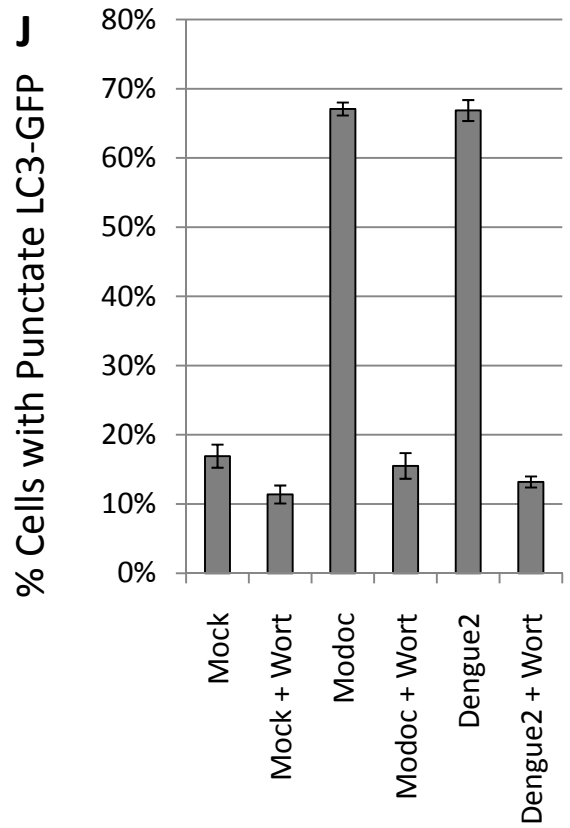
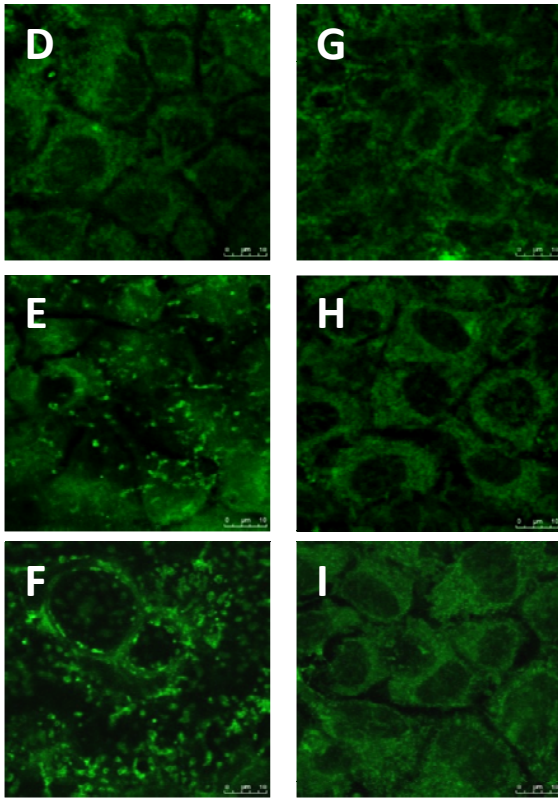
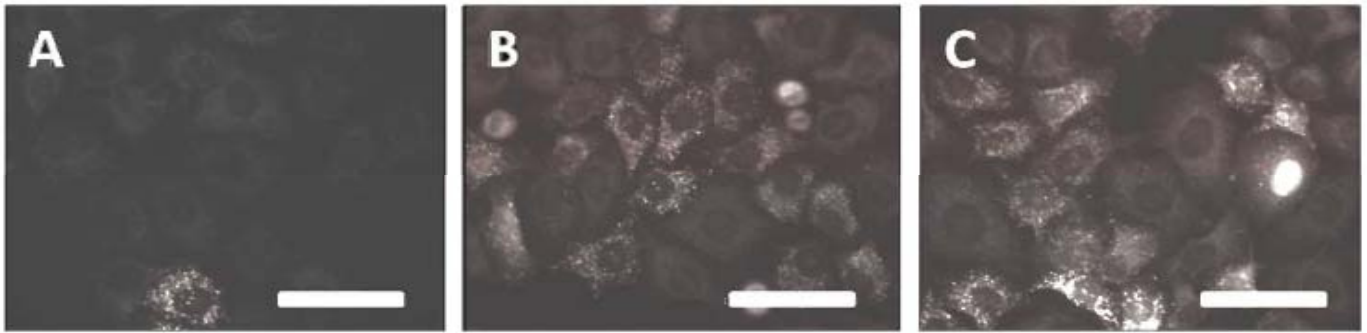


