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# Flaviviruses manipulate mitochondrial processes to evade the innate immune response

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Mitochondria are essential eukaryotic organelles that regulate a range of cellular processes, from metabolism to calcium homeostasis and programmed cell death. They serve as essential platforms for antiviral signaling proteins during the innate immune response to viral infections. Mitochondria are dynamic structures, undergoing frequent fusion and fission processes that regulate various aspects of mitochondrial biology, including innate immunity. Pathogens have evolved sophisticated mechanisms to manipulate mitochondrial morphology and function to facilitate their replication. In this review, we examine the emerging literature on how flaviviruses modulate mitochondrial processes.

Mitochondria are essential eukaryotic organelles that regulate different cellular processes from energy production to calcium homeostasis and innate immune signaling. Their function, or dysfunction, has been implicated in neurodegenerative diseases, cancer, ageing, and metabolic disorders<sup>1</sup>. Mitochondria are key regulators of innate immune signaling in response to infection by various pathogens i.e., viruses, bacteria, or fungi. Pathogens have evolved and possess elegant mechanisms to modulate aspects of mitochondrial morphology and function to benefit their replication.

Flaviviruses are one group of pathogens that are known to target mitochondria. There are over 70 viruses in the *Flavivirus* genus which are globally distributed<sup>2,3</sup>. Currently, licensed vaccines for flaviviruses are limited to yellow fever virus, Japanese encephalitis virus, dengue virus, Kyasanur forest disease virus, and tick-borne encephalitis virus. There are no approved antiviral treatments for any flavivirus. With over 3 billion people at risk of infection by one or more flaviviruses annually, and the expected geographic expansion of flaviviruses with climate change, these viruses pose a substantial public health burden<sup>4</sup>. Understanding how flaviviruses interact with host mitochondria will provide insight into the pathogenic mechanisms of flavivirus diseases, improve our understanding of host responses to infection, and may potentiate the development of novel host-acting therapeutics that counter the effects of flaviviruses on mitochondria. In this Review, we briefly introduce the functions of mitochondria, highlighting their role in antiviral responses, followed by a detailed discussion of how specific flaviviruses target mitochondria function and morphology and the effects on viral replication.

## Mitochondria: functions and regulatory mechanisms

Mitochondria, the powerhouses of cells, are essential double-membraned organelles in eukaryotes. They play significant roles in almost every major cellular process, including metabolic regulation, apoptosis, calcium homeostasis, signaling pathways and reactive oxygen species (ROS) production<sup>5–8</sup>. Additionally, mitochondria function in host defense against pathogens by regulating signaling pathways and promoting apoptosis in response to stimuli and stress conditions<sup>6,9,10</sup>.

The primary function of mitochondria is to generate adenosine triphosphate (ATP), the energy currency of the cell, through oxidative phosphorylation (OXPHOS). This process occurs at the inner mitochondrial membrane, where the electron transport chain (ETC) and ATP synthase are embedded<sup>11</sup>. Mitochondrial metabolic pathways, including the tricarboxylic acid (TCA) cycle, fatty acid oxidation (FAO), the ETC, and OXPHOS, convert substrates into ATP<sup>12</sup>. Mitochondria regulate these pathways by sensing and controlling metabolites and responding adaptively to metabolic stresses. The integration of metabolic pathways with gene expression is crucial for regulating various cellular functions such as growth, survival, differentiation, and immune recognition<sup>13</sup>.

Mitochondria also play an essential role in the intrinsic pathway of apoptosis by regulating caspase activation through mitochondrial outer membrane permeabilization (MOMP)<sup>14</sup>. Upon pro-apoptotic stress, proteins such as BAX and BAK induce MOMP, leading to the release of cytochrome-C into the cytosol. Cytochrome-C then binds to apoptosis protease activating factor 1 (APAF1), forming the apoptosome and activating initiator caspase-9. This activation subsequently triggers effector caspases like caspase-3 and caspase-7, leading to controlled cellular demolition<sup>15</sup>.

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Mitochondria are a major source of ROS, which are byproducts of the ETC formed during oxidative phosphorylation. While low levels of ROS function in cell signaling and homeostasis, excessive ROS can cause oxidative damage to proteins, lipids, and DNA. To mitigate this damage, mitochondria have antioxidant systems, including superoxide dismutase (SOD) and glutathione<sup>16</sup>. Oxidatively stressed mitochondria can induce autophagy and mitophagy, and ROS also activate inflammatory signaling pathways<sup>17</sup>.

Mitophagy is the selective autophagic degradation of damaged mitochondria, crucial for maintaining mitochondrial quality control. The process is mediated by PTEN-induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin. When mitochondria are damaged, PINK1 accumulates on their outer membrane, recruiting Parkin. Parkin then ubiquitinates mitochondrial surface proteins, signaling the autophagic machinery to degrade the damaged mitochondria<sup>18</sup>. This process ensures the removal of dysfunctional mitochondria, preventing cellular damage and maintaining mitochondrial function.

**Mitochondrial morphology**

Mitochondria are dynamic organelles whose morphology is regulated by a delicate balance between fusion/elongation and fission/fragmentation processes. These processes are crucial for maintaining mitochondrial function, distribution, and quality control within the cell. The dynamic nature of mitochondria is governed by specific proteins that facilitate these morphological changes.

