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Role of autophagy-related proteins and cellular microRNAs in chikungunya and dengue virus infection

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CHAPTER 1

General introduction and scope of the thesis

Introduction

1) Epidemiology and clinical characteristics of dengue and chikungunya virus infections

Viruses transmitted to humans by mosquitoes of the genus *Aedes sp*. are one of the most important causes of febrile illness worldwide, especially in the developing world. Dengue virus (DENV) is the most common arthropod-borne virus, a virus which belongs to the *Flavivirus* genus of the *Flaviviridae* virus family. DENV is antigenically divided into four serotypes (DENV-1 to DENV-4) and all serotypes cause human disease and are widespread around the globe (1). DENV has a global estimate of 390 million infections annually, mainly in tropical and sub-tropical regions in Asia and the Americas (2). DENV infections, however, also occur in Africa and the Eastern Mediterranean, and recently autochthonous dengue cases have been reported in different areas of Europe and the United States (3,4). It has been estimated that in 96 million cases, infection leads to a clinically apparent disease, and 500,000 to 1 million individuals develop severe dengue (2). The disease is usually characterized by an acute high temperature (≥38.5°C), vomiting, nausea, aches, macular transitory rash and sometimes mild haemorrhagic manifestations (5). In some cases, the disease develops to a more severe form, particularly amongst children experiencing a heterotypic secondary infection (6,7). Severe dengue involves marked plasma leakage and severe haemorrhagic manifestations, which may lead to organ impairment and hypovolemic shock (5,8–10). In 20,000 cases per year dengue is fatal. Chikungunya virus (CHIKV) is a member of the *Alphavirus* genus of the *Togaviridae* virus family, and the causative agent of mosquito-borne chikungunya fever. The virus was first isolated in 1952, during an outbreak in southern Tanzania of a dengue-like illness accompanied by severe arthralgia (11). CHIKV became endemic in many countries in Africa and Asia (12). Following a large outbreak in 2005 in La Reunion island, the virus spread more globally leading to recurrent outbreaks in the Americas and the Caribbean, and also in more temperate locations in southern Europe among others (13). For CHIKV, three distinct genotypes are defined: West African, East Central South African (ECSA), and Asian (14). The current outbreaks in many tropical and sub-tropical areas of Africa, Asia, Europe, the Pacific archipelago, and the Americas involve either the ECSA genotype, the Asian genotype, or both (15). From 2014 to 2016, it was estimated that 2 million people have been infected with CHIKV (16). CHIKV infection is characterized by a high symptomatic attack rate, especially in areas with no prior history of infection, where 50% - 97% of individuals develop a clinically apparent disease and 35-52% of these individuals develop more severe chronic manifestations that can last from months to years (17–19). Chikungunya fever is characterized by an acute fever, joint and muscle pain, headache, nausea, fatigue and rash. The joint pain is often more debilitating than that of dengue fever, and it can persist for several months or even years after the infection has been resolved (20,21).

2) Dengue virus and Chikungunya virus pathogenesis

Upon a mosquito-bite, virus particles are released directly into the bloodstream and the layers of the skin tissue, resulting in infection of different types of cells. In the case of DENV, immature Langerhans cells, epidermal dendritic cells and keratinocytes have been reported to be the first cells to become infected (22). During primary infection, attachment of DENV particles to the cell surface is mediated by various receptors, depending on the cell type. DC-SIGN and the mannose receptor are fundamental for the infection of dendritic cells and macrophages, respectively (23). Other attachment factors include heparan sulfate, lectins, heat shock proteins, the laminin receptor and the CD14-associated protein (24). The replicative cycle of DENV is described in detail in **Chapter 2**. After the first rounds of replication, DENV-infected Langerhans cells migrate from the site of infection to the lymph nodes, where macrophages and monocytes become infected and amplify the viral load (22). DENV is then disseminated through the lymphatic system ultimately leading to the infection of several organs and tissues, including the spleen, liver and bone marrow (25,26). Infection is typically resolved within 1 week after the onset of the fever, predominantly due to the action of the innate immune system (27). Both type I and II interferons (IFN-I and IFN-II) have been described as critical cytokines in the control of DENV infection (28). DENV-specific B and T cells, which are only generated after approximately 6 days post-infection, help to fully control viral replication (29). The immune response triggered provides life-long protection against re-infection with the same serotype and several months of cross-protection against infection with the other serotypes (30). Re-infection with another serotype after prolonged time has been associated with severe disease development as a consequence of original antigenic sin of B and T cells, and a phenomenon referred to as antibody-dependent enhancement of infection (31). Third and fourth DENV infections are typically already controlled early in infection and as such do not lead to clinical apparent disease (32).

During CHIKV infection, the first cells that are infected are presumed to be fibroblasts, keratinocytes, melanocytes and monocytes (33). Although the identity of the cellular factors involved in CHIKV entry remains unknown, various proteins have been proposed as candidate receptors. For example, prohibitin, phosphatidylserine-mediated virus entry-enhancing receptors, and glycosaminoglycans have been suggested as potential CHIKV entry factors in mammalian cells (34). Recently, the matrix-remodelling associated 8 protein was also reported to function as a receptor for multiple arthritogenic alphaviruses, including CHIKV (35). The downstream steps involved in the replicative cycle of CHIKV are described in detail in **Chapter 2**. CHIKV initially replicates in dermal fibroblasts, migrating monocytes/macrophages and endothelial cells (34). Subsequently, the virus is transported to secondary lymphoid organs, where it infects and replicates in migratory cells leading to high viremia. Subsequently, CHIKV disseminates to the joints, skeletal muscles, heart, kidney, liver, and more rarely, the brain (36). In these tissues, the infection is associated with a marked infiltration of mononuclear cells. After virus dissemination, fibroblasts remain the main target cells, followed by macrophages, especially in tissue sanctuaries such as the synovial membrane that surrounds specific joints (37). The acute phase of infection is characterised by high virus titers in blood that trigger a robust systemic activation of