Figure 2.5



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Figure 2.6

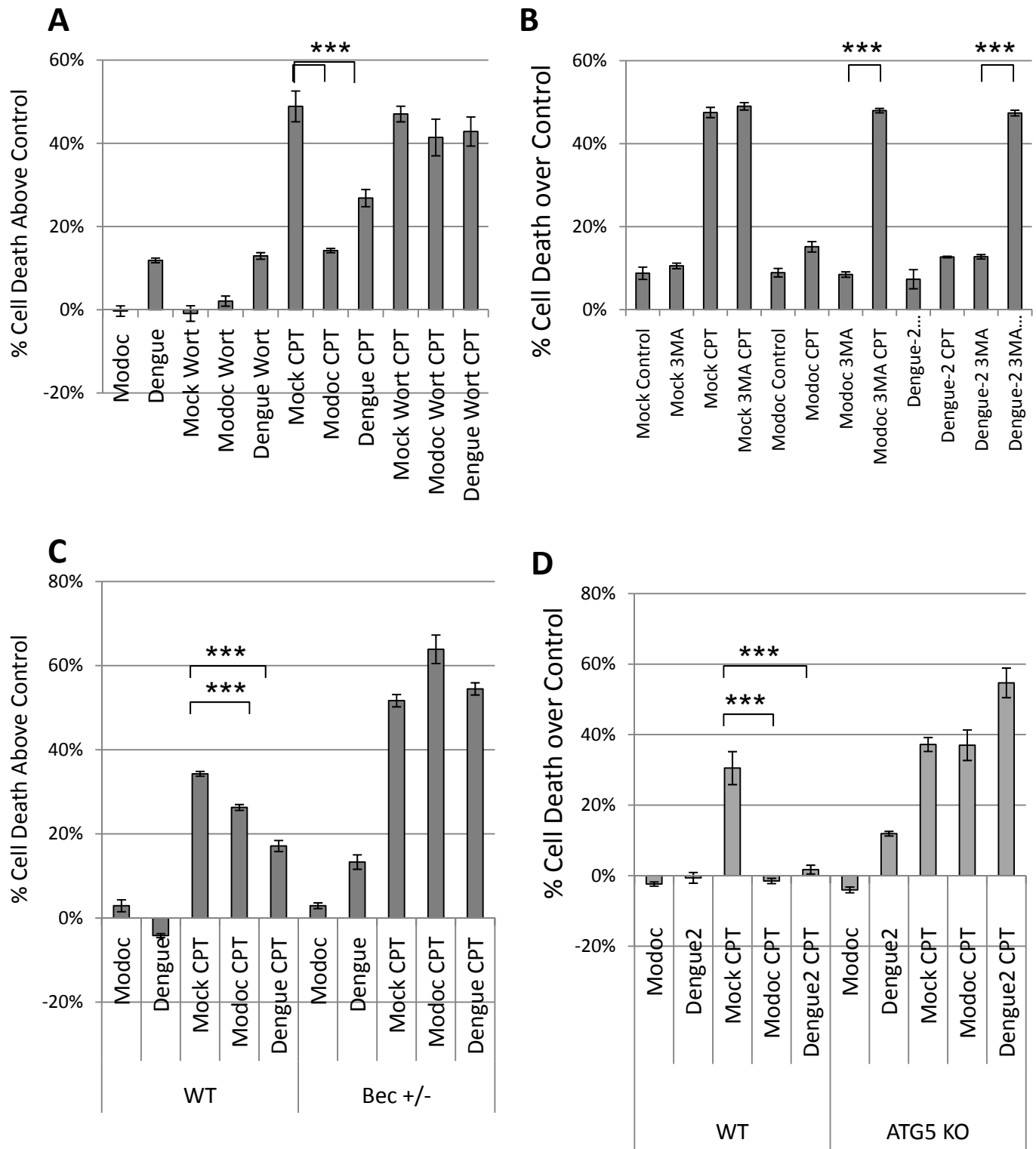


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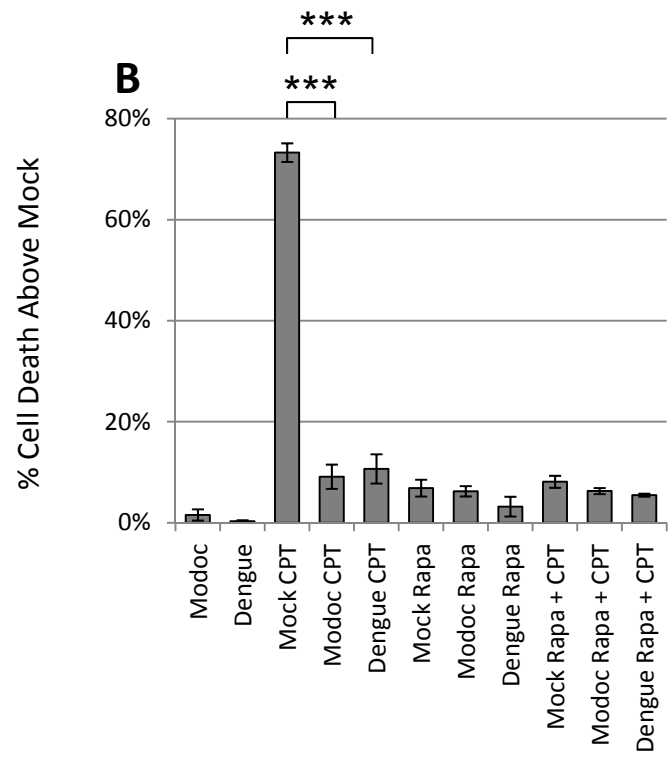
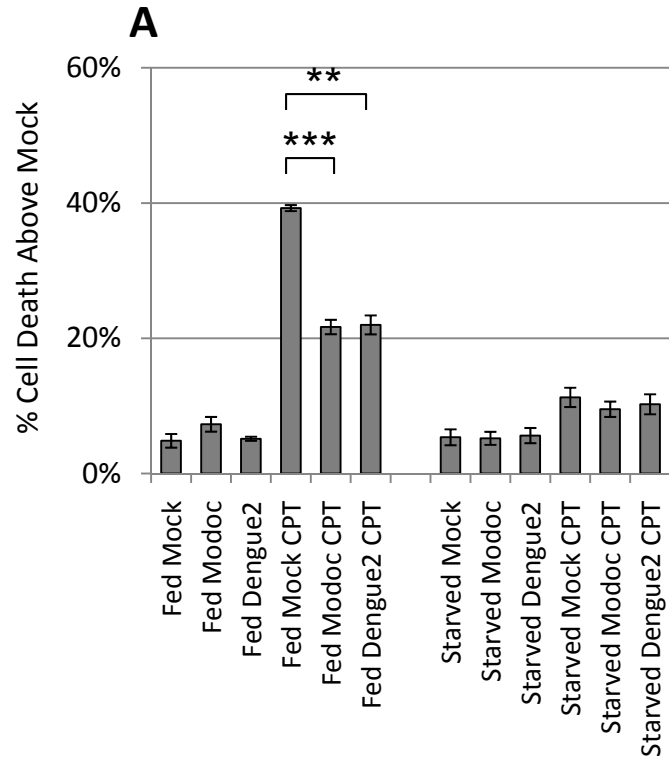