**Fission/fragmentation proteins.** Mitochondrial fission describes the division of one mitochondrion into two or more mitochondria. This process is essential for several cellular functions, including the distribution of mitochondria during cell division, the transport of mitochondria to different cellular regions, and the removal of damaged mitochondria through mitophagy. The key protein involved in mitochondrial fission is dynamin-related protein 1 (Drp1), a cytosolic protein that translocates to the outer mitochondrial membrane (OMM) upon activation. This protein self-assembles into polymers to form constriction sites on mitochondria in a GTP-dependent manner, which in turn facilitates fission<sup>19</sup>. This often occurs at mitochondria–endoplasmic reticulum (ER) contact sites in a Ca<sup>2+</sup>-dependent mode<sup>20</sup>.

**Fusion/elongation proteins.** Fusion allows the mixing of mitochondrial contents, which is essential for maintaining mitochondrial function and genetic stability, especially under stress conditions, by diluting defective components within the mitochondrial network. Fusion is mediated by proteins located on the outer and inner mitochondrial membranes. The mitofusins (Mfn1 and Mfn2) are GTPases located on the OMM. These proteins mediate the initial tethering and fusion of the OMM between adjacent mitochondria<sup>21</sup>. Optic Atrophy Factor 1 (Opa1) is located on the inner mitochondrial membrane (IMM) to facilitate and regulate the fusion between the OMM and IMM. Opa1 also helps maintain cristae structure and is involved in apoptosis regulation<sup>19,21</sup>. Together these proteins facilitate the fusion of mitochondrial membranes, allowing for the sharing of mitochondrial DNA, proteins, and metabolites. Mitochondrial elongation occurs in response to certain stress conditions. For example, during starvation, an increase in cyclic adenosine monophosphate (cAMP) levels activates protein kinase A (PKA), causing phosphorylation of Drp1 at Ser637, resulting in the retention of Drp1 in the cytosol and inhibition of fission. This promotes mitochondrial elongation<sup>22</sup>.

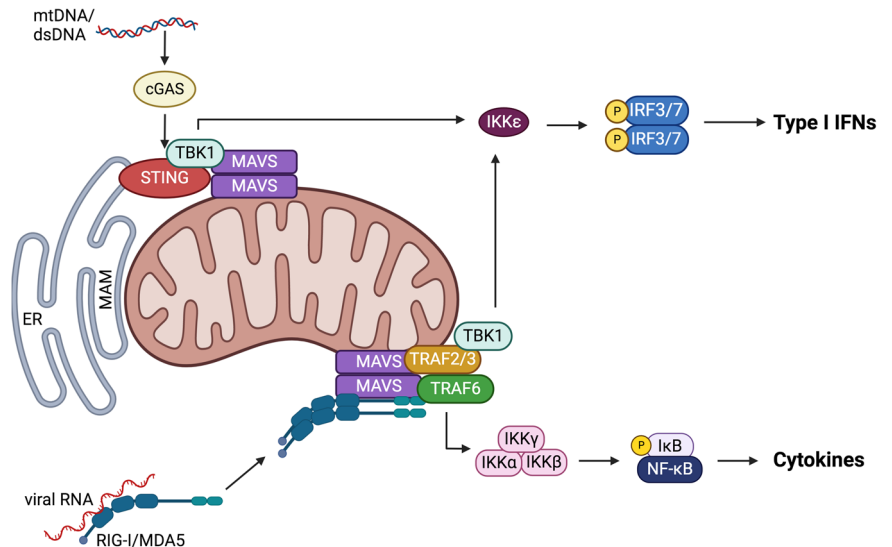
The mitochondrial shape depends on the balance between fission and fusion, which is crucial for maintaining mitochondrial integrity and function. Unbalanced fusion leads to mitochondrial elongation, while unbalanced fission results in excessive mitochondrial fragmentation, both of which impair mitochondrial function<sup>23</sup>. The occurrence of fragmentation leads to pathological phenotypes and cell death.

**Mitochondria and innate immunity**

Another major function of mitochondria is their role in innate immunity (Fig. 1). The release of mitochondrial DNA (mtDNA) into the cytosol activates the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway, which induces the production of type I interferons (IFNs) and other inflammatory cytokines, enhancing the immune response to infections and causing cellular stress. Mitochondria are also platforms for propagating RIG-I-like receptor (RLR) signaling in response to sensing of cytosolic RNA.

**cGAS-STING pathway activation.** Under stress conditions, both nuclear and mitochondrial DNAs can be released into the cytoplasm. The

**Fig. 1 | Mitochondria play important roles in innate antiviral immune responses.** Under stress conditions, such as those induced by viral infection, DNA is released from the mitochondria (mtDNA) and nucleus (dsDNA). These DNAs are detected by cGAS, which, upon DNA binding and activation, catalyzes the synthesis of cGAMP. cGAMP binds to STING, which is associated with the endoplasmic reticulum (ER). Mitochondria interface with the ER at mitochondria-associated membranes (MAMs), where activated STING binds to MAVS and recruits TBK1, which phosphorylates IKKε. IKKε phosphorylates IRF3/7, which dimerizes and translocates to the nucleus to activate the transcription of type I interferons (IFNs). Viral RNA is detected in the cytosol by RIG-I-like receptors (RLRs), including RIG-I and MDA5. Upon RNA binding, RLRs oligomerize and associate with MAVS on the mitochondrial membrane. MAVS recruits TRAF2/3 and TRAF6. TRAF2/3 activates TBK1 and downstream interferon transcription as described above. TRAF6 activates the IκB kinase complex (IKK, composed of IKKγ, IKKα, and IKKβ), which phosphorylates the NF-κB inhibitor-α (IκB), causing NF-κB to translocate to the nucleus where it activates the transcription of pro-inflammatory cytokines.