monocyte-driven innate immune response, principally involving the production of IFN-I as well as many pro-inflammatory cytokines and chemokines (38). This is followed by the engagement of the adaptive immunity through activation and proliferation of CD8-positive and CD4-positive T cells in the late stages of the acute disease (39,40). After the first week of infection, CHIKV-specific antibodies produced by B cells are readily detected, which results in the control of the infection and life-lasting immunity (41). Chronic CHIKV disease is characterised by aberrant activation of the immune system, which features elevated levels of pro-inflammatory cytokines and activated CD8-positive and NK cells (42,43). Although the reasons behind this immune activation are still not well defined, one of the most studied hypothesis is based on the presence of CHIKV antigens in joint and muscle samples several months after an acute infection (37,44,45).

3) Vaccines and treatment options for infections caused by dengue and chikungunya virus

Despite the high viral burden, there are no licensed antivirals available to treat the diseases caused by DENV and CHIKV. However, there are a vast number of therapeutic strategies under development. The goal of these strategies is to lower the viral load with the aim to alleviate the disease symptoms. For DENV, different antiviral compounds, including antibodies, entry inhibitors, non-specific molecules or drugs targeting specific viral proteins have been described (46). Only a small number of antivirals have been developed further, mainly due to limited efficacy towards all serotypes or to adverse effects in animal models (47). To date, only few compounds have been tested in human clinical trials and thus no positive effect has so far reported. For example, the nucleoside-analogue balapiravir, which inhibited DENV replication *in vitro* and was proven to be safe in humans, did not lead to significant changes in viral load in DENV-infected patients (48). Similarly, other drugs such as lovastatine, prednisolone, celgosivir and cloroquine, had no efficacy against DENV infection when assessed in clinical trials (49). For CHIKV, the studied approaches have been based on the use of antibodies, antimicrobial compounds and traditional antivirals like ribavirin, IFN-α, and niclosamide among many others (50). Despite the existence of all these potential antiviral candidates, only chloroquine has been evaluated in clinical studies, yet, there is a lack of consistency in the data gathered so far about the benefits for patients receiving this drug (51,52). Interestingly, drugs targeting host cellular pathways and proteins important for viral replication have also been developed, including an inhibitor of protein kinase C and mitogen-activated protein kinase signalling for CHIKV, and molecules that interfere with fatty acids metabolism in the case of DENV (53–55). These drugs, however, have not been studied in animal models and in the clinic yet (53–55).

Despite the urgent need of specific antivirals, research was more centred around the development of a safe and efficacious vaccine in the last decades. In 2015, a tetravalent DENV vaccine based on a live chimeric yellow fever/dengue virus (Dengvaxia) was licensed in several countries, to be administered exclusively to ≥9 year-olds in regions with high endemicity (56). As a consequence of mass immunization programs, however, the vaccine was shown to have potential adverse effects, as there is a higher risk of developing severe disease in vaccinated seronegative individuals (57–59). Therefore,

the World Health Organization currently advises to use Dengvaxia only in people with pre-existing DENV immunity (60). Other vaccine candidates are currently in phase I and phase II clinical trials (61). In the case of CHIKV, a vaccine candidate was evaluated in phase I and phase II clinical trials in 1998 with promising results, but the research on this vaccine was stopped and a licenced product remains unavailable (62). Due to the recent re-emergence of CHIKV, however, research on vaccine development has been intensified. Current vaccine candidates for CHIKV include virus-like particles, subunit vaccines, vectored/chimeric vaccines, nucleic acid vaccines, and live attenuated vaccines, which are in different stages of development (50,63). A live attenuated virus vaccine (VLA1553) is in the recruitment stage of a phase I clinical trial and two other vaccines based on virus-like particles and a measles virus-based chimera have successfully completed phase II trials (64–66).

4) Cellular host factors and pathways involved in dengue and chikungunya virus infections

Infected cells try to cope with the virus by activating an array of signalling pathways to maintain/restore cellular homeostasis and to control/inhibit viral replication. These pathways can be activated directly due to the presence of a viral genome and proteins, or can be driven indirectly by other cellular processes that are activated during viral infection. Viruses, on the other hand, have evolved strategies to hijack/manipulate cellular pathways to their benefit. It is, however, beyond the scope of this introduction to describe the numerous pathways that aid in controlling DENV and CHIKV replication. Instead, we will focus on those pathways studied in the subsequent chapters of this thesis. In the following sections, we will therefore introduce the main molecular aspects regarding the post-transcriptional regulation of gene expression by microRNAs (miRNAs) and the autophagy pathway; and describe their role in viral infection. Furthermore, due to the links that have been described between autophagy and other cellular pathways, the unfolded protein response, mitochondrial-function and cell death will also be addressed.