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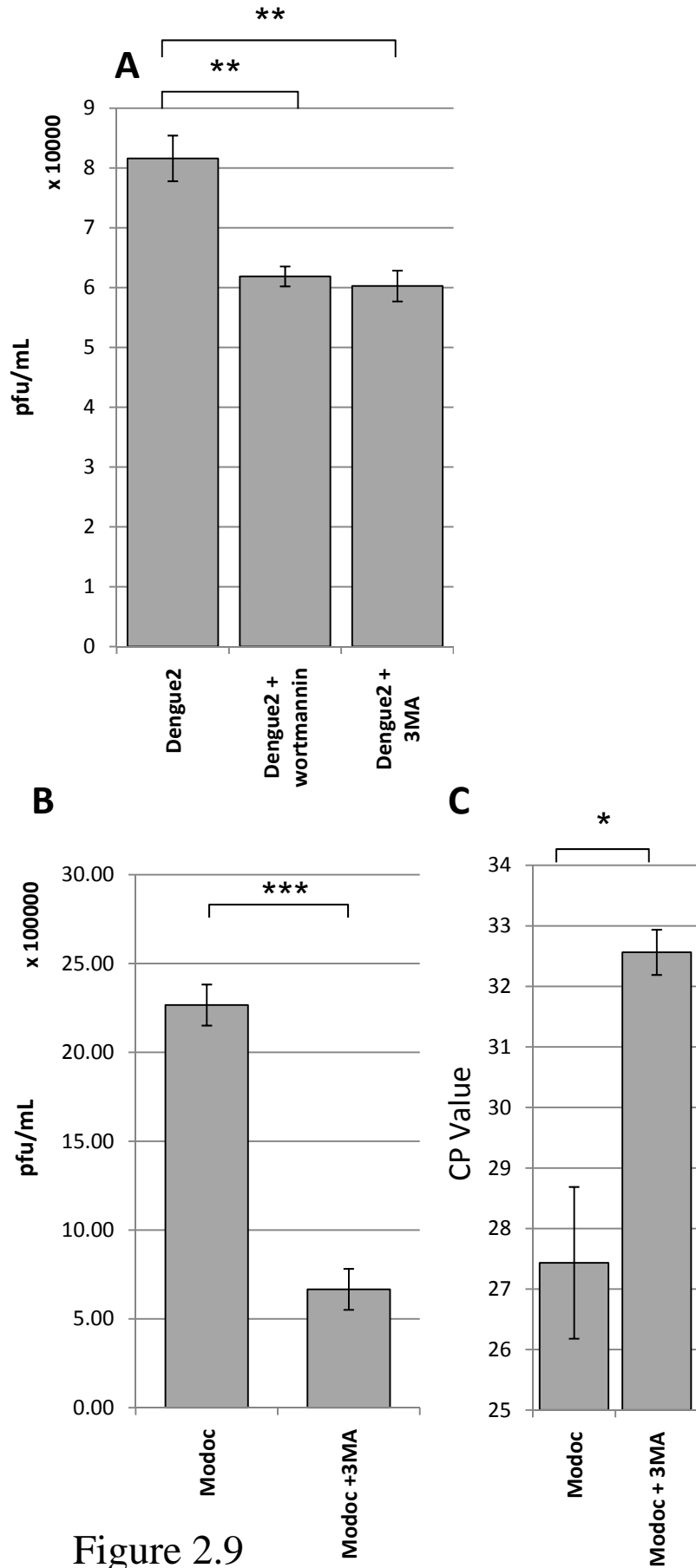
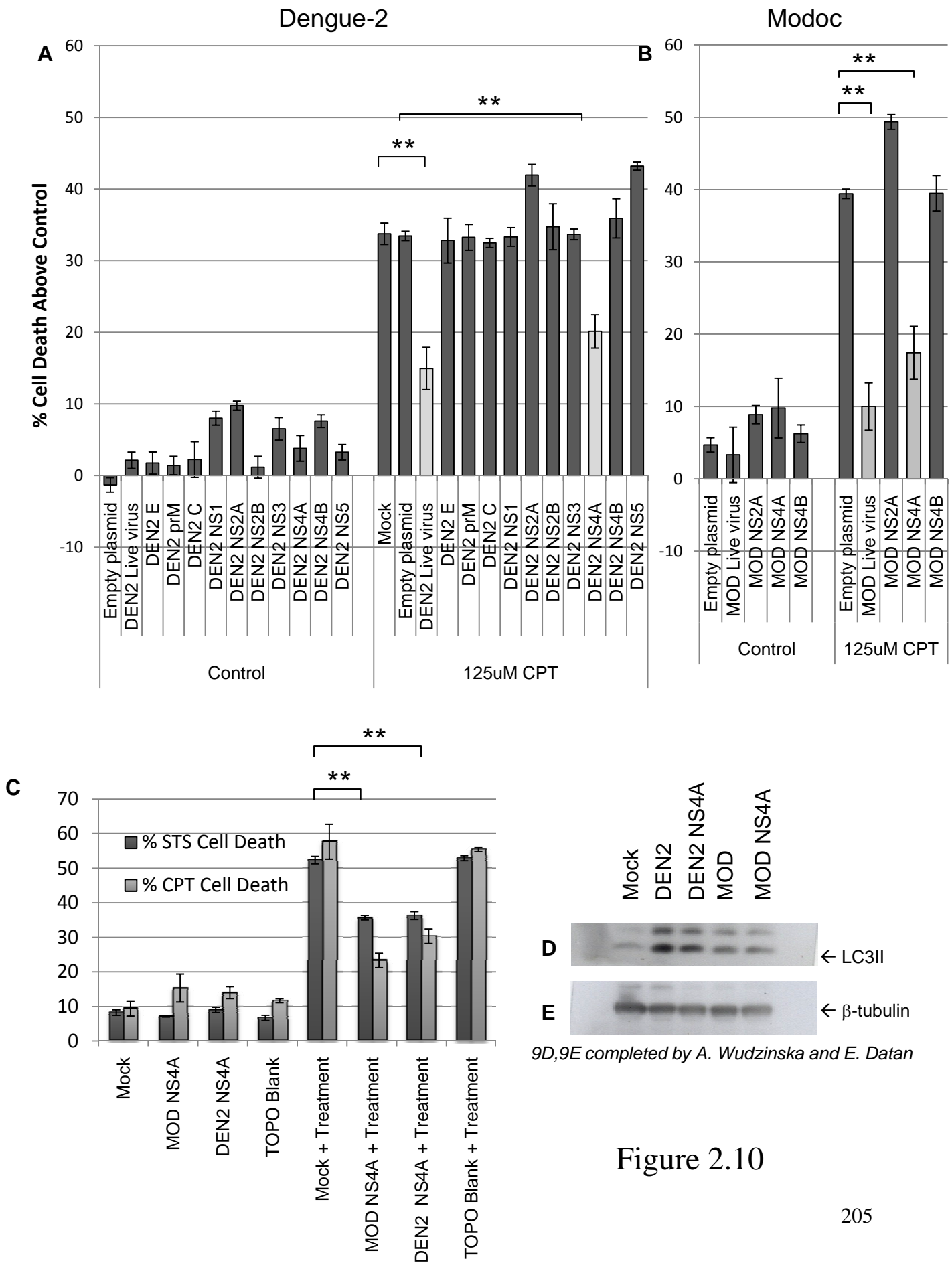
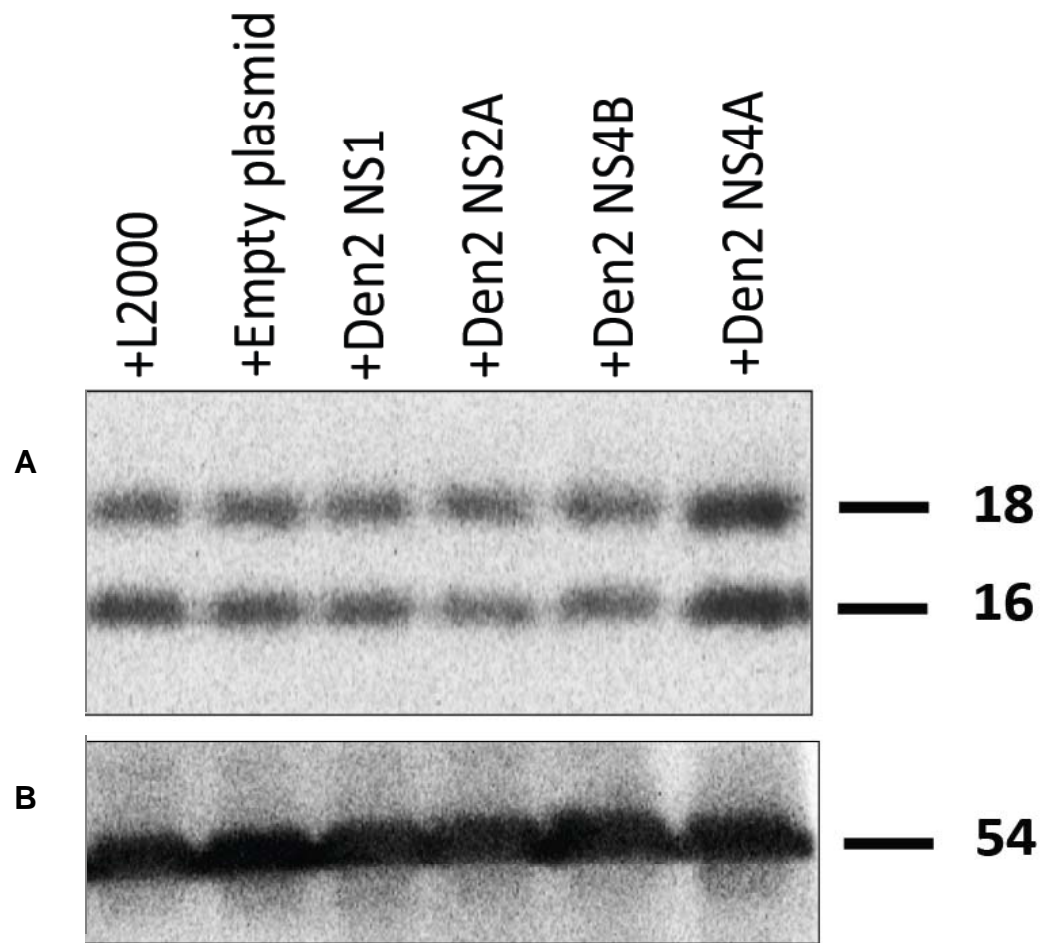