presence of these DNAs is detected by the cGAS-STING pathway, an inflammatory signaling mechanism. cGAS recognizes cytoplasmic double-stranded DNA (dsDNA). Upon binding to DNA, cGAS dimerizes and catalyzes the synthesis of cyclic dinucleotide cGAMP from ATP and GTP<sup>24</sup>. Next, cGAMP binds to STING, causing its oligomerization and translocation from the ER to the Golgi apparatus. Activated STING then recruits TANK-binding kinase 1 (TBK1), leading to the phosphorylation of interferon regulatory factor 3 (IRF3). Phosphorylated, activated IRF3 translocates to the nucleus, inducing the expression of type I IFNs and other inflammatory mediators<sup>25–27</sup>. This pathway is crucial for sensing intracellular pathogens, including *Mycobacterium tuberculosis*, herpesviruses, dengue virus, norovirus, influenza A virus, encephalomyocarditis virus, and severe acute respiratory syndrome coronavirus 2<sup>28–34</sup>.

The release of mtDNA into the cytosol is a marker or indication of mitochondrial dysfunction. When this occurs, a mitochondrion will first try to repair itself by degrading, clearing, or expelling damaged proteins and lipids. However, when this fails, mitochondria will release their entire contents, including mtDNA, into the cytosol, thus causing a sterile activation of various innate immune signaling pathways such as TLR-9, NLRP3 inflammasome, and cGAS-STING pathways. These pathways trigger the production of cytokines or regulate cell death. This self-destruction can occur in the absence of pathogens, although pathogens that induce mitochondrial stress can contribute to this process<sup>35</sup>.

**MAVS and RLR signaling.** Cytosolic and membrane-associated pathogen recognition receptors (PRRs) are key players in activating early innate immune signaling in response to infection. In relation to viral infections, the RLRs detect viral RNA in the cytoplasm. RLR binding to RNA causes oligomerization of RLR and translocation to the mitochondria, where they interact with mitochondrial antiviral signaling protein (MAVS). MAVS then activates downstream signaling factors, including TBK1 and IκB kinase (IKK), or tumor necrosis factor receptor-associated factor 2/6 (TRAF2/6), which in turn activate IRF3 and NF-κB, respectively, resulting in the expression of IFNs and cytokines<sup>36,37</sup>. RLR activation has been shown to promote mitochondrial elongation and enhance IFN and cytokine production, while fragmentation dampens RLR signaling<sup>37,38</sup>.

Given the roles that mitochondria play in energy production and metabolism, calcium and redox balance, apoptosis, and innate immune

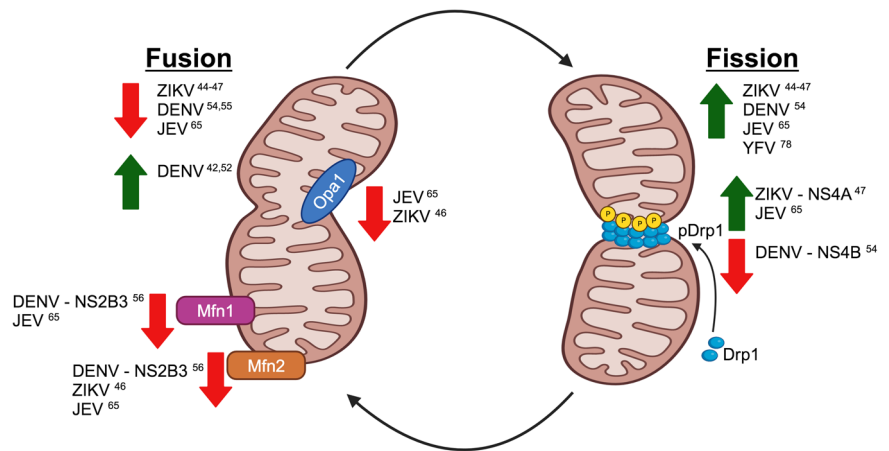
signaling, it is no surprise that pathogens have evolved ways to interfere with mitochondrial dynamics and function for their own benefit. Multiple pathogenic bacteria, encode virulence factors that modulate mitochondrial fission-fusion dynamics<sup>39</sup>. Viruses such as dengue, severe acute respiratory virus (SARS), human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza also manipulate fission and fusion to enhance their replication. Additionally, viruses and bacteria can induce or suppress different arms of mitochondrial metabolic pathways<sup>39</sup>. Flaviviruses are no different in this regard. Yellow fever, dengue, Zika, and West Nile virus have all been reported to modulate some aspect of mitochondrial dynamics.

**Mitochondrial dynamics during flavivirus infection**

Flaviviruses are single-stranded positive-sense RNA viruses. Their approximately 11-kb genome encodes a polyprotein that is processed by viral and host proteases into three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5)<sup>40</sup>. Following entry and uncoating, initial polyprotein translation occurs at ER-associated ribosomes. Newly synthesized viral proteins, including NS2B/3, NS4A, and NS4B, remodel ER membranes to form convoluted membranes where viral genome replication and transcription will occur<sup>40,41</sup>. The induction of convoluted membranes disrupts ER-mitochondria contacts, perturbing normal signaling between these compartments<sup>42</sup>. Below we review how mitochondria, which are key regulators of innate antiviral signaling, are manipulated by flaviviruses to evade host responses and enhance replication.