A. Post-transcriptional regulation of gene expression by microRNAs

RNA interference (RNAi) pathways comprise diverse biological mechanisms of small RNA-mediated gene regulation and genome defence, which are conserved across eukaryotes, from yeast to humans (67). Although the regulatory function of small RNAs was recognized in the early 90s in plants, fungi and nematodes (68–70), it was not until 1998 when an explanation for these phenomena was described in *Caenorhabditis elegans* and the term RNAi was coined by Fire and Mello (71). RNAi was quickly identified to occur in other animals like insects and mammals, and the pathways and proteins associated to this silencing mechanism were proficiently described in the following decade (72–74). As the name indicates, RNAi is mediated by the interaction of non-coding RNA molecules with target mRNA transcripts, to induce post-transcriptional gene silencing (75). RNAi has been described as a natural mechanism to control vital processes, such as cell growth, tissue differentiation, heterochromatin formation, cell proliferation, cell death and metabolic control (76). Therefore, it is not

surprising that RNAi dysfunction is linked to multiple infectious diseases, neurological disorders, and many types of cancer (77–80).
In most animals, three types of small RNA molecules are recognized to be central to the RNAi pathways: piwi-interacting RNAs (piRNA), small interfering RNAs (siRNAs) and miRNAs (67). Within those, only miRNAs and piRNAs are genome-derived; whereas siRNAs are usually from exogenous origin, although some exceptions exist (74,81). The piRNAs participate in repression of transposons and germ line genome integrity, although the post-transcriptional and transcriptional processes involved are still poorly described (82). In contrast, the miRNA-based pathways control endogenous gene expression (83,84). In humans, it has been estimated that at least ~6,000 genome sequences encode for miRNAs and these are thought to control most mammalian protein-coding genes (85,86).

miRNA biogenesis and modes of action

miRNA expression is initiated by RNA Pol II in the nucleus, which transcribes ~1000 nucleotides long primary miRNAs (pri-miRNAs) (Fig. 1). The pri-miRNA folds into a double-stranded RNA structure lacking full complementarity, which results in a stem loop that bears single or clustered hairpins and terminal 5′ and 3′ overhangs (77). Pri-miRNAs are subsequently trimmed by the RNase III enzyme Drosha to generate precursor miRNAs (pre-miRNAs, Fig. 1), which are subsequently exported into the cytoplasm (87). These pre-miRNAs are then processed by the endoribonuclease-containing protein Dicer (Fig. 1), and gives rise to a 21-25 nucleotides RNA duplex that are further loaded onto an Argonaute (AGO) protein to form the pre-miRNA silencing complex (pre-RISC) (88). Following miRNA duplex loading, AGO quickly removes one of the strands of the miRNA duplex (Fig. 1), forming the mature RISC. The selected miRNA strand subsequently guides the selection of an mRNA target on the basis of base complementarity (89). Nucleotides 2-6 of the guide strand constitute the 'seed sequence', which complementarity with the target is critical in determining the silencing efficacy (77). Once the mRNA target is loaded in the mature RISC, several mechanisms have been described to participate in the gene silencing step (Fig. 1). Although the translation of mRNAs targeted by RISC can be blocked, the most common miRNA-silencing mechanism is associated with mRNA decay (89). In the latter case, mRNA degradation occurs as a consequence of decapping and of 5′to 3′ decay (89). While mRNA endonucleolytic cleavage is common in plants, this phenomenon is rare in animals (89). In mammals, miRNAs are usually regarded as 'fine-tuners' of gene expression, leading to subtle but important changes in the cell proteome at a given time (90).

The role of miRNAs in viral infections

In 2005, it was published for the first time that a mammalian miRNA, miR-32, had antiviral properties against the retrovirus primate foamy virus type 1 in human cells (91). This finding, set the path for a series of publications supporting the notion that mammalian miRNAs might directly target the genome of RNA viruses (91–96). In parallel, it was also discovered that miRNAs are involved in the regulation of cellular

responses such as innate immunity, apoptosis and the oxidative stress response (97). For example, IFN-induced miRNAs, such as miR-155 and miR-29b, suppress vesicular stomatitis virus and Japanese encephalitis virus (98–101). In contrast, IFN-induced miR-146a, down-regulates the IFN response, thereby enhancing the replication of hepatitis C virus, CHIKV, and DENV (102–104). More specifically, miR-146a favours DENV replication by targeting TRAF6, thereby decreasing IFN-β response in the monocytic cell line THP-1 and in human primary monocytes (104). CHIKV exploits miR-146a in a similar fashion in human synovial fibroblasts (102). In contrast, induction of miR-30e* in DENV-infected HeLa, U-937 cells and human peripheral blood mononuclear cells hyperactivate nuclear factor-κB (NF-κB), which in turn leads to the production of IFN-β thereby suppressing DENV replication (105). Another investigation revealed that overexpression of miRNA let-7c inhibits DENV replication in Human hepatoma cells (Huh7) and in the macrophage-monocytic cell line U-937-DC-SIGN (106). This miRNA contributes to the oxidative stress response in DENV-infected cells (106). The above studies therefore clearly show that miRNAs have pro and antiviral functions, depending on the role of the 'target protein' in infection and pathogenesis.

B. Autophagy

Autophagy, from the Greek words for 'self' and 'eating', refers to a set of pathways that converge in the degradation of cytoplasmic components, from aggregate-prone proteins to organelles like mitochondria (107). Different types of autophagy are currently recognized (108,109), the main type, i.e. macroautophagy, involves the generation of double-membrane vesicles or autophagosomes, which sequester cytoplasmic material before its delivering to the lysosome (110). The term autophagy was initially coined by Christian de Duve while characterizing the existence of double-membrane vesicles containing cytoplasmic content in various states of degradation (111,112), which is currently known as macroautophagy. The molecular machinery of macroautophagy was later characterized by Yoshinori Ohsumi, who received the Nobel prize for 'Medicine or Physiology', for his contribution to the identification of the autophagy-related (*ATG*) genes (113). Macroautophagy contributes to the elimination of cytotoxic protein aggregates, limits microbial proliferation and promotes cell survival during periods of stress, including nutrient deprivation. The mechanism and regulation of autophagy, and how alphaviruses and flaviviruses interact/interfere with ATG machinery is described in detail in **Chapter 2**.