Figure 2.9





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Figure 2.11

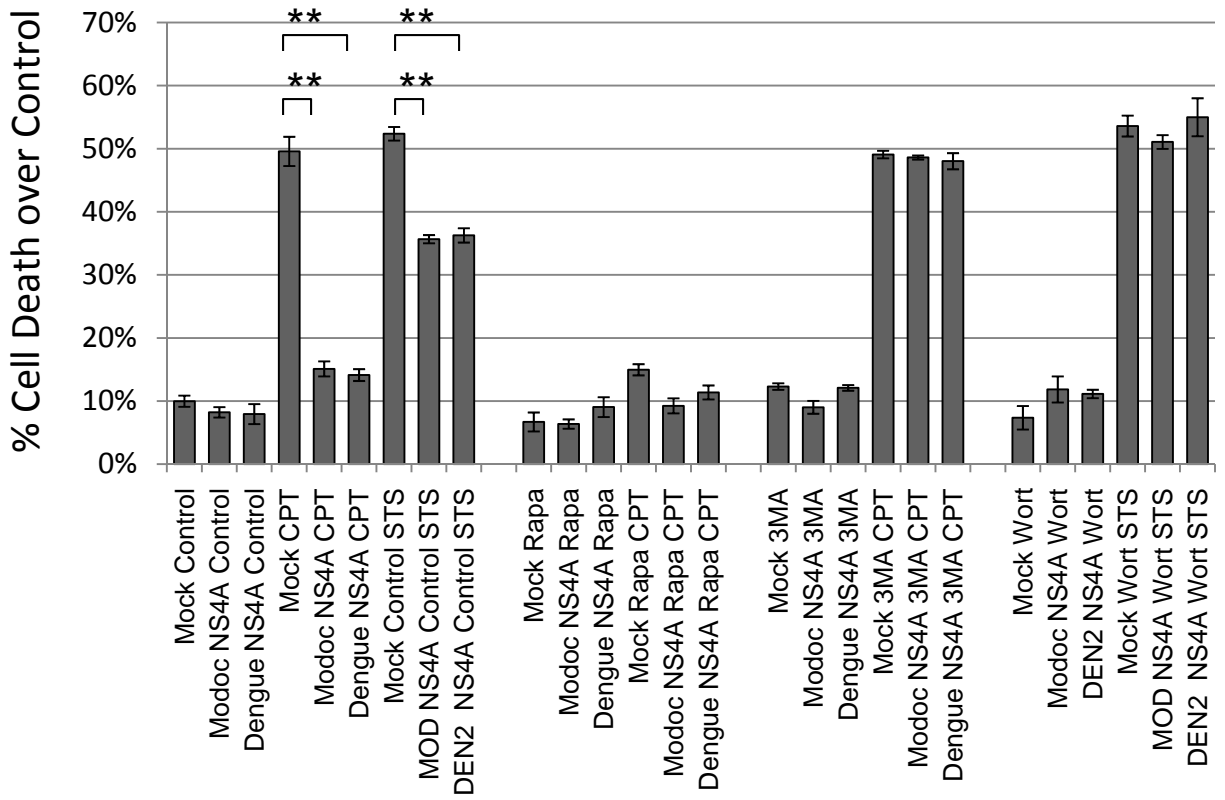


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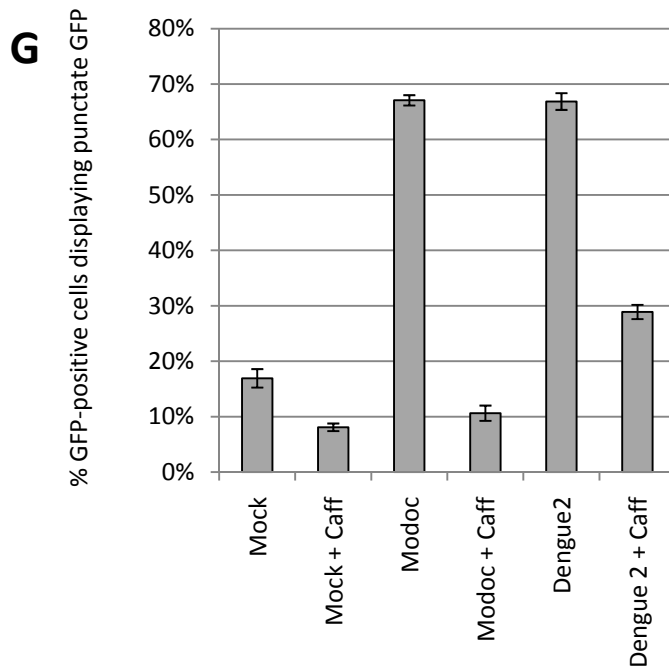
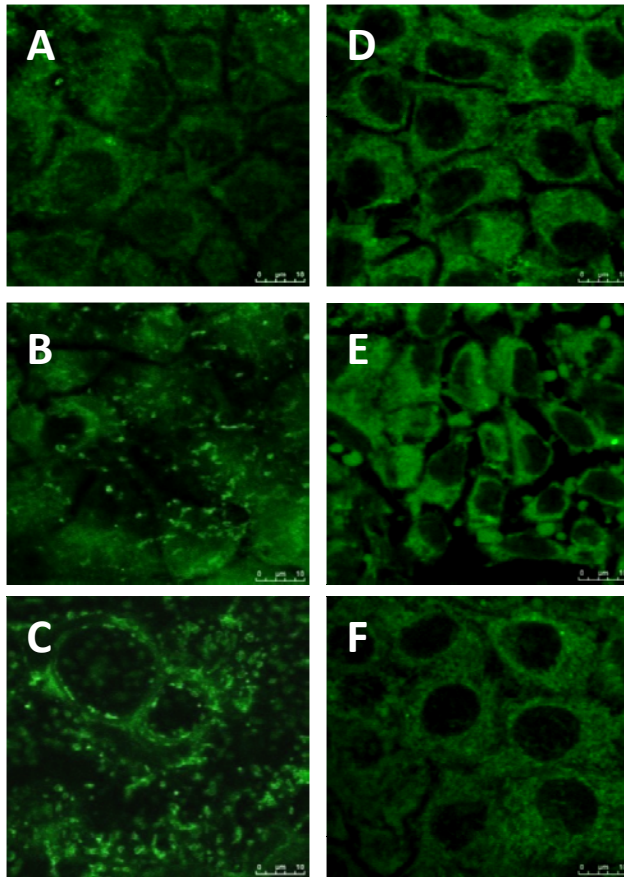


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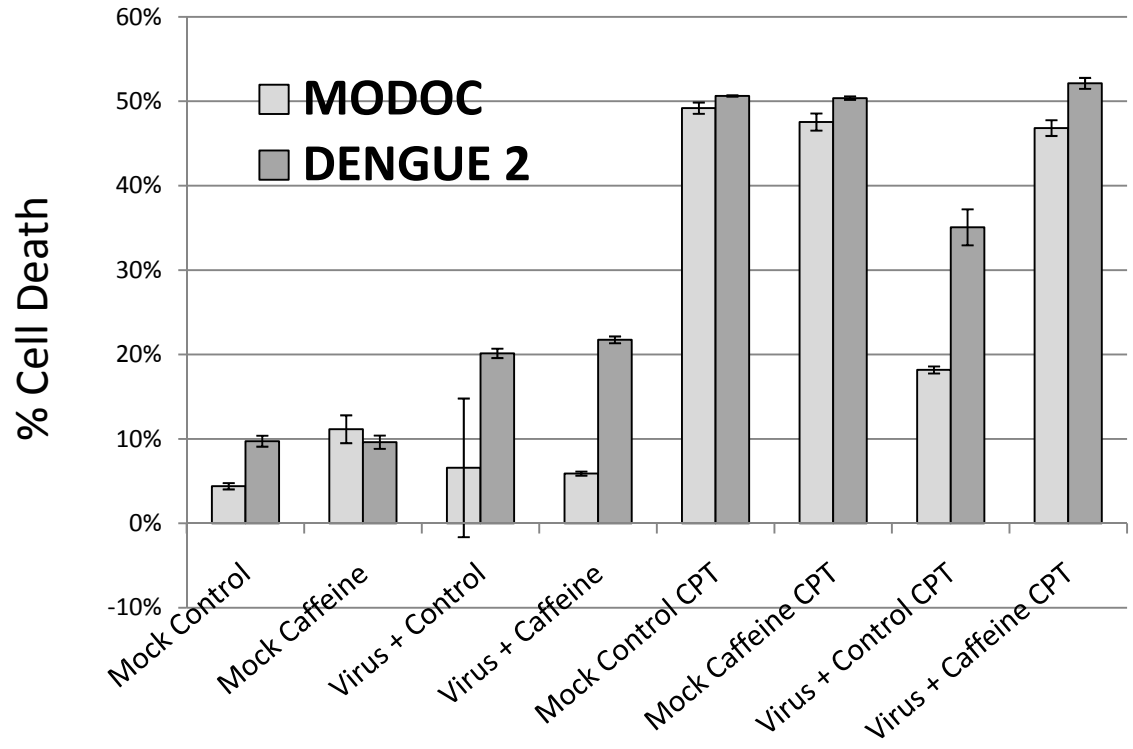