**Zika virus.** Mitochondrial elongation is known to enhance innate antiviral signaling, specifically RLR-mediated and cGAS-STING signaling, by increasing the surface area for interactions between the ER and mitochondria. Mfn1 and Mfn2 interact with MAVS to regulate RLR-signaling<sup>38,43</sup>. To counteract IFN and interferon-stimulated gene (ISG) production, ZIKV has been reported to induce mitochondrial fragmentation (Fig. 2). Using immunofluorescence (IFA) and quantitative analysis of mitochondrial characteristics (length, networks, branches), Garcia et al. found that ZIKV replication in two different human retinal cells induced mitochondrial fragmentation, a phenotype also observed in human lung adenocarcinoma cells, A549 cells, using transmission electron microscopy (TEM)<sup>44,45</sup>. This is supported by findings from more physiologically relevant cell lines, placental JEG-3 cells, neuronal stem cells, and neuronal SNB-19 cells, where infection-induced fragmentation

**Fig. 2 | Flaviviruses modulate mitochondrial fission and fusion dynamics.** Mitochondria are highly dynamic and are maintained in a cycle of fusion and fission. Fusion can lead to mitochondrial elongation, which augments innate antiviral signaling downstream of RLRs, thereby increasing interferon production. Fission leads to fragmented mitochondrial networks and can reduce mitochondria-endoplasmic reticulum contacts, which dampens innate antiviral signaling. Zika virus (ZIKV), dengue virus (DENV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) enhance mitochondrial fission. JEV and the NS4A protein of ZIKV promote fission by increasing the levels of the activated form of fission factor Drp1 (pDrp1). ZIKV and JEV enhance fission by reducing the protein levels of fusion factors Mfn1, Mfn2, and/or Opa1. Some reports found that DENV enhances fusion, leading to mitochondrial elongation, in part through NS4B-mediated reduction in levels of the fission factor pDrp1. Other studies found that DENV NS2B3 cleaves the fusion factors Mfn1 and Mfn2, leading to their proteasomal degradation, and enhancing fission. Superscripts refer to reference numbers.



and mitochondrial swelling as early as 4 h post infection<sup>46,47</sup>. NS4A increased the levels of activated fission factor pDrp1 and was sufficient to induce fragmentation<sup>47</sup>. This suggests that NS4A-mediated enhancement in pDrp1 shifts the balance of fission-fusion in favor of fission, although how NS4A increases pDrp1 levels remains unknown. Other viral factors or processes are likely involved, as NS4A alone did not induce the same degree of fragmentation observed during ZIKV infection<sup>47</sup>. In this same study, NS4A and NS4B inhibited IFN signaling, with NS4A reducing MAVS oligomerization and MAVS-mediated IFN- $\beta$  production. Together the results point to NS4A-enhanced mitochondrial fission, which abrogates IFN and ISG production through impacts on MAVS signaling. The protease NS3 also interacts with MAVS and promotes poly-ubiquitination and proteasomal degradation of MAVS, which reduces IFN production<sup>48</sup>. Overall, it is evident that while ZIKV induces mitochondrial fission, the inhibition of IFN and ISG production results from a combination of fission effects and the targeting of specific mitochondrial-associated proteins by viral proteins.

The fusion factors Mfn1, Mfn2, and Opa1 are also targeted by ZIKV. Yang et al. found a reduction in Mfn2 levels in ZIKV-infected neuronal stem cells (NSCs) and SNB-19 cells<sup>46</sup>. Interestingly, there were strain and cell-type specificities to the reduction of other fusion factors, as Opa1 was reduced in NSCs by the Uganda strain, but not in SNB-19 cells or by the Puerto Rico strain. Overexpression of both Mfn1 and Mfn2 reduced ZIKV-induced mitochondrial fragmentation, implicating ZIKV-induced reduction of Mfn2 in augmenting fragmentation. Fragmentation is pro-viral for ZIKV replication, as siRNA knockdown of Mfn2 increased viral RNA levels, whereas silencing of Drp1 dramatically reduced viral RNA<sup>47</sup>. Future studies should aim to elucidate how ZIKV causes these reductions, investigating the reason behind virus strain and cell type differences, and determining whether these differences underlie neurological symptoms. The strain differences provide a useful starting point for identifying viral proteins involved in this process.

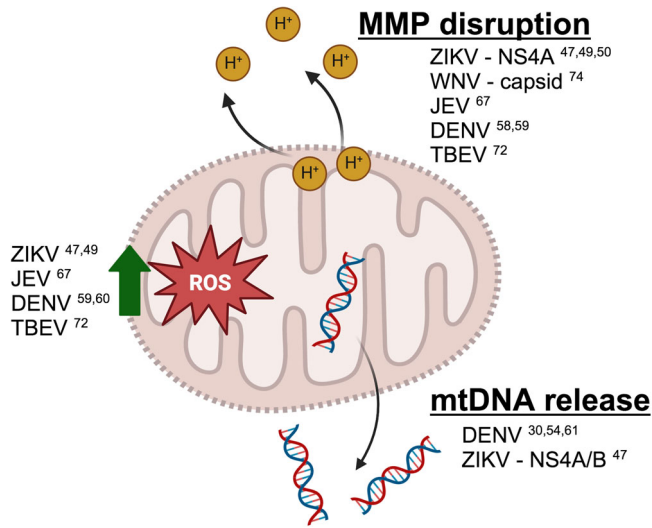
Besides altering fission-fusion dynamics, ZIKV also modulates mitochondrial respiration, ROS accumulation, and mtDNA release (Fig. 3). In astrocytes, ZIKV infection caused an initial burst in oxidative phosphorylation, with a subsequent reduction in basal respiration and reserve capacity at later timepoints<sup>49</sup>. These changes were accompanied by an accumulation of mitochondrial ROS and loss of mitochondrial membrane integrity. Similar findings of ROS accumulation, mitochondrial depolarization, and reduction in basal respiration were observed in placental JEG-3 cells<sup>47</sup>. Yau and colleagues performed a particularly detailed analysis of ZIKV-induced alterations to oxidative phosphorylation and glycolytic flux. In MRC-5 cells, virulent strains reduced MMP, glycolytic capacity, and oxidative phosphorylation, while attenuated strains did not affect these measures<sup>50</sup>. Importantly, these modulations appear to play a role in viral pathogenesis. Pre-treatment of cells or dams with glycolytic intermediates aimed at restoring normal metabolism resulted in reduced expression of inflammatory genes and largely rescued congenital defects. Further investigation is needed to determine how ZIKV causes these changes, including whether there are specific viral proteins involved or if more systemic factors are at play. Nevertheless, these findings may pave the way for novel therapeutics and interventions that can abrogate aspects of ZIKV pathology.