C. Mitochondria

Mitochondria are organelles that play central roles in ATP production, regulation of cellular metabolism, proliferation, apoptosis, stress response, calcium signalling, ROS signalling, synthesis of phospholipids and others (114–116). In mammals, mitochondria have a highly conserved small, closed, circular, dsDNA genome of approximately 16,6 kb in length. (117). Mitochondrial DNA (mtDNA) contains 37 genes coding for two rRNAs, 22 tRNAs and 13 proteins (118). Protein translation of mitochondrial genes is mediated by mitochondrial ribosomes located in the matrix of the organelle (117). The proteins required for transcription and translation of mtDNA are encoded in the nucleus

and imported to mitochondria (119,120). Mitochondria have an outer and inner membrane with an intermembrane space between them. The proteins encoded by mtDNA make part of the mitochondrial respiratory complexes I to IV, and function together with the citric acid cycle in maintaining an electrochemical gradient in the mitochondrial intermembrane space (121). The electric potential is important for multiple of mitochondrial functions, especially ATP synthesis, and changes herein can eventually dictate cell survival (118).

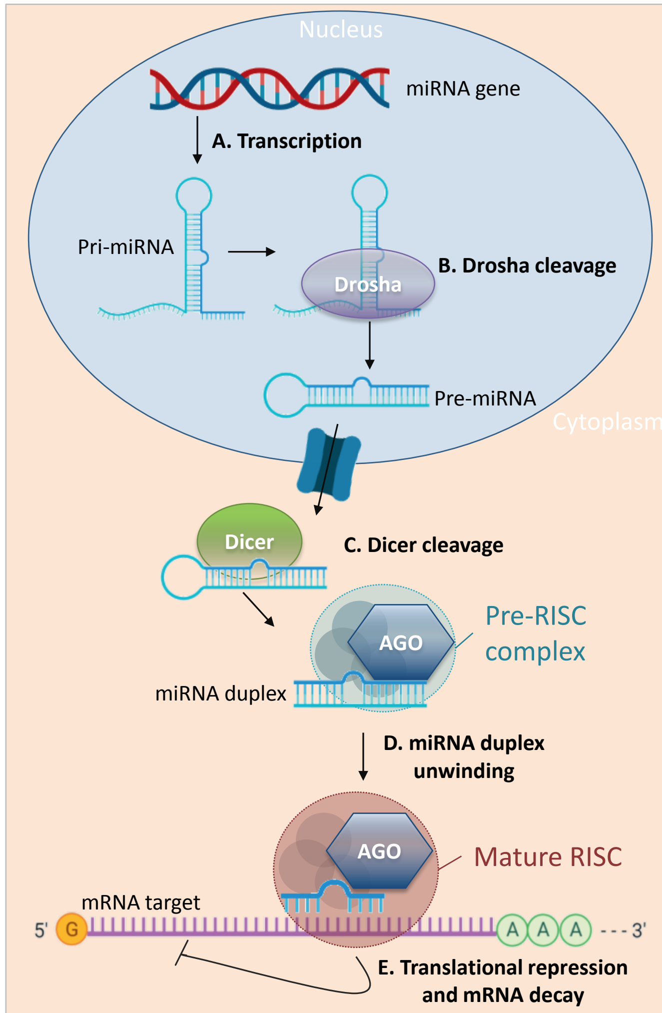

Figure 1. miRNA biogenesis in mammals. Pri-miRNAs are transcribed from miRNA-encoding genes and processed by the endonuclease Drosha to generate pre-miRNAs. Dicer cleavage generates a miRNA duplex that is recognized and loaded into an AGO protein to form a pre-RISC complex. AGO mediates miRNA duplex unwinding and strand selection forming the mature RISC in which the target mRNA will be recognized by base-complementarity and loaded. This will lead to the repression of translation or mRNA decay.

Mitochondria constitute a highly dynamic network and their function is tightly linked to their external structure and distribution within cells. Proper mitochondrial morphology, function and location are regulated by mitochondrial fission, fusion and clearance via mitophagy. Mitochondrial fission involves the fragmentation of mitochondria into single organelles and is enhanced during apoptosis, cell division and hypoxia (122). Fusion of mitochondria, on the other hand, is decreased by all these

events, high loads of mtDNA mutations, and loss of membrane potential (118,123). Fusion promotes complementation between damaged mitochondria, by allowing the exchange of mtDNA, proteins and lipids thereby maximizing the oxidative capacity in response to toxic stress (124). Lastly, mitophagy entails the clearance of mitochondria through the autophagy pathway. Mitophagy is activated by oxidative damage of mitochondrial lipids, mtDNA or proteins; in order to eliminate defective mitochondria (125). Mitophagy is assisted by diverse mitochondrial proteins, including PTEN-induced putative kinase 1, Parkin ubiquitin ligase, BCL2 interacting protein 3 (BNIP3) and NIX (125). These proteins, however, also participate in other mitochondrial processes. BNIP3, for example, has been found to promote mitochondrial fragmentation and apoptosis (126).