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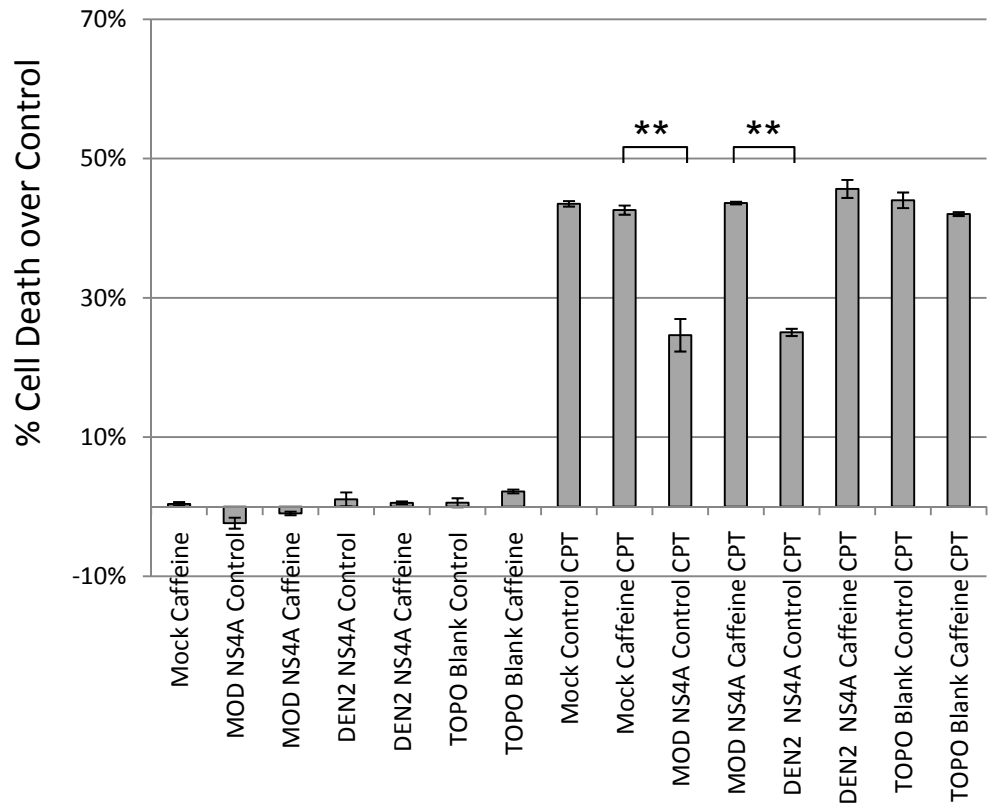


Figure 2.15

## Figure Captions

### Figure 1.1 Influenza A virus induces apoptosis through mitochondrial permeabilization via

**Bax activation.** (A) Influenza A virus induction of MDCK cell death was assessed using Trypan blue exclusion. Cell killing by influenza A/WSN/33 virus is pronounced by 24 hpi, with nearly total population death by 48 hpi (right axis, line). Nuclear alterations typical of apoptosis were observed by Hoechst staining and fluorescence microscopy. Dramatic nuclear fragmentation was seen by 24 hpi, and almost all cells exhibited nuclear fragmentation by 48 hpi (left axis, bars), indicating that influenza A virus-induced cell death is primarily apoptotic. (B) Cytochrome *c* release was assessed by centrifugal separation of mitochondria from the cytoplasm followed by western blotting for cytochrome *c*. Cytochrome *c* was seen to shift from an exclusively mitochondrial localization (P) to the cytosolic compartment (S) by 18 hpi. (C) Bax and Bak expression in MDCK cells following infection was assessed by Western blotting. Bak, a protein that constitutively associates with the outer mitochondrial membrane, is severely downregulated following infection in MDCK cells. Bax is slightly downregulated by 24 hpi.  $\beta$ -Tubulin was used as a loading control. (D and E) Bax activation and translocation were assessed by immunocytochemistry and confocal microscopy. (D) Mock-infected cells show a diffuse staining pattern indicating inactive Bax located throughout the cytoplasm. (E) Upon activation by influenza A virus, Bax was seen to shift from its inactive cytoplasmic distribution to a punctate distribution (arrows) as activated Bax was translocated to the mitochondria following infection. In this figure, DAPI rather than Bax stains the nuclei. Bars, 50  $\mu$ m.

## Figure 1.2 Cell death and nuclear and mitochondrial alterations follow influenza

**A/WSN/33 virus infection in cells lacking Bax and/or Bak.** (A) Total influenza virus-induced cell death was analyzed by Trypan blue exclusion. While influenza A virus induces excessive cell death in Bak KO cells compared to the WT at 24 hpi and 48 hpi ( $P < 0.0001$  and  $P < 0.0002$ , respectively), Bax KO cells do not differ from WT cells in the response to infection. Bax/Bak DKO cells show increased rates of death compared to the WT at 24 hpi and 48 hpi ( $P = 0.03$  and  $P = 0.000001$ , respectively), indicating that Bax and Bak activities modulate death during infection. (B) Influenza A virus induced nuclear condensation and fragmentation were analyzed by Hoechst staining. Bax KO cells exhibited a drastic reduction in nuclear alterations during influenza A virus infection compared to the WT at both 24 hpi and 48 hpi ( $P < 0.00003$  and  $P = 0.00015$ , respectively). In contrast, Bak KO cells showed significant increases in influenza A virus-induced changes in nuclear morphology compared to the WT at 24 hpi and 48 hpi ( $P = 0.04$  and  $P = 0.0002$ , respectively), while no significant difference was seen between Bax/Bak DKO and WT cells. These results suggest that influenza A virus may employ Bax as a means to trigger mitochondrial apoptosis during infection and that Bak antagonizes Bax in this situation. (C to J) Changes in nuclear morphology during influenza A virus infection were assessed by fluorescence microscopy at 48 hpi. Arrows mark nuclear condensation and fragmentation during influenza A virus infection, indicating apoptotic cell death. (C) WT mock; (D) WT plus influenza A virus; (E) Bax KO mock; (F) Bax KO plus influenza A virus; (G) Bak KO mock; (H) Bak KO plus influenza A virus; (I) Bax/Bak DKO mock; (J) Bax/Bak DKO plus influenza A virus. Bars, 50  $\mu\text{m}$ . (K to M) KO of Bax and/or Bak was confirmed by Western blotting. (K and

*L*) Bax is present only in WT and Bak KO cells (*K*), while Bak is present only in WT and Bax KO cells (*L*), confirming knockout in Bax KO, Bak KO, and Bax/Bak DKO cells. (*M*)  $\beta$ -Tubulin was used as a loading control.

**Figure 1.3 Influenza A virus establishes infection and replicates in WT, Bax KO, Bak KO, and Bax/Bak DKO cells.** Actin was stained by phalloidin-TRITC (*A, D, G, and J*). Immunocytochemical staining of influenza A virus-infected cells at 24 hpi with anti-WSN whole-virus primary antibody, followed by Alexa Fluor 488 staining (*B, E, H, and K*). Reveals cytoplasmic influenza A virus-induced vesicles containing mature virions in all infected cells regardless of Bax and/or Bak activity. (*C, F, I, and L*) Merge. Bars, 10 $\mu$ m.