Damaged mitochondria release mtDNA which is detected by the cGAS-STING pathway to activate IFN production. Mitophagy, which begins with mitochondrial fragmentation, removes damaged mitochondria from the cell and prevents the activation of inflammatory pathways. As described, ZIKV promotes mitochondrial fragmentation, and it is unsurprising that the virus would also modulate mitophagy (Fig. 4). However, there are conflicting reports on this. Ponia et al. found that ZIKV NS5 binds to Ajuba, a host factor that promotes mitophagy. This interaction suppresses

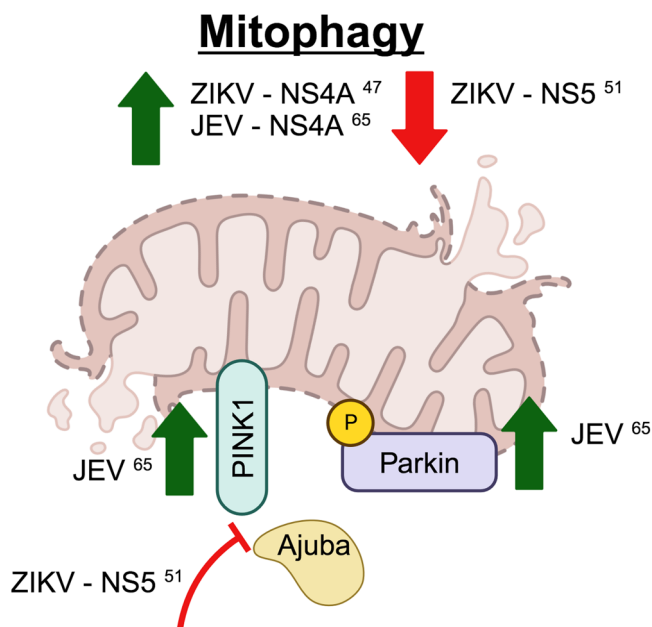
the recruitment of Ajuba to mitochondria, thereby reducing mitophagy<sup>51</sup>. Infection of Ajuba null MEFs resulted in higher IFN- $\beta$  and chemokine production, with reduced viral titers. These results imply that ZIKV, or at least the NS5 protein, reduces mitophagy, yet this negatively impacts viral replication. Reduced mitophagy also inhibited ZIKV replication in A549 cells<sup>45</sup>. In contrast to Ponia, Lee et al. found that multiple ZIKV strains induce mitophagy in JEG-3, HeLa, and neural progenitor cells and that NS4A is sufficient to promote mitophagy<sup>47</sup>. Silencing of PINK1, a key regulator of mitophagy, rescued replication. The differences between the Ponia and Lee studies are likely due to the different methods used to measure or characterize mitophagy. Additionally, variations in the cell types, as well as the differences in viral proteins each group investigated likely contributed to the differences in these studies. Lee et al. used a pH-sensitive reporter plasmid that allows visualization and quantification of mitochondria within lysosomes. Ponia et al. relied on western blots to probe the levels of mitophagy-associated proteins in mitochondrial cell fractions, a less precise method. Overall, it is clear that ZIKV infection affects mitophagy, but additional work is needed to determine how different viral proteins affect mitophagy and whether the overall effects on mitophagy are beneficial to the virus or the cell.

**Dengue virus.** While the evidence for ZIKV-induced mitochondrial fission is clear, there are conflicting reports on how DENV affects fission and fusion. Barbier et al. reported increased mitochondria length, indicating enhanced fusion, or elongation, in Huh7 cells. Infection of Huh7 and dendritic cells reduced pDrp1 and mitochondrially-associated Mfn1 and Mfn2<sup>52</sup>. Inhibiting fusion through Mfn2 silencing, or chemically and genetically inducing fission, reduced DENV protein levels and titers. In agreement with Barbier et al., Chatel-Chaix et al. reported that DENV infection induced mitochondrial elongation in Huh7, and that NS4B was sufficient for this. Infection reduced mitochondrially localized fission factors Drp1 and pDrp1, and silencing of Drp1 enhanced DENV replication<sup>42</sup>. Also, in agreement with Barbier, silencing of Mfn2 resulted in fragmented mitochondria and inhibited DENV replication. Given that mitochondrial elongation has been shown to promote innate antiviral signaling, and to restrict the replication of ZIKV, it is surprising that DENV promotes a process that would be expected to be antiviral. Chatel-Chaix et al. offer an explanation involving reduced ER-mitochondria contacts resulting from elongation and subsequently impaired innate antiviral signaling. They support this by demonstrating reduced ER-mitochondria contacts in transmission electron microscopy images, DENV-induced reductions in ER-mitochondria tether proteins, and showing that enforced elongation reduced DENV-induced IFN and ISG production in Huh7 and Huh7.5 cells<sup>42,53</sup>.