The role of mitochondria in dengue and alphavirus virus infection

Mitochondria play integral roles in the control of viral replication, mainly due to their participation in immune signalling, metabolism and cell death; which are major determinants of viral replication and pathogenesis (127). For example, RIG-I-like receptors (RLR), which recognize viral RNAs, signal through the downstream adaptor mitochondrial antiviral signalling protein (MAVS) to induce NF-κB, IFN regulatory factor 3 (IRF3) and IRF7 (128). Consequently, MAVS-mediated activation of these transcription factors induces the production of pro-inflammatory cytokines and IFN-I leading to a cellular antiviral state (129,130). Frequently, these immunomodulatory functions are regulated by mitochondria dynamics and morphology. MAVS signalling, for instance, is facilitated by mitochondrial fusion (130). Likewise, mitochondrial fusion supports the assembly of the inflammasome, a multi-protein complex that leads to the production of pro-inflammatory cytokines during RNA virus infection (131). Interestingly, several viruses have evolved strategies to manipulate these cellular responses. For example, DENV infection has been shown to supress mitochondrial fusion thereby interfering with MAVS-mediated signalling and restricting the RIG-I-dependent IFN response (132–134). Furthermore, DENV infection of the hepatoma cell line HepG2 has also been associated with alteration in the bio-energetic function of mitochondrial morphology, leading to the loss of the mitochondrial membrane potential (135). In some instances, the mitochondrial abnormalities associated with viral infection can lead to apoptosis. In the case of alphaviruses, for example, infection of cells with Semliki Forest virus (SFV) not only induces MAVS, thereby eliciting an IFN antiviral response, but also leads to cell death by recruiting CASP8 (136). Furthermore, infections with Sindbis virus (SINV) and Venezuelan equine encephalitis virus (VEEV), two other alphaviruses, induce an anomalous perinuclear distribution of mitochondria (137,138). For VEEV, this is associated with a reduction in mitochondrial activity and increased fission and mitophagy, thereby contributing to apoptosis (138). When viral infection leads to cell death, it can have both beneficial and detrimental roles for virus proliferation, as will be discussed below.

D. The unfolded protein response

In eukaryotes, folding and glycosylation of secretory and membrane-associated proteins principally occurs in the ER (139). When the influx of nascent, unfolded polypeptides exceeds the processing capacity of the ER, the Unfolded Protein Response (UPR) is activated as a stress response to return the ER to its normal physiological state (140). UPR signalling in metazoans starts with the activation of ER-resident transmembrane proteins that operate as sensors in the ER lumen and respond to the accumulation of unfolded and misfolded proteins (141). The ultimate function of the UPR is to mitigate the stress; by delaying protein synthesis, stimulating ER-associated protein degradation (ERAD), and increasing chaperone transcription to up-regulate the ER folding capacity (142). Prolonged UPR induction can, however, also lead to the stimulation of other stress responses such as apoptosis or autophagy (143).

The activation of the UPR is tightly regulated by the immunoglobulin heavy-chain binding protein (BIP), also known as GRP78 and HSP5A, which is an ER-resident chaperone bound to the luminal domain of three different UPR receptors (Fig. 2) (144). When unfolded proteins accumulate in the lumen of the ER, BIP specifically binds to exposed hydrophobic regions of the nascent polypeptides, thereby uncoupling itself from the UPR receptors (145). This disassociation triggers activation of one or more UPR branches, depending on the type and source of ER-stress (146). One branch of the UPR is mediated by inositol requiring enzyme 1 (IRE1), a kinase/endoribonuclease that is activated by dimerization of its kinase luminal domains that is followed by autophosphorylation (Fig. 2) (147). Activated IRE1 subsequently mediates cytoplasmic splicing of the mRNA encoding for the x-box binding protein 1 transcription factor (*Xbp-1*) (148). The spliced, and therefore active version of *Xbp-1*, activates the transcription of genes encoding for ER chaperones and ERAD components, contributing to the mitigation of the protein load in the ER (149). Another branch of the UPR is initiated by double-stranded RNA-activated protein kinase (PKR)–like ER kinase (PERK) phosphorylation in a similar fashion as for IRE1 (Fig. 2) (145). Subsequently, PERK phosphorylates the eukaryotic translation initiation factor 2 subunit α (eIF2α), thereby stalling protein synthesis and counteracting ER protein overload (150). Furthermore, eIF2α phosphorylation facilitates the selective translation of activating transcription factor 4 (ATF4), which induces the expression of ATG genes and C/EBP homologous protein (CHOP), leading to activation of autophagy and apoptotic signalling respectively (151). The third UPR pathway is initiated by the activating transcription factor 6 (ATF6), through BIP-mediated activation, in a way that remains unclear (Fig. 2) (142). In response to ER stress conditions, transmembrane ATF6 is transported to the Golgi complex, where it undergoes a series of proteolytic cleavages that result in the cytoplasmic release of its N-terminal domain (142). Cytosolic ATF6 is then translocated into the nucleus where it mediates transcription of multiple UPR target genes, including chaperones, CHOP, and of the transcription factor XBP1 (141). ATF6 also induces the transcription of proteins involved in lipid biosynthesis, ultimately leading to expansion of the ER volume necessary to accommodate the extra enzymes produced by the UPR (152).

