Rewiring cellular networks by members of the Flaviviridae family

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Abstract | Members of the Flaviviridae virus family comprise a large group of enveloped viruses with a single-strand RNA genome of positive polarity. Several genera belong to this family, including the Hepacivirus genus, of which hepatitis C virus (HCV) is the prototype member, and the Flavivirus genus, which contains both dengue virus and Zika virus. Viruses of these genera differ in many respects, such as the mode of transmission or the course of infection, which is either predominantly persistent in the case of HCV or acutely self-limiting in the case of flaviviruses. Although the fundamental replication strategy of Flaviviridae members is similar, during the past few years, important differences have been discovered, including the way in which these viruses exploit cellular resources to facilitate viral propagation. These differences might be responsible, at least in part, for the various biological properties of these viruses, thus offering the possibility to learn from comparisons. In this Review, we discuss the current understanding of how Flaviviridae viruses manipulate and usurp cellular pathways in infected cells. Specifically, we focus on comparing strategies employed by flaviviruses with those employed by hepaciviruses, and we discuss the importance of these interactions in the context of viral replication and antiviral therapies.

As obligate intracellular parasites, viruses strictly depend on their ability to manipulate the machinery of host cells to propagate. Consequently, viruses have evolved numerous strategies to manipulate infected cells by triggering a series of metabolic and structural changes that facilitate viral replication.

The Flaviviridae family provides many fascinating examples of virus-driven cellular reprogramming. This family is composed of four genera: Flavivirus (with 53 species); Hepacivirus (with 14 species); Pegivirus (with 11 species) and Pestivirus (with 4 species). Several members within the Hepacivirus and Flavivirus genera have a substantial impact on human health. Chronic infection by hepatitis C virus (HCV), the prototypic hepacivirus, is the leading cause of liver disease worldwide, with ~71 million individuals at risk of developing liver cirrhosis and hepatocellular carcinoma. Recently, highly effective direct-acting antiviral drugs targeting essential viral processes have become available for clinical use; however, a prophylactic vaccine to control the HCV pandemic is still missing (reviewed in REF. 3). In contrast to the predominantly persistent infection by HCV, human infections with flaviviruses are acute and self-limiting and are either asymptomatic or present as an undifferentiated febrile illness that can, in specific cases, lead to more severe symptoms, such as vascular leakage, severe haemorrhage, shock or serious neurological complications, such as encephalitis and meningitis. Dengue virus (DENV), the aetiologic agent of dengue fever and dengue haemorrhagic fever, or dengue shock syndrome, is responsible for an estimated 60 million symptomatic infections annually, causing approximately 10,000 deaths per year. Although a DENV vaccine has recently been licensed, its overall efficacy is limited, especially in immunologically naive individuals, and its administration is not recommended for young children or elderly people, both of whom have a higher risk of serious disease.

Examples of neurotrophic flaviviruses include West Nile virus (WNV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV) and Zika virus (ZIKV). The latter has recently spread worldwide, and infections have been linked to Guillain–Barré syndrome in adults and multiple neurodevelopmental defects, including microcephaly in infants born to mothers infected during the first trimester of pregnancy (reviewed in REF. 6). Although ZIKV is rarely neuro-invasive in adults, it can infect human neural progenitor cells (hNPC), likely resulting in the congenital disorders mentioned above (reviewed in REF. 7). At present, no approved antiviral drugs are available for the treatment of Flavivirus infections.
In addition to the marked differences in tropism and pathogenesis, viruses within the *Flavivirus* and *Hepacivirus* genera, while sharing similarities in their overall genome organization, differ in several respects, such as their mechanism of translation: a canonical, cap-dependent pathway in the case of flaviviruses or an internal ribosome entry site (IRES) in the case of HCV (FIG. 1). Along the same line, the general principle of the replication cycles of flaviviruses and hepaciviruses is similar (BOX 1 and BOX 2, respectively), but multiple
Microcephaly
A neurological condition of abnormal brain development that causes substantially smaller infant head circumferences relative to age-matched controls.

Tropism
Tissue specificity of a virus. Tropism is determined primarily by the presence of membrane receptors that can be exploited by the virus to gain access to host cells.