In contrast to these two groups, Singh et al. found that all four DENV serotypes induced mitochondria fragmentation and swelling in Huh7 cells, with interesting serotype-specific differences in the extent and rate of fragmentation<sup>54</sup>. Yu et al. demonstrated support for this by using A549 cells and observing that DENV infection also inhibited fusion<sup>55</sup>. Consistent with current knowledge about elongation and innate antiviral signaling, overexpression of Mfn1 in DENV-infected cells rescued mitochondria length, increased IRF3 activation and IFN- $\beta$  production, and suppressed DENV replication. Mfn2 did not affect IFN production but did inhibit DENV-induced MMP depolarization, highlighting the multiple roles that mitochondrial proteins play. The mechanism through which DENV augments fragmentation remains unclear. While Yu et al. showed that DENV NS2B3, which is imported to the mitochondria<sup>56</sup>, cleaved Mfn1 and Mfn2, leading to their proteasomal degradation, Singh and colleagues observed reductions not only in Mfn1 and Mfn2 levels but also in the fission factor Drp1/pDrp1. Mitochondria are highly dynamic, and it is likely that these protein levels will fluctuate during the course of infection but overall tip the balance in favor of fission (Fig. 2).



**Fig. 3 | Flavivirus infection induces markers of mitochondrial stress.** Accumulation of reactive oxygen species (ROS), disruption of mitochondrial membrane potential (MMP), and mtDNA release are markers of mitochondrial stress. ZIKV, WNV, JEV, DENV, and TBEV infection disrupt the MMP (H<sup>+</sup> proton leakage), leading to mitochondrial depolarization and downstream effects on ATP synthesis. For ZIKV, this is mediated by NS4A, and for WNV, by the capsid protein. Infection by ZIKV, JEV, DENV, and TBEV also leads to the production and accumulation of mitochondrial ROS. ZIKV and DENV infection promotes the release of mtDNA into the cytoplasm, where it is sensed by pattern recognition receptors like cGAS-STING, leading to innate immune response activation. Superscripts refer to reference numbers.



**Fig. 4 | Flaviviruses modulate mitophagy.** Mitophagy, the controlled degradation of damaged mitochondria from the cell, prevents the release of proteins and other components that can activate inflammatory pathways. Flavivirus infection induces mitochondrial stress, and flaviviruses target this process to evade innate immune responses. The NS4A proteins of ZIKV and JEV enhance mitophagy. For JEV, this involves increasing levels of key regulators of mitophagy, the kinase PINK1 and the ubiquitin E3 ligase Parkin. In contrast, the NS5 protein of ZIKV abrogates mitophagy by inhibiting the interaction between PINK1 and Ajuba, another host factor that promotes mitophagy. Superscripts refer to reference numbers.

Other aspects of DENV-induced alterations to mitochondria function are more similar to ZIKV. Infection of HepG2 cells increased basal respiration at early time points but there was reduced ATP synthesis and reduced energy charge at later timepoints<sup>57</sup>. This was accompanied by increased membrane permeability and MMP depolarization, explaining the reduced efficiency of ATP synthesis (Fig. 3). Increased permeability and reduced MMP were also observed in donor platelet cells from DENV-infected patients<sup>58</sup>. Meuren et al. measured energy production in infected human brain microvascular endothelial cell line (HBMECs) and also found a reduction in ATP and energy reserves, accompanied by MMP depolarization<sup>59</sup>. In a recent pre-print, Freppel et al. performed very detailed quantitative analyses of different aspects of mitochondrial respiration in DENV-infected or NS4B-expressing cells and found reduced basal, maximal, and ATP respiration/production<sup>53</sup>. All of these studies reported DENV-induced alterations to mitochondrial respiration that would be expected to harm the cell and create a low-energy environment for continued replication, albeit following an initial flux in energy production. Further mechanistic studies are needed to elucidate how DENV proteins or replication processes modulate mitochondrial respiration and to understand the impact of the dysregulation of these processes on DENV replication.

As with ZIKV, DENV infection induces ROS accumulation, and this has antiviral effects. For example, Meuren et al. used flow cytometry to quantify ROS accumulation in HBMECs, revealing that DENV infection increased overall ROS levels and, specifically, mitochondrial ROS levels<sup>59</sup>. Inhibition of ROS scavenging the mitochondria significantly reduced DENV replication and progeny production. Similar results were observed in DENV-infected Caco-2 cells<sup>60</sup>. The mechanism by which ROS accumulation in mitochondria inhibits replication has not been determined. However, considering the connections between ROS, antiviral signaling, and mitophagy, these pathways present possible avenues to explore. For example, oxidatively-stressed mitochondria may release mtDNA, and DENV infection has been reported to increase cytoplasmic and extracellular mtDNA<sup>30,54,61</sup>. mtDNA, and DENV, activates antiviral cGAS-STING, and increased cGAMP and IFN production were also reported in these studies<sup>30,54</sup>.

There is a myriad of other examples of DENV-mitochondria interactions that impact IFN and ISG production. DENV infection of THP-1 cells induced CMPK2 expression, an ISG whose product is localized to the mitochondria and makes substrate for the ISG RSAD2/viperin<sup>61</sup>. CMPK2 restricted DENV replication and was important for DENV-induced activation of ISGs. Interestingly, CMPK2 was also reported to restrict ZIKV replication by inhibiting protein translation, independently of its involvement with viperin and dependent on its mitochondrial localization<sup>62</sup>. DENV NS4A has been reported to physically interact with MAVS in mitochondria-associated membranes (MAMs), and this inhibited RIG-I-MAVS interactions and downstream pIRF3, IFN-β, and ISG activation<sup>63</sup>. NS2B, which degrades cGAS<sup>31</sup>, has also been reported to interact with MAVS and to inhibit RIG-I mediated signaling at the levels of MAVS, although in this case the mechanism of inhibition does not appear to be through NS2B cleavage of MAVS<sup>64</sup>. The overall picture that emerges is one where DENV infection targets multiple aspects of mitochondria, including mitochondrial dynamics and proteins involved in innate immune signaling, to establish a more permissive replication environment. Considering the multifaceted roles that mitochondria play in cell biology, this strategy of modulating multiple aspects allows DENV to circumvent some, though not all, of the host's defense strategies.