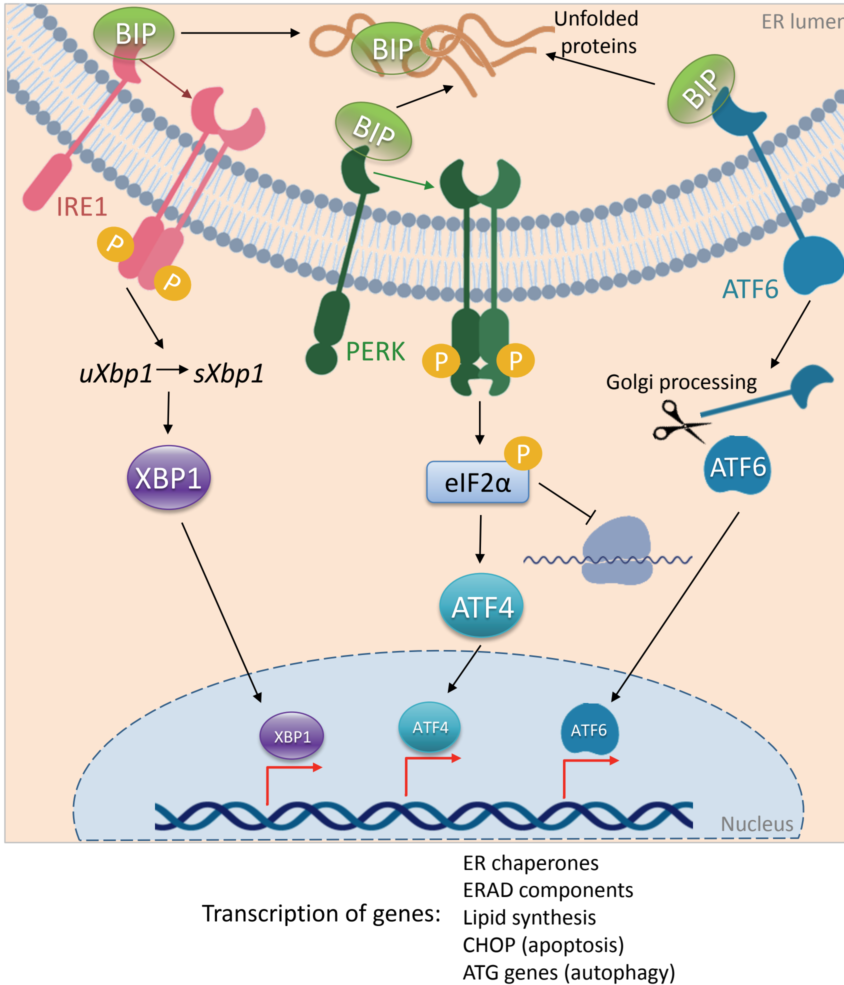

Figure 2. Unfolded protein response. Uncoupling of the ER-resident chaperone BIP from the luminal UPR receptors IRE1, PERK and ATF6, initiates the UPR. IRE1 activations reuslts in *Xbp1* spicing and its activation. PERK activation leads to eIF2α phosphorylation, which arrests general translation and favours unconventional translation of ATF4. The ATF6 branch of the UPR starts with the processing of ATF6 in the Golgi, which is required for its activation. XBP1, ATF4 and ATF6 transcription factors are translocated into the nucleus to induce the transcription of multiple sets of genes that counteract ER stress, or lead to autophagy and/or apoptosis stimulation.

The role of the unfolded protein response in dengue and chikungunya virus infection

Viral infections are often associated with the induction of the UPR pathways, mainly because viral protein translation and replication cause a significant increase in ER stress. In specific instances, UPR activation is favourable for viral replication by either inducing expansion of the ER or increasing ER-resident chaperones. Flaviviruses like DENV, WNV, Japanese encephalitis virus (JEV), Tick-borne encephalitis virus, and Usutu virus, activate multiple branches of the UPR (153–156). DENV infection was found to induce PERK activation and eIF2α phosphorylation early in infection, whereas IRE1-XBP1 and

ATF6 are activated in the later stages of the replication cycle (157). Activation of the PERK- eIF2α branch of the UPR has been associated with CHOP expression and induction of apoptosis (158). *Xbp1* splicing after infection with DENV and also JEV, depends on the expression of the viral protein NS2B/3 (153). WNV, on the other hand, preferentially activates the ATF6 pathway, and it has been demonstrated that NS4A and NS4B increase *Xbp1* transcript levels (154). Furthermore, DENV and WNV are known to increase BIP protein expression (145). For alphaviruses, it has been shown that the expression of the glycoproteins of SFV and CHIKV activate the UPR (159,160). CHIKV infection leads to eIF2α phosphorylation and *Xbp1* splicing, although the viral protein nsP2 prevents an effective UPR response by inhibiting translocation of the XBP1 protein to the nucleus and the expression of ATF4 and other UPR targeted genes (159). In addition, CHIKV-induced activation of the IRE1-XBP1 pathway, has been associated with autophagy initiation (161). In sum, both flaviviruses and alphaviruses cause extensive ER stress, thereby activating different UPR pathways, however, diverse strategies are employed by these viruses to control and benefit from this cellular response.

E. Cell death

Cell death occurs through numerous mechanisms, which include either coordinated cellular death programs, i.e., regulated cell death (RCD), or uncontrollable processes due to extreme environmental conditions, i.e., accidental cell death (162,163). RCD involves defined signalling cascades and effector mechanisms, and includes pathways such as necroptosis, pyropotosis, ferroptosis and apoptosis which is the best studied RCD pathway (163). Apoptosis consists of two main pathways: intrinsic apoptosis and extrinsic apoptosis (Fig. 3). Extrinsic apoptosis is mediated by environmental cues that activate cell surface death receptors, such as apoptosis antigen 1 (APO1, also known as FAS) and tumor necrosis factor receptor (TNFR) (Fig. 3) (162). These environmental stimuli drive the activation of initiator caspases, CASP8 and CASP10 (162). Intrinsic apoptosis, on the other hand, is triggered by intracellular cues like DNA damage, which activate BH3-only proteins and induce mitochondrial-outer membrane permeabilization (MOMP) (Fig. 3) (164). BH3-only proteins, such as BCL2 associated agonist of cell death (BAD), BH3 interacting-domain death agonist (BID), Bcl-2-like protein 11 (BIM), and the aforementioned BNIP3 and NIX; interact with anti-apoptotic B-cell lymphoma 2 (BCL-2) and B-cell lymphoma-extra-large (BCL-X_L), thereby leading to the oligomerization of BCL-2-associated X protein (BAX) and/or BCL-2 antagonist or killer (BAK), which form channels in the outer mitochondrial membrane (Fig. 3) (165). Thereafter, mitochondrial soluble proteins such as cytochrome C and EndoG are released into the cytoplasm activating CASP9 (166). At this stage, intrinsic and extrinsic apoptosis converge by activating CASP3, CASP6 and CASP7, which are considered as the ultimate cell death effector molecules, as they cleave the substrates responsible for the morphological characteristics associated with apoptosis (Fig. 3) (167,168).