**Figure 1.4 Influenza A virus-induced cytochrome *c* release and subsequent caspase activation are dependent primarily on Bax signaling.** (*A to H*) Immunocytochemical staining of cytochrome *c* release in cells lacking Bax and/or Bak following influenza A virus infection. (*A to D*) Cytochrome *c* staining reveals a punctate distribution consistent with mitochondrial localization in healthy, mock-infected cells of each type. (*E and G*) Following influenza A virus infection at 24 hpi, cytoplasmic cytochrome *c* is evident in WT (*E*) and Bak KO (*G*) cells. (*F and H*) Bax KO (*F*) and Bax/Bak DKO (*H*) cells show no appreciable cytochrome *c* release by 48 hpi and retain a punctate cytochrome *c* staining pattern. These results indicate that Bax is necessary for influenza A virus-induced cytochrome *c* release. (*I to P*) Immunocytochemical staining of active caspase-3 in cells lacking Bax and/or Bak following influenza A infection. (*I to L*) Staining for active caspase-3 yields no signal in healthy, mock-infected cells. (*M, O, and P*) Following influenza A virus infection at 48 hpi, widespread caspase-3 activation is

evident in WT (*M*), Bak KO (*O*), and Bax/Bak DKO (*P*) cells. (*N*) Bax KO cells do not show appreciable caspase-3 activation following influenza A virus infection, indicating that Bax, in the presence of Bak, is necessary for caspase-3 activation by influenza A virus. The activation of caspase-3 in Bax/Bak DKO cells indicates that there is an alternative pathway to caspase activation during influenza A virus infection in the absence of both Bax and Bak.

**Figure 1.5 Executioner caspase activation does not occur after infection of Bax KO cells.** (*A to E*) Western blotting for executioner caspase activation in influenza A virus-infected cells lacking Bax and/or Bak. Immunoblotting was performed with antibodies specific for active caspase-3 (*A*), cleaved caspase-7 (*B*), Bcl-2 (*C*), influenza A virus NP (*D*), and  $\beta$ -tubulin (*E*), which was used as a loading control. An activation of caspase-3 and caspase-7 was not seen in Bax KO MEFs and was impaired in Bax/Bak DKO cells, whereas caspase activation was evident in WT and Bak KO cells that constitutively expressed Bax. Bcl-2 expression was constant across all cell types in both mock- and influenza A virus-infected cells. (*F*) Executioner caspase activity (caspase-3 and caspase-7) was assessed using and Apo-One caspase activity kit, and cleavage of fluorogenic substrate was assessed at 495 nm by use of a fluorescence microplate reader (Bio-Tek). Data are expressed as arbitrary units (milliunits [mUnits]), (fluorescence from influenza A virus-infected cells) x (fluorescence from mock-infected cells), in individual wells. The rate of activated executioner caspase substrate cleavage was high in Bak KO cells compared to the WT ( $P = 0.03$ ), but the substrate was largely uncleaved in Bax KO cells following infection ( $P = 0.007$ ), indicating that Bax in the presence of Bak is required for executioner caspase activation in response to influenza A virus.

**Figure 1.6 Influenza A virus induces autophagy-like death in the absence of Bax.**

(A) Lysosomal acid phosphatase activity in influenza A virus-infected cells was assessed by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate substrate. In cells constitutively expressing Bax, acid phosphatase activity decreases following infection, as these cells follow an apoptotic path to death. In cells lacking Bax, acid phosphatase activity increased, indicating an increase in lysosomal activity, a common marker for autophagic death. One unit equals 1  $\mu$ mol of 4-nitrophenylphosphate per minute under the experimental conditions. (B) Cells were infected for 48 hpi and stained with LysoTracker Red DND-99 for 30 min prior to collection and immediate analysis by FACS. In WT, Bak KO, and Bax/Bak DKO cells, influenza A virus infection results in an increase in lysosomal volume by 48 hpi. In Bax KO cells, a slight decrease in lysosomal volume was observed by 48 hpi. (C to J) Cells were transfected with a construct expressing LC3-GFP prior to infection and observed by confocal microscopy for LC3 and translocation following infection. (C to F) The diffuse LC3-GFP expression pattern in mock-infected cells indicates a cytoplasmic, inactive distribution of LC3 in each cell type. (G and I) In cells constitutively expressing Bax, LC3-GFP expression remains diffuse following infection, indicating a lack of LC3 activation as these cells undergo apoptosis. (H and J) In cells lacking Bax (Bax KO and Bax/Bak DKO cells), LC3 GFP staining shifts to a punctate pattern indicating LC3 activation and translocation to autophagosomes, a process that occurs solely during autophagy. Bars, 25  $\mu$ m.

**Figure 1.7 Influenza A virus replication is dependent upon opposite virus-induced effects**

**on Bax and Bak activity that are unlikely to be interferon related.** (A) Influenza A virus replication was analyzed by plaque assay. Virus replication is severely attenuated in

Bax KO cells, resulting in a 2-log decrease in PFU/ml compared to the WT. Bak KO cells allow a maximum replication similar to that of the WT, while Bax/Bak DKO cells show a slight elevation of infectious titers during infection. These results indicate that Bax is proviral during infection, while Bak is dispensable for replication. **(B)** Bax was transiently expressed in all cell types by Lipofectamine 2000 transfection of a C2-Bax-GFP construct prior to infection, and supernatant samples were collected for plaque assay at 48 hpi. Baseline virus replication in each cell type was evaluated using empty C2-GFP plasmid transfection. Bax reconstitution in Bax KO cells resulted in a fivefold increase in infectious titers compared to the control ( $P = 0.0007$ ). A minimal effect on the virus titer was seen after Bax overexpression by transient transfection in WT cells compared to empty plasmid controls. **(C)** Influenza A virus replication was assessed by reverse transcription-PCR. Serial dilutions of stock virus at known concentrations were also analyzed to generate a standard curve to which experimental samples were compared, thus calculating the approximate number of influenza A virus particles/ml in each sample. By 24 hpi, Bax KO, Bak KO, and Bax/Bak DKO cells all showed significantly higher levels of influenza A virus RNA released into the cell culture supernatant than did WT cells. **(D)** Interferon activity was assessed by infecting mock- and influenza A virus-infected cells with interferon-sensitive, GFP-linked NDV and quantifying the mean GFP expression levels of 10,000 events per condition by FACS analysis. Each assay was run in triplicate, and data are expressed as the ratio of the numbers of influenza A virus-infected to mock-infected cells per cell type. After influenza A virus infection, Bak KO cells exhibited a slight decrease in ratio compared to the WT, representing a 30% increase in interferon activity ( $P = 0.002$ ). Bax KO and Bax/Bak DKO cells both showed

similar fluorescence changes compared to the WT after infection. Due the high degree of similarity between cell types, these results suggest that the interferon response in infected cells is modulated by viral replication in the presence of Bak and is only slightly modified by Bax activity during influenza A virus infection. As an elevated interferon response typically leads to a reduced virus replication capacity, these results also suggest that it is unlikely that the observed trends in infectious virus titer are due to virus-induced interferon signaling.

**Figure 1.8 Influenza A virus NP exhibits increased nuclear retention in Bax KO cells.**

Nuclear retention of NP due to a lack of caspase activity has been linked to decreased titers of virus. DAPI staining was used for nuclear localization (*A, E, I, and M*), lysosomes were stained with LysoTracker Red DND-99 (*B, F, J, and N*), and influenza A virus NP localization was determined using an NP-specific antibody (*C, G, K, and O*). Images were taken using a confocal microscope. (*D, H, L, and P*) Merge. Lysosomal localization of influenza A virus NP was not observed in WT cells or any of the KO cells. NP showed near-complete cytoplasmic localization in WT cells (*D*) and Bax/Bak DKO cells (*P*). A slight nuclear retention of NP was observed in Bak KO cells (*L*), while in Bax KO cells (*H*), a nuclear retention of influenza A virus NP is obvious in all infected cells. Bars, 10  $\mu\text{m}$ . (*Q*) Nuclear retention of NP in infected Bax KO and, to a lesser extent, Bak KO cells was confirmed by western blotting of cytoplasmic (*C*) and nuclear (*N*) fractions of infected cells. (*R*)  $\beta$ -Tubulin was used to ensure efficient fractionation.