Figure 1 | Flavivirus and Hepacivirus genome organization and membrane topology of mature viral proteins. The ORF encoding the dengue virus (DENV) (part a) or hepatitis C virus (HCV) (part b) polyprotein and the predicted secondary structures of the 5′ and 3′ non-translating regions (NTR) are depicted on the top of each panel. a The DENV genome contains a type 1 cap structure at the 5′ end. Polyprotein cleavage by cellular signal peptides is indicated by scissors. Arrows denote the cleavage by the viral protease, whereas the black vertical arrow indicates cleavage by the Golgi apparatus-resident protease furin. The question mark denotes a DENV polyprotein cleavage performed by an unknown protease. The DENV structural proteins capsid protein C, prM and envelope protein E are constituents of the virion; NS1, the only non-structural protein residing in the lumen of the endoplasmic reticulum (ER), and NS2A are essential for virus replication and production of infectious particles; serum protease subunit NS2B acts as a cofactor for serine protease NS3 and recruits NS3 to ER membranes; NS3 is a multifunctional protein with protease, nucleotide 5′ triphosphatase (NTPase), RNA 5′ triphosphatase and helicase activities; NS4A is an integral membrane protein with membrane curvature-inducing activity; the 2K peptide serves as a signal peptide for co-translational NS4B insertion into the ER membrane; NS4B is a protein with no reported enzymatic activity that interacts with NS3 and is absolutely required for virus replication; NS5 consists of an N-terminal domain that possesses guanylyltransferase, guanine-N7-methyltransferase and nucleoside-2′-O-methyltransferase activities involved in 5′-RNA capping and methylation of the viral genome, and a C-terminal domain with RNA-dependent RNA polymerase activity responsible for viral RNA synthesis. b The HCV RNA genome is ~9.6 kb long, uncapped and flanked by highly structured 5′ and 3′ NTRs. The 5′ NTR contains a type III internal ribosome entry site (IRES) that directs the cap-independent translation of the viral RNA genome. Polyprotein cleavage by the viral protease is indicated by arrows, whereas cleavage by cellular signal peptides is indicated by scissors. The cleavage by the cellular signal peptide peptidase resulting in the removal of the HCV core carboxy-terminal region is indicated by an asterisk. The core protein and the envelope glycoproteins E1 and E2 constitute the viral particle, whereas p7 and NS2 support particle assembly yet are not incorporated into virions; the NS2 C-terminal domain contains a cysteine protease that catalyses the cleavage of the NS2–NS3 junction; NS3 contains serine protease, RNA helicase and NTPase activities; NS4A acts as cofactor for the NS3 protease and anchors NS3 to ER membranes; NS4B is involved in the formation of the HCV replication organelle; NS5A is a phosphoprotein with an intrinsically unfolded C-terminal region that mediates interactions with numerous cellular proteins; NS5B is the viral RNA-dependent RNA polymerase responsible for RNA synthesis. Note that only the DENV capsid and NS1 as well as HCV NS5A are shown as dimers, but additional viral proteins form homodimers and heterodimers or oligomeric complexes, AUG, methionine (start codon); D1, domain 1. Part b is adapted from REF. 39, Macmillan Publishers Limited.

Architectural replication organelles
Replication of the genome of positive-strand RNA ((+)-RNA) viruses occurs in close association with cellular endomembranes within organelle-like structures defined as replication organelles (ROs). These ROs serve to increase the local concentration of cellular and viral factors that are required for genome replication, coordinate the different steps of viral replication through their compartmentalization and to shield genomic RNA from cellular innate immune sensors. ROs can be grouped into two morphologically distinct classes designated as invaginated/spherule-type ROs or protrusion-type ROs. Invaginated/spherule-type ROs are generated by invaginations of the donor membrane into the organelle lumen. The protrusion-type ROs are composed of clusters of single-membrane vesicles (SMVs), double-membrane vesicles (DMVs), multimeric vesicles (MMVs) and often multimembrane tubules. Interestingly, although donor membranes can be provided by different organelles, ROs from all (+) RNA viruses can be classified into one of these two morphotypes, suggesting evolutionarily conserved mechanisms of their biogenesis.