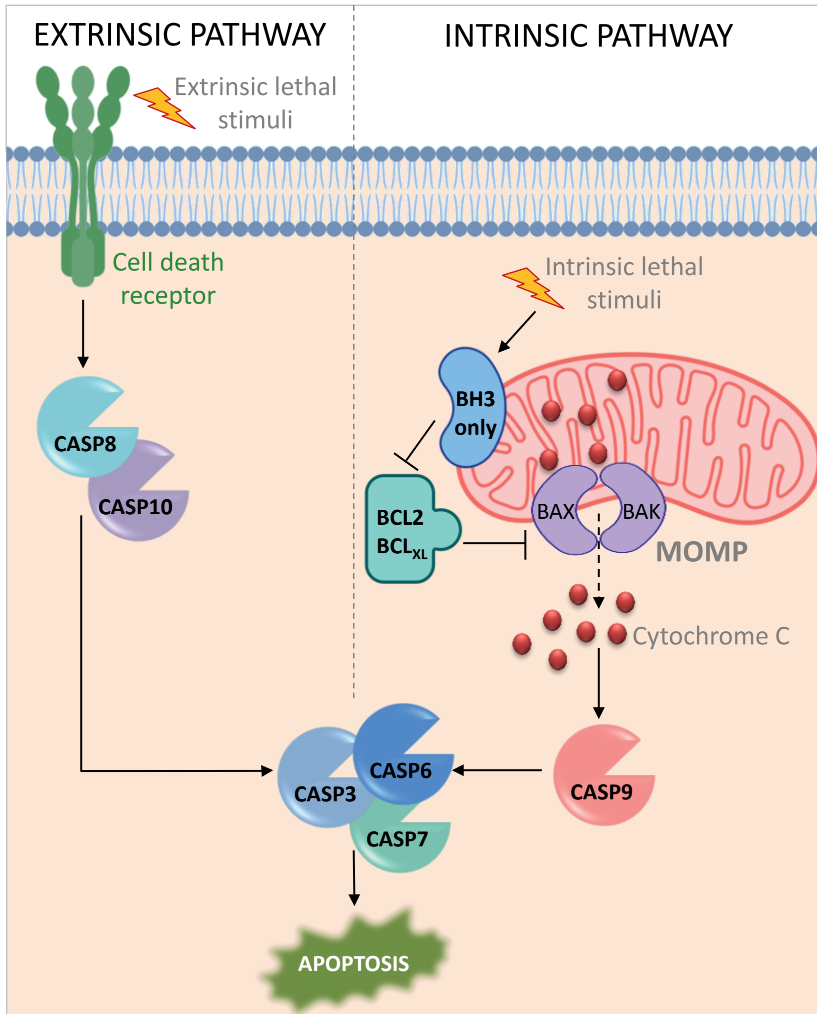

Figure 3. Apoptosis signalling pathways. Extrinsic apoptosis is initiated by external stimuli that activate cell death receptors on the extracellular surface of the plasma membrane, which activate the initiator caspases, CASP8 and CASP10. Intrinsic apoptosis is initiated by intracellular cues that activate BH3-only proteins, which interact with anti-apoptotic BCL-2 or BCL_{XL} leading to the oligomerization of BAX/BAK to form pores in the mitochondrial outer membrane. This drives MOMP and cytochrome C release, which downstream activates CASP9. Extrinsic and intrinsic apoptosis converge in the activation of executioner caspases, such as CASP3, CASP6 and CASP7.

Interplay between autophagy and apoptosis

It is generally assumed that autophagy and apoptosis are mutually exclusive pathways, however, different lines of evidence suggest that although autophagy induction prevents cell death and vice versa, these pathways can also occur at the same time or trigger one another. For example, selective autophagy of mitochondria in which MOMP causes loss of the inner mitochondrial transmembrane potential, reduces the likelihood of cells to undergo apoptosis (169,170). Similarly, activation of effector caspases during

apoptosis leads to cleavage of cellular proteins, including those related to autophagy, thus inhibiting the autophagic response (171). On the other hand, autophagy-mediated degradation of iron storages triggers a specific type of RCD known as ferroptosis (172,173). Autophagy can be followed by activation of cell death, as both pathways are under the control of common upstream signals (174). Among those, BH3-only proteins are able to promote autophagy via the same interactions required to induce intrinsic apoptosis, e.g., via association with BCL-2 or BCL-X_L (175). Through this mechanism, several BH3-only proteins, including BAD, BID, BNIP3, NIX, Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) and p53 upregulated modulator of apoptosis (PUMA), mediate the dissociation of BCL-2 from for the ATG protein BECLIN1 thereby activating autophagy (175). Moreover, BNIP3 and NIX can stimulate the selective removal of damaged mitochondria via mitophagy, by interacting with the microtubule-associated protein 1A/1B-light chain 3 (LC3) pool, which is located in the interior of autophagosomes (176,177). Altogether, these studies underline the importance of autophagy and apoptosis in the cellular response to stress, and show the vast interplay between these two pathways.