**Figure 1.9 Influenza A infection causes Bax and Bak-dependent changes in mitochondrial**

**function.** (*A*) Mitochondrial respiration activity following infection was assessed by MTT assay at 24 and 48 hpi. Influenza A infection causes only a slight reduction in

mitochondrial activity in wild-type and Bax/Bak DKO MEF's at both times. In contrast, Bax KO and Bak KO cause a substantial decline in mitochondrial function following infection. *(B)* A similar pattern of mitochondrial failure during Influenza A infection is seen when assessed by MitoTracker RedCMXRos staining and FACS analysis, indicating that Bax and Bak activity assist in maintaining mitochondrial function during infection.

**Figure 1.10 Influenza A infection causes Bax and Bak-dependent changes in mitochondrial morphology.** *(A)* Mock-infected wild-type MEF's show robust, ovoid mitochondria, an appearance that is maintained following influenza A infection *(E)*. *(B)* Bax KO causes the production of much smaller mitochondria, arranged end-to-end in a 'beads on a string' morphology. *(F)* Following Influenza A infection, the 'beads on a string' morphology of Bax KO cells is replaced with larger, ovoid mitochondria similar to those found in wild-type cells. *(C)* The mitochondria of mock-infected Bak KO cells are also robust and ovoid, similar to wild-type. *(G)* Following Influenza A infection, the mitochondria of Bak KO cells changes to small 'beads on a string' morphology similar to mock-infected Bax KO cells. *(D)* Mock-infected Bax/Bak DKO cells show robust, ovoid mitochondria similar to wild-type, morphology that is maintained following infection *(H)*.

**Figure 2.1 Flaviviruses establish a productive infection in renal epithelial cells.**

Dengue-2 and Modoc virus infected MDCK cells were fixed at 48hpi, stained with phalloidin-TRITC (red, actin) and antibody Di-4G2-15 (green, flavivirus Envelope protein). Flavivirus Envelope protein accumulation in cytoplasmic vesicles within the ER-rich perinuclear region of both Dengue-2 virus *(A)* Mock-infected cells lack positive staining for virus protein. Massive flavivirus Envelope protein accumulation in

cytoplasmic vesicles within the ER-rich perinuclear region of both Dengue-2 virus (**B**) and Modoc virus (**C**) -infected cells demonstrates the establishment of flavivirus translation and assembly. (**E**) RT-PCR analysis of extracellular viral RNA reveals that both Modoc and Dengue-2 viruses release newly synthesized virions from infected cells to relatively high titer by 96 hpi.

**Figure 2.2 Flavivirus does not kill epithelial cells or fibroblasts.** (**A**) MDCK renal epithelial cells infected with Modoc virus at various MOI show no significant death when analyzed by trypan blue exclusion, even very late following infection with high MOI. (**B**) No significant death is observed following infection with Modoc virus at MOI=10 in several other cell types as late as 144 hpi.

**Figure 2.3 Flavivirus infection confers resistance to death in renal epithelial cells.**

(**A**) MDCK cells infected at MOI=10 with either Modoc or Dengue-2 virus demonstrate a significant reduction in cell killing compared to mock when exposed to a variety of biochemically distinct death stimuli. Infection with either Dengue-2 or Modoc virus leads to protection against death induced by DNA damage (75 $\mu$ M CPT; 39%, 83% decline, respectively), kinase inhibition (20 $\mu$ M STS: 40%, 72% decline, respectively), protein synthesis inhibition (100mM CHX: 46%, 51% decline, respectively), and cytopathic viral infection (MOI=5 Influenza A: 31%, 36% decline, respectively).

**Figure 2.4 Flavivirus infection kills primary macrophages but protects species-matched fibroblasts.** (**A**) Both Dengue-2 and Modoc virus infection leads to cell killing in Swiss Webster primary macrophages with levels of death roughly equal that of CPT-treated cells (~25% above control). (**B**) No cell killing of Swiss Webster primary embryonic fibroblasts is observed after Dengue-2 or Modoc infection. Instead, these cells are

significantly protected against CPT-induced death by infection with either virus, yielding a 59% and 70% decline in CPT-induced death, respectively.

**Figure 2.5 Flavivirus-infected MDCK cells are protected from death, while non-infected**

**bystander cells are not.** (A) No staining for virus particles is seen in mock infected cells. (B,C) Caspase-3 activation is evident following CPT treatment in mock-infected cells. (D) Strong positive staining for the Dengue-2 envelope protein indicates virus production in a subset of cells following infection. (E) While caspase-3 activation is evident in infected cell populations following Dengue-2 infection, it is restricted to uninfected cells (F).

**Figure 2.6 Flavivirus infection leads to PI3K-dependent autophagy.** (A) Cytoplasmic

distribution of LC3-GFP observed in mock infected HeLa cells indicates a low level of autophagy typical of healthy cells. However, following infection of HeLa cells with either (B) Dengue-2 virus or (C) Modoc virus, LC3-GFP shifts to a punctate pattern, indicating upregulation of autophagy and accumulation of LC3-GFP at autophagosomal membranes. (D) Mock-infected MDCK cells transiently expressing LC3-GFP display little LC3-GFP punctation, indicating a low level of autophagy. Both (E) Dengue-2 and (F) Modoc virus-infected MDCK cells display LC3-GFP punctation, indicating upregulation of autophagy following infection. (G) Inhibition of PI3K by exposure to Wortmannin has no effect on LC3-GFP punctation in mock-infected cells, but abolishes LC3-GFP punctation induced by both (H) Dengue-2 virus and (I) Modoc virus infection, indicating that flavivirus-induced autophagy is PI3K-dependent. (J) Cells were scored for LC3-GFP punctation and expressed as the percent cells with punctate LC3-GFP. A limited amount of LC3-GFP punctation is seen in mock control cells, while infection by

either Dengue-2 or Modoc virus increases punctation dramatically (to ~70%), indicating flavivirus-induced upregulation of autophagy. PI3K inhibition by Wortmannin completely eliminates both Modoc and Dengue-2 virus-induced upregulation of autophagy. **(K)** Analysis of whole-proteins lysates of Dengue-2 infected cells at 0, 3, 6, 12, 24, 36 and 48hpi demonstrates a steady increase in LC3 cleavage to 36hpi, indicating Dengue-2 induced autophagy in infected cells. The decline in LC3 cleavage at 48hpi suggests complete LC3 turnover at late hpi. **(L)**  $\beta$ -tubulin was used as a loading control.

**Figure 2.7 Inhibition of autophagy abolishes flavivirus-induced protection against death.**

**(A)** Non-specific PI3K inhibition by Wortmannin eliminates protection by both Dengue-2 and Modoc virus in MDCK renal epithelial cells. **(B)** Inhibition of class III PI3K activity by 3-methyl adenine (3MA) also eliminates protection against death in MDCK cells, indicating class III PI3K involvement in flavivirus-induced protection. **(C)** Inhibition of autophagy by Beclin-1 knockdown eliminates protection by both Dengue-2 and Modoc virus against CPT-induced death. **(D)** Inhibition of autophagy by ATG5 knockout also eliminates protection by both Dengue-2 and Modoc virus.

**Figure 2.8 Stimulation of autophagy provides protection in epithelial cells regardless of**

**flavivirus infection.** **(A)** Autophagy upregulation by nutrient deficiency in MDCK renal epithelial cells provides protection against CPT-induced death with or without flavivirus infection. **(B)** Upregulation of autophagy signaling by mTOR inactivation using rapamycin treatment allowed no significant death by high levels of CPT with or without infection by either virus each, allowing only ~10% cell death by CPT.