In the case of flaviviruses, electron microscopy analysis of cells infected with DENV or ZIKV revealed the formation of clusters of vesicles ~90 nm in diameter, defined as vesicle packets (VPs), that are created by the invagination of rough endoplasmic reticulum (ER) membranes (FIG. 2A). Additionally, bundled smooth ER membranes, termed convoluted membranes (CMs), are often observed in close proximity to mitochondria (see below) and VPs. CMs and ROs are interconnected and form a single endomembrane network. A pore-like opening of ~11 nm connects the vesicle interior with the cytosol, presumably to allow for the exchange of metabolites and other molecules (FIG. 2A, inset). The detection of viral replicase components and double-stranded RNA (dsRNA) replication intermediates within invaginated vesicles suggests that VPs are the site of viral genome amplification. Virions bud within ER cisternae opposed to the pores of invaginated vesicles, and clusters of virions can often be observed in paracrystalline arrays in enlarged ER cisternae in close proximity to VPs.

The specific function of CMs in viral infection is still unclear. An enrichment for viral proteins but not dsRNA in these cellular structures suggests that CMs are sites of polyprotein maturation. CMs could also serve as lipid storage sites or interfere with innate immune responses by disrupting the mitochondria-associated membranes (MAMs), an important interface for innate immune signalling, or by sequestering innate immune pattern recognition receptors (PRRs). However, the absence of such structures in either DENV-infected mosquito cells or ZIKV-infected hNPCs questions a general function for CMs in viral replication and argues for a cell-type-specific role. In addition to VPs and CMs, tightly juxtaposed ER cisternae with limited luminal area, termed zipper ER (zER) membranes, are often observed in ZIKV-infected hepatoma cells. Interestingly, in hNPCs infected with
Internal ribosome entry site (IRES). A folded RNA element capable of recruiting the small ribosomal subunit that also mediates cap-independent initiation of RNA translation. IRES elements require only a subset of the canonical translation initiation factors, which are determined by the specific type of IRES.

Polyprotein
A polypeptide composed of individual domains that are released both co-translationally and post-translationally by proteolytic cleavage to produce functionally distinct proteins.

ZIKV, neither CMs nor zERs are formed, and the average diameter of VPs is considerably smaller (~60 nm versus ~90 nm in hepatoma cells), suggesting that cell type-specific factors help determine the architecture of ZIKV ROs.

In contrast to flaviviruses, HCV induces the production of ~150 nm diameter DMVs, forming clusters designated as the membranous web (FIG. 2B). Membranous web formation can be induced by the synthesis of the viral replicate proteins non-structural protein 3 (NS3) to

### Box 1 | Flavivirus replication cycle

Virus particles bind to the surface of susceptible cells, which include monocytes, skin dendritic cells (for example, Langerhans cells in the case of dengue virus (DENV)), neurons (for example, in the case of West Nile virus (WNV) and Zika virus (ZIKV)), placental macrophages (Hofbauer cells), trophoblasts, human neural progenitor cells, testicular cells, uterine fibroblasts and eye-associated tissues (for example, in the case of ZIKV). The current view is that viral particles first interact with attachment factors that are required to tether the virion onto the cell surface, which is followed by further specific interactions with secondary receptors, mediating endocytic internalization and likely defining tissue specificity. Receptors or attachment factors that were identified in relevant target cells include the C-type lectin CD209 antigen (also known as DC-SIGN), which is exploited by all four DENV serotypes and WNV to attach to dendritic cells; the mannose receptor, described as a DENV receptor in macrophages; and members of the TIM (T cell immunoglobulin mucin domain protein 1) and TAM (tyrosine protein kinase receptor 3 (TYRO3)–AXL–MER) family of phosphatidylserine receptors, facilitating DENV infection of primary epithelial cells and ZIKV entry into placenta cells, skin fibroblasts and glial cells.

Flavivirus particles predominantly enter via a clathrin-dependent entry pathway, exposing viral particles to an acidic endosomal compartment that triggers conformational rearrangements of the envelope glycoproteins to allow fusion of viral and endosomal membranes, resulting in the release of the viral RNA into the cytosol. The released positive-sense RNA (RNA) is recognized by ribosomes, initiating translation at the rough endoplasmic reticulum (ER) membrane and producing a single polyprotein. Viral and cellular proteases catalyse the co-translational and post-translational cleavage of the polyprotein into three structural and seven non-structural proteins, almost all of which are associated with intracellular membranes (see the figure). Whereas the structural proteins are constituents of the virion, the non-structural proteins orchestrate ER membrane invaginations, which are the presumed site of RNA genome amplification via the synthesis of a negative-sense RNA (RNA) intermediate. All Flavivirus non-structural proteins are essential for RNA replication, with DENV non-structural protein 1 (NS1) having a dual role in RNA replication and virus particle production. The (+)RNA molecules that are generated by the viral replicase complex can be incorporated into viral particles, a process involving RNA encapsidation and budding into the lumen of the ER, which occurs predominantly in regions opposite to the replication sites. Maturation of nascent DENV particles containing prM occurs along the secretory pathway by furin-mediated cleavage of prM. Flavivirus particles are presumed to exit the cell through the conventional secretory pathway, although the details of how this process occurs and whether lipid droplets, as well as apolipoproteins, are involved in virus production, as is the case of hepatitis C virus infection, is still uncertain. HSPG, heparin sulfate proteoglycans.
Very-low-density lipoprotein (VLDL). A liver-produced plasma lipoprotein particle ~30–70 nm in diameter involved in cholesterol and triglyceride transport. Low-density lipoprotein and VLDL contain distinct sets of apolipoproteins.