The role of regulated cell death pathways in dengue and chikungunya virus infection

Virus infections often trigger cell death-associated pathways, yet, viruses have evolved mechanisms to interfere with these pathways to ensure their chance to produce viral progeny and dissemination (178). Indeed, several mammalian viruses were found to induce pro-survival mechanisms whereas other mammalian viruses were shown to trigger pro-apoptotic cell programs to promote virus progeny (179,180). DENV has been shown to induce extrinsic and intrinsic apoptosis in primary cells and laboratory cell lines (181–183). During active DENV replication in human cells, increased levels of pro-inflammatory cytokines such as TNF-α, interleukin-10 and TNF-related apoptosis-inducing ligand (TRAIL) are detected; which in turn activate TNFR and FAS receptors thereby triggering extrinsic cell death (183,184). Intrinsic apoptosis in DENV-infected cells is mediated by ER stress or by high production of mitochondrial reactive oxygen species (ROS) generated by viral replication and translation (185–187). However, it has also been shown that ER-stress signalling during DENV infection leads to cell protection mechanisms (188). DENV also increases cellular respiration and causes a decrease in membrane potential, leading to a decrease in cellular ATP content, which usually precedes cell death (135). Overall, although DENV induces cell death, infection also results in the activation of other cellular pathways that counteract apoptosis and promote cell survival, ensuring successful infection. For alphaviruses, both cellular and viral mechanisms are involved in the induction of apoptosis. Several alphaviruses were shown to actively inhibit host cell gene expression through downregulation of cellular transcription and phosphorylation of eIF2α (189,190), and this has been primarily linked to the induction of apoptosis. Moreover, in the case of SFV and SINV, it has been suggested that the nsP2 viral protein downregulate RNA polymerase I- and II-dependent cellular transcription, which inhibits the antiviral response and ultimately induces cell death (191). For CHIKV, suppression of eIF2α phosphorylation in the early phase of virus replication appears to depend on nsP4 (190). The shutoff of host protein translation is responsible for the high cytopathic effect observed upon alphavirus

infection (191). In addition, the 6K protein of SINV has been shown to induce apoptosis via the formation of cation-selective ion channels in lipid bilayers (137,192,193). In case of CHIKV-induced apoptosis, the apoptotic blebs were found to contain infectious virus particles and therefore it has been suggested that they could have a role in viral spread from cell to cell (194). In sum, these data demonstrate that cell death contributes to viral pathogenesis, but also that depending on the modality of cell death, viral progeny production can be boosted by this process.

5) Scope of this thesis

The high clinical impact of dengue and chikungunya infection, and the limited resources available to treat and prevent these infections prompted us to further delineate the virus-host interactions that occur during infection with the aim to better understand the replication cycle of these viruses and to identify new avenues for therapeutic intervention. We put special detail in two main topics and their associated molecular mechanisms: 1) contribution of autophagy and ATG proteins to DENV and CHIKV infection, and 2) miRNA-mediated regulation of cellular protein expression and its effect on DENV replication.

In **Chapter 2** we review the most recent advances related to autophagy induction and modulation by a set of common flaviviruses and alphaviruses: DENV, Zika virus, WNV and CHIKV. Furthermore, we provide insight into the links that have been described between autophagy, cell death and the UPR pathway in the context of the infections caused by these viruses. We also describe the challenges that the field of autophagy currently faces and call for special attention when interpreting autophagy-related literature and data.

In **Chapter 3** we evaluate which ATG proteins play a role in DENV infection by performing an siRNA screen. To this end, we employed an image-based approach, in which the extend of infection of ATG-depleted cells with a GFP-tagged DENV is determined by automated fluorescence microscopy. We describe the optimization of the screen conditions and the analysis of the data. Preliminary results about the validation of two independent hits are shown. This chapter constitutes the basis of future studies aiming to fully validate the hits found in this screen, and to describe their specific function in DENV infection.

In **Chapter 4** we evaluate which ATG proteins influence CHIKV infection using a similar approach as described to **Chapter 3**, but adapted to this virus. Analysis of the siRNA screen data and validation experiments revealed that BNIP3 is an antiviral protein. Next, we investigated the role of BNIP3 in viral infection. First, using a diverse set of techniques, we studied the involvement of autophagy, mitophagy and cell death in the antiviral function of BNIP3. Secondly, we analysed the step of the CHIKV replication cycle that is hampered by BNIP3. We also assessed the antiviral role of BNIP3 across multiple CHIKV genotypes and SFV. In summary, this chapter unveils a previously unknown function of the autophagy-associated receptor BNIP3 in controlling alphavirus infection.

In **Chapter 5**, we evaluate the changes that occur in the miRNAs expression landscape of monocyte-derived-macrophages (MDMs) infected with DENV. To this end, we determine the miRNA profile in mock-treated cells, DENV-infected cells and in

bystander cells that have been exposed to DENV using an Illumina-based platform for deep-sequencing of small RNAs. By performing a series of complementary experiments in different cell lines, we identify miR-3614-5p as a miRNA that controls DENV-2 infection. Furthermore, to elucidate the potential targets of miR-3614-5p, we performed *in silico* prediction and mass spectrometry-based proteomic analyses, and identified Double-stranded RNA-specific adenosine deaminase 1 (ADAR1) as a protein that could be involved with the antiviral mechanism. We then performed diverse studies aiming to validate this hypothesis. Collectively, this chapter describes the changes that occur in the miRNAs landscape upon DENV infection and unravels the mechanism by which a cellular miRNA can fine-tune viral replication in infected cells.

Finally, the results obtained in this thesis are summarized and discussed in depth in **Chapter 6**.

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