**Figure 2.9 Inhibition of autophagy reduces extracellular flavivirus titer (A)** Plaque assay

analysis of Dengue-2 virus replication in MDCK renal epithelial cells with and without inhibitors of autophagy Wortmannin or 3-methyladenine (3MA) demonstrates a role for autophagy in Dengue-2 replication. Inhibition of autophagy by either Wortmannin or 3MA reduces viral titer by 27% at 96 hpi. **(B)** Plaque assay analysis of Modoc virus replication in MDCK cells with and without 3MA reveals a similar role for autophagy in Modoc replication. Inhibition of autophagy by 3MA leads to a 71% decline in Modoc virus replication in MDCK cells at 96hpi. **(C)** RT-PCR quantification of extracellular Modoc viral RNA at 96 hpi also demonstrates that autophagy inhibition by 3MA reduces replication. Note that CP value indicates number of rounds of replication to measurability, and thus lower initial levels result in higher CP values.

**Figure 2.10 Individual expression of flavivirus NS4A leads to protection similar to live**

**flavivirus infection.** **(A)** Infection with Dengue-2 virus and individual expression of Dengue-2 NS4A protect against CPT-induced cell killing. Expression of any of the ten Dengue-2 structural and non-structural genes does not lead to significant death in MDCK renal epithelial cells (each <10% cell death above control). Challenge by 75 $\mu$ M CPT leads to ~35% cell death above control in mock-infected MDCK cells, while live Dengue-2 pre-infection allows only ~15% cell death above control by CPT, a 57% decline. Individual expression of Dengue-2 NS4A is the only gene sufficient to induce protection against cell killing by CPT, yielding a ~45% decline in death when compared to mock-infected controls. NS2A and NS5 each allow slightly more death by CPT than mock-infected cells. Individual expression of empty plasmid vector, C, prM, E, NS1, NS2B, NS3 and NS4B have no effect on CPT-induced death and are similar to mock.

**(B)** Infection with live Modoc virus infection or individual expression of Modoc NS4A also protects cells against CPT-induced death. Expression of NS2A, NS4A or NS4B does not lead to significant cell killing in MDCK cells (<10%). Challenge by CPT kills ~40% of mock-infected cells, while live Modoc pre-infection protects against CPT-induced death, allowing only 10% cell death above control. Modoc NS4A expression leads to similar protection causing a 75% decline in cell death by CPT when compared to mock-infected controls. Modoc NS2A expression allowed 50% cell killing above control by CPT, slightly more than in mock-infected cells. Expression of the empty plasmid vector or Modoc NS4B had no effect on CPT-induced killing, each allowing 40% cell death by CPT. **(C)** NS4A expression from either Dengue-2 or Modoc virus leads to protection against multiple death stimuli. Control MDCK cells exhibit ~50% cell death when exposed to either a DNA damaging agent (75 $\mu$ M CPT) or a kinase inhibitor (20 $\mu$ M STS). However, expression of Dengue-2 NS4A or Modoc NS4A provided protection against both insults, causing a ~50% decline in death by CPT and a 30% decline in death by STS when compared to mock-infected controls, indicating that NS4A-induced protection against cell death is not pathway-specific. **(D)** western blotting using a specific antibody for cleaved LC3II reveals an increase in LC3II cleavage following both live Dengue-2 and Modoc infections, as well as after individual expression of NS4A from either virus. **(E)**  $\beta$ -tubulin was used as a loading control. Together, these results indicate that flavivirus NS4A expression is sufficient to induce both flavivirus-induced autophagy upregulation and flavivirus-induced protection against death. *(D,E completed by Aleksandra Wudzinska and Emmanuel Datan)*

**Figure 2.11 Flavivirus NS4A expression is sufficient to induce LC3 cleavage.** Whole-protein lysates were collected from MDCK cells transiently expressing Dengue-2 non-structural proteins NS1, NS2A, NS4A and NS4B at 24 hours post-transfection, as well as cells transfected with empty plasmid and cells exposed to the transfection agent as controls. All conditions stimulate a moderate amount of LC3 cleavage, likely owing to stress occurring during our transfection protocol as indicated by LC3 cleavage after exposure to the transfection agent alone. NS4A expression leads to a substantial increase in LC3 cleavage compared to controls and expression of other Dengue-2 non-structural genes, indicating NS4A expression alone is uniquely sufficient to upregulate autophagy in MDCK cells.

**Figure 2.12 Flavivirus NS4A-induced protection in epithelial cells is dependent upon autophagy signaling.** Mock-infected control MDCK cells experienced 50% cell death when exposed to either 125mM CPT or 20 $\mu$ M STS for 24 hours. Expression of either Dengue-2 or Modoc NS4A leads to protection against cell death by both CPT and STS, each leading to a ~30% decline in cell death following treatment with either death inducer, similar to live virus infection. Upregulation of autophagy signaling by rapamycin treatment conferred protection against death by CPT regardless of expression of NS4A from either virus, allowing less than 15% death in all conditions. Inhibition of autophagy signaling by 3-methyl adenine (3MA) treatment leads to the abolition of Dengue-2 and Modoc NS4A-induced protection, allowing ~50% death by CPT, similar to mock. Inhibition of autophagy signaling by Wortmannin treatment also eliminates protection against STS-induced cell death induced by NS4A from either virus, allowing ~50% cell death, similar to mock.

**Figure 2.13 Flavivirus infection induces ATM-dependent autophagy upregulation.**

(A) Cytoplasmic distribution of LC3-GFP observed in mock infected cells indicates an overall lack of autophagy upregulation in control cells. However, following infection with either (B) Modoc virus or (C) Dengue-2 virus, LC3-GFP shifts to a punctate pattern, indicating autophagy upregulation and accumulation of LC3-GFP at autophagosomal membranes. ATM inhibition by caffeine treatment has no effect on LC3-GFP localization in mock-infected cells (D), and eliminates LC3-GFP punctuation induced by both Modoc virus (E) and Dengue-2 virus (F), indicating that flavivirus-induced autophagy is ATM-dependent. (G) Cells were scored for LC3-GFP punctuation and expressed as the percent cells with punctate LC3-GFP. A limited amount of LC3-GFP punctuation is seen in mock control cells (~12%), presumably a result of normal housekeeping activity. Infection by either Dengue-2 or Modoc virus increases punctuation dramatically (~68%), indicating flavivirus-induced autophagy upregulation. ATM inhibition by caffeine completely abolishes both Modoc and Dengue-2 virus-induced autophagy upregulation, yielding levels of punctuation similar to that of mock control cells (~12%).

**Figure 2.14 Flavivirus-induced protection is ATM-dependent.** ATM inhibition by caffeine

eliminates protection induced by both Dengue-2 and Modoc virus in MDCK renal epithelial cells. CPT-induced death in mock-infected cells reached ~50% above control, while Dengue-2 and Modoc virus infected cells were resistant to cell killing by CPT (18% and 35% above control, respectively). ATM inhibition eliminated the protection conferred by infection with either virus, allowing CPT-induced death to reach levels similar to mock CPT (~50% above control).

**Figure 2.15 Flavivirus NS4A-induced protection is ATM dependent.** Mock-infected control

MDCK cells experienced ~45% cell death when exposed to either 75 $\mu$ M CPT or 20 $\mu$ M STS for 24 hours. Expression of either Dengue-2 or Modoc NS4A leads to protection against cell death by both CPT and STS, each causing a ~43% decline in cell death following treatment with either death inducer. Inhibition of ATM by caffeine treatment eliminates NS4A-induced protection, allowing CPT-induced death to reach levels similar to mock-infected cells.

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