NS5B independent of viral RNA replication. The DMV outer membrane is often linked to the ER, suggesting that DMVs originate as protrusions that extend from the ER towards the cytosol16 (Fig. 2B, inset). In addition to DMVs, SMVs and MMVs can also be observed within the membranous web (Fig. 2B). Though the contribution of SMVs to the viral replication cycle is still unclear, MMVs have been shown to form after the formation of DMVs and might represent a replication by-product or arise as the result of a cellular stress response17.

Box 2 | Hepatitis C virus replication cycle

Hepatitis C virus (HCV) particles are closely associated with host cell lipoproteins and have a high lipid content. These lipoviroparticles primarily target hepatocytes and enter cells through stepwise binding to several host cell receptors that eventually localize viral particles to tight junctions where they are internalized (see the figure). In this case, heparan sulfate proteoglycans (HSPGs) together with low-density lipoprotein receptors (LDLRs) and other cell surface molecules mediate the initial binding to the cell. Viral particles then interact with the scavenger receptor class B member 1 (SRB1) and the tetraspanin CD81, which guide the lateral diffusion of bound virions towards the apical membrane. There, CD81 associates with the tight junction proteins claudin 1 (CLDN1) and occludin (OCLN), a step that is critical for endocytic internalization via the clathrin-dependent entry pathway.

After release of the viral RNA into the cytosol, the viral genome is translated at the rough endoplasmic reticulum (ER), and viral proteins together with host cell factors form the replication organelle, which is composed primarily of double-membrane vesicles (DMVs), a fraction of which remain open towards the cytosol. DMVs either remain linked to the ER or are released. HCV particle assembly occurs in close association with cytosolic lipid droplets at sites most likely enriched in core protein; envelope glycoproteins E1 and E2; p7 and non-structural protein 2 (NS2)163. Formation and possibly also secretion of newly formed virions occur in association with components of the very-low-density lipoprotein (VLDL) machinery. Secreted HCV particles exhibit an unusually low buoyant density (≤1.055 g/ml in serum and ~1.1 g/ml in cell culture), are highly pleomorphic, incorporate cellular lipoproteins (most notably apolipoprotein E (APOE)) and have a lipid composition resembling low-density lipoprotein (LDL) particles (reviewed in REF. 119). APOE might be acquired during or after engulfment of HCV particles and contribute to particle maturation, particle release or acquisition of infectivity164. Interestingly, APOE, the secreted glycoprotein E NS3 of pestiviruses and NS1 of flaviviruses were reported to fulfil the same function in virus maturation and can even rescue infectivity of HCV particles that are produced in APOB–APOE double knockout cells165. Most recent morphological and biochemical analyses have shown that HCV forms lipoviroparticles (that is, particles resembling VLDL)166 but can also bind to VLDL particles after release from cells, thus forming particle complexes167,168. Progeny HCV virions are presumed to exit the cell via secretory pathways.

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The correlation between DMV abundance and RNA amplification, as well as the presence of replicase activity in isolated DMVs, suggests that DMVs constitute the site of HCV RNA replication. However, it is not yet possible to unambiguously localize the site of de novo RNA synthesis to either the lumen or to the outer membrane of DMVs. Biochemical studies have shown that HCV RNA and replicase activity reside within a nucleoseresistant and protease-resistant environment, supporting the hypothesis that replication occurs within the membrane-protected luminal side of DMVs. Exchange of metabolites and factors that are required for replication, as well as the exit of newly synthesized genomes from DMVs, could occur through pore-like openings, which were observed in ~10% of the vesicles. This would suggest that only a minor proportion of the DMVs supports active replication at a given time point and that replication might cease when membrane openings are closed. Alternatively, proteinaceous transport complexes, such as nuclear pore complex-like structures, might enable traffic in and out of a closed membrane compartment.