**Japanese encephalitis virus.** Japanese encephalitis virus is less studied than DENV or ZIKV, but from available studies it is clear that JEV infection also targets mitochondria. JEV-infected Huh-7 cells showed reduced mitochondrial footprints as measured by immunofluorescence

analysis, indicative of mitochondrial fragmentation<sup>65</sup>. This mechanism likely involves both the inhibition of fusion and activation of fission, as evidenced by reduced levels of *Mfn1*, *Mfn2*, and *Opa1* with an increase in activated pDrp1. Augmented fragmentation was accompanied by increased mitophagy in infected cells, and this was confirmed in vivo from the livers of infected mice. NS4A, which was localized exclusively to the mitochondria and microsomes, interacted with the mitophagy factor PINK1, and was sufficient to enhance mitophagy. As with ZIKV, inhibiting fission through Drp1 silencing reduced JEV titers by approximately 50%, while silencing of PINK1, and hence mitophagy, decreased JEV titers by 90%. These results suggest that JEV infection actively promotes mitochondria fragmentation and, subsequently, mitophagy for pro-viral effects. Further research is needed to determine how the fusion factors are degraded, and how enhanced mitophagy promotes replication. It is likely that fragmentation and mitophagy inhibit innate antiviral signaling that depends on mitochondrial proteins and function, as has been suggested for ZIKV.

In agreement with the enhanced fission and mitophagy reported by Agarwal et al., Singh and colleagues found reductions in mtDNA copy number in the hippocampus, cortex, and brain stem of JEV-infected BALBc mice, suggesting a reduction in mitochondria<sup>66</sup>. However, transcript levels of *Mfn1*, *Mfn2*, and *Opa1* increased following infection, while mRNA of *Drp1* and *FIS1* were significantly reduced at 3- and 7-days post infection, which are signatures of increased fusion, not fission. The protein levels of these factors were not measured, and this may explain the discrepancy between the observed effect on mitochondrial quantity and fission-fusion transcript results. If so, this suggests that viral proteins may influence post-transcriptional and post-translational modulations of these host proteins. For example, DENV NS2B3 has been reported to cleave *Mfn1* and *Mfn2*<sup>55</sup>, and this may be a shared strategy used by flaviviruses to enhance their replication. It is also plausible that other effects on the mitochondria caused by JEV infection could lead to a reduction in mitochondrial numbers. For example, in the same study, researchers observed a dramatic accumulation of ROS in brain tissue homogenates from infected mice. Additionally, another group found that NS2B3 was sufficient to reduce MMP and enhance ROS accumulation in TE671/RD cells<sup>67</sup>.

JEV also targets specific mitochondrial proteins involved in innate antiviral signaling. Zhou and colleagues found that JEV NS1', an extended NS1 protein produced from a ribosomal frameshift, was sufficient to inhibit IFN- $\beta$  production at the levels of MAVS in RLR signaling<sup>68</sup>. Both MAVS mRNA and protein levels were reduced by the expression of NS1'. An NS1' deficient virus was defective in tissue culture replication and attenuated in a mouse model, however replication and virulence were restored upon silencing of MAVS. This is a novel role for NS1 and NS1', and these results highlight the need for additional studies of the flavivirus non-structural proteins which will reveal new mechanisms by which these viruses hijack host processes and exert their pathogenic effects.

**Tick-borne encephalitis virus.** Tick-borne encephalitis virus is widespread in the Baltic region and throughout Central Asia, and infections are increasing in frequency<sup>69</sup>. There are few studies on TBEV and mitochondria. From the available research, it is evident that TBEV modulates mitochondrial characteristics, although the underlying mechanisms remain unclear. The mitochondria in some infected astrocytes and glioblastoma cells appeared to be swollen at late time-points post infection (9 days), and some had deformed cristae<sup>70,71</sup>. Infected PMJ-2 macrophage cells also exhibited alterations to mitochondria, in this case, dysregulated fluctuations in MMP and accumulation of ROS over 72 h<sup>72</sup>. The non-structural proteins alone, without viral replication, were sufficient to induce ER-remodeling to form viral replication compartments, which were in close contact with mitochondria<sup>73</sup>. This is similar to what has been seen with DENV and ZIKV replication compartments, where it is better understood that infection causes dysregulated ER-mitochondria contact with

consequences for viral replication<sup>42,53</sup>. Given these intriguing yet limited findings, further mechanistic studies are warranted.

**West Nile virus.** West Nile virus and mitochondria have mostly been studied through the lens of apoptosis. WNV infection of Vero and HeLa cells induces mitochondrial cytochrome-C release, MMP disruption, and cleavage of caspase 3, caspase 9, and PARP, followed by apoptosis<sup>74-76</sup>. Both capsid and NS3 are sufficient to activate caspase cleavage, however, the role that mitochondria per se play in regulating WNV replication has not been extensively explored.

**Yellow Fever virus.** Yellow fever virus is also understudied regarding its effects on mitochondria, possibly because of the vaccine's high effectiveness and the perception that the virus poses less of a threat, despite estimates indicating tens of thousands of deaths annually<sup>77</sup>. A recent preprint found that YFV infection of A549 cells did not significantly affect total mitochondrial mass, but slightly increased the number of networks, structures, and nodes quantified from IFA images, which suggests mitochondrial fragmentation<sup>78</sup>. These alterations were only observed at early time points post infection and were normal by 3 dpi. Chemical induction of fragmentation reduced supernatant titers, suggesting that fragmentation is antiviral. This would appear to contradict the finding that YFV enhanced fragmentation. The discrepancy may be due to the use of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which causes MMP depolarization and thus affects other mitochondrial functions besides inducing fragmentation.

#### Open questions and future directions

The examples reviewed here clearly demonstrate that flaviviruses target mitochondria to enhance their replication and pathogenesis (Table 1). Mitochondria are essential for host defense and utilize redundant mechanisms to protect the host against viral infection. While there has been some progress toward elucidating the mechanisms by which specific flaviviruses exert these effects, much more work is needed in this area. Future studies could address several key questions such as (1) which specific viral proteins alter mitochondrial morphology, MMP depolarization, or any other aspect of mitochondria affected by infection, (2) how these proteins interact with host factors to induce these changes, (3) what the effects of each alteration on specific steps in viral replication are and (4) how do specific examples of mitochondrial dysregulation cause the pathogenic effects of flavivirus infections. Validating these findings in animal models will be essential for translating and applying our knowledge to human infections. Furthermore, more research is needed to determine whether DENV infection induces mitochondrial elongation or fragmentation. As discussed above, variations in cell lines, methods for observing and measuring mitochondrial morphology, and tools used to study the effects of fission-fusion on replication differ across studies. Focusing on physiologically relevant cells, such as monocytes, macrophages, and liver cells, will be important.

Future studies should also expand the scope of flaviviruses that are studied. While there are multiple groups working on DENV, ZIKV, and mitochondria, other medically relevant flaviviruses such as YFV, JEV, WNV, TBEV, and Powassan virus (POWV) are much less studied in this regard, or not at all in the case of POWV. Understanding the detailed mechanisms through which each flavivirus targets mitochondria for pro-viral effects and identifying pathways shared by these viruses will facilitate the development of novel broadly acting host- or virus-targeted antivirals. For example, a drug might inhibit flavivirus-induced mitochondrial fragmentation to reverse the dampening effects on innate antiviral responses and tip the balance in favor of the host. If such a drug acted on host factors, it would concomitantly avoid the threat of evolved viral resistance.

**Table 1 | Comprehensive table summarizing how different flaviviruses modulate mitochondrial processes to enhance their replication**

Virus	Effect on mitochondria	Viral protein?	Reference
Dengue virus	Fragmentation/reduced fusion		Singh 2022, Yu 2015
	Reduced Mfn1 protein levels	NS2B3	Barbier 2017, Yu 2015, Singh 2022
	Reduced Mfn2 protein levels	NS2B3	Barbier 2017, Yu 2015, Singh 2022
	Reduced pDrp1		Barbier 2017, Singh 2022
	mtDNA release		Sun 2017, Singh 2022, Lai 2021.
	Elongation	NS4B	Barbier 2017, Chatel-Chaix 2016
	ROS accumulation		Meuren 2022, Khan 2021
	MMP disruption		El Bacha 2007, Hottz 2013
Zika virus	Fragmentation		Garcia 2020, Ledur 2020, Yang 2020, Lee 2023, Huang 2023
	Reduced Mfn2 protein levels		Yang 2020
	Increased pDrp1	NS4A	Yang 2020
	Reduced Opa1		Yang 2020
	MMP disruption		Yau 2021,
	ROS accumulation		Ledur 2020, Lee 2023
	Increased mitophagy	NS4A	Lee 2023
	Reduced mitophagy	NS5	Ponia 2021
mtDNA release		Lee 2023	
Japanese encephalitis virus	Fragmentation		Agarwal 2022
	Reduced Mfn1 protein levels		Agarwal 2022
	Reduced Mfn2 protein levels		Agarwal 2022
	Increased pDrp1		Agarwal 2022
	Increased Parkin		Agarwal 2022
	Increased mitophagy	NS4A	Agarwal 2022
	MMP disruption		Yang 2009
	ROS accumulation		Yang 2009, Singh 2023
West Nile virus	MMP disruption	capsid	Yang 2002
Tick-borne encephalitis virus	Enlarged, with deformed cristae		Palus 2014, Ruzek 2009
	MMP disruption		Berankova 2024
	ROS accumulation		Berankova 2024
Yellow fever virus	Fragmentation		Gomez 2023

**Conclusions**

With their multifaceted roles in cell function, mitochondria are understandably targeted by flaviviruses and other pathogens to promote replication and spread. In this Review, we highlighted specific examples of how flaviviruses modulate mitochondrial morphology, energy production, mitophagy, oxidative stress, and mitochondrial roles in innate antiviral signaling. Future work will improve our understanding of the mechanisms underlying these effects and how mitochondrial dysregulation contributes to flavivirus pathogenesis and disease severity.

**Data availability**

No datasets were generated or analysed during the current study.

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Conceptualization: R.B. and M.L.-R.; Data curation: R.B., K.K., and M.L.-R.; Writing—original draft preparation: R.B. and K.K.; Preparing figures: R.B.; Writing—reviewing and editing: K.K., J.B.P., R.B., and M.L.-R.; Supervision: M.L.-R.; all authors have read and agreed to the published version of the paper.

### Competing interests

The authors declare no competing interests.

### Additional information

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