**Biogenesis of replication organelles**

Whereas the morphology and the architecture of Flavivirus ROs are well-defined, relatively little is known about the molecular mechanisms governing the biogenesis of VPs and CMs. In the case of DENV, available evidence argues for a prominent role of the two small non-structural proteins NS4A and NS4B in the formation of ROs. Both proteins possess multiple transmembrane spanning α-helices and a single amphipathic α-helix that is partially embedded into the luminal leaflet of the ER (Figs 1a,2A). The lipid bilayer asymmetry that is produced by the α-helix insertion might act as a wedge and induce negative membrane curvature.

Moreover, membrane bending might be increased by the formation of NS4A and NS4B homo-oligomers and hetero-oligomers. However, the individual or combined expression of NS4A and NS4B outside the context of viral infection does not induce the formation of VPs, suggesting that the membrane remodeling functions of these proteins are not sufficient to phenocopy DENV ROs and that additional viral factors are required. Owing to its analogy to alphaviruses and the Flock House virus, viral RNA could be one candidate factor.

Several reports have also suggested roles for non-structural proteins NS1 and NS2A in the formation of Flavivirus VPs. NS1 interacts with both NS4A and NS4B (Fig. 2A), and recombinant NS1 interacts with and remodels lipids in vitro. NS2A is a small hydrophobic protein with five transmembrane α-helices that can alter membrane permeability. In addition, NS2A is enriched in subcellular regions containing viral dsRNA and may interact with replicase proteins. Together, these results support a model in which oligomers of NS4A and NS4B, and possibly NS2A, induce negative membrane curvature, with NS1 dimers present within the ER lumen promoting positive membrane curvature and assisting in the formation of invaginated vesicles (Fig. 2A, inset). Additionally, host factors such as components of the endosomal sorting complex required for transport (ESCRT) machinery appear to have a role in the formation of VPs, for example, by participating in the assembly of the vesicle pore.

The concerted action of the viral replicase complex proteins, together with tightly regulated polyprotein cleavage, is required for the formation of the HCV membranous web (reviewed in Ref. 8). Key contributors to its biogenesis are NS4B and NS5A. The complex topology of NS4B, which is comprised of four transmembrane α-helices flanked by two amphipathic α-helices on either side (Fig. 1b), together with its oligomerization capabilities, may support and promote positive membrane curvature. Moreover, essential residues for DMV formation and RNA replication have been identified within the NS4B carboxy-terminal domain. Regarding NS5A, it is the only HCV protein able to induce DMVs, a process that is facilitated by determinants that are located within the membrane-associated amino-terminal amphipathic α-helix of NS5A domain 1 (Ref. 31). Furthermore, NS5A recruits several host factors that are essential for membranous web formation. Finally, NS5A inhibitors as well as antagonists of cyclophilin A, a chaperone that binds to NS5A, block membranous web formation, highlighting its essential role in the biogenesis of HCV ROs.
Cyclophilin inhibitors to treat HCV infections

Cyclophilin A (CYPA) is a highly abundant cytosolic peptidyl-prolyl isomerase (PPIase) that catalyzes the cis–trans isomerization of peptide bonds at proline residues and hence facilitates protein folding. The initial observation that the immunosuppressive drug cyclosporin A (CsA) inhibits HCV replication in cell culture led to the discovery of CYPA as an essential host factor that interacts with the viral non-structural protein 5A (NNSA) protein. In addition to CsA derivatives such as alisporivir (formerly known as Debo 025), NIM811 and SCY635 that retain high-affinity CYP binding but lack immunosuppressive effects, are potent HCV inhibitors. More recently, alisporivir has been advanced into phase II and III clinical trials for HCV therapy and has demonstrated promising efficacy with an acceptable safety profile.27,28

α-Glucosidase inhibitors to treat DENV infections

The endoplasmic reticulum-resident α-glucosidase I and II catalyze the trimming of glucose residues on N-linked oligosaccharides, a step that is essential for protein recognition and folding by the chaperones calnexin and calreticulin. Several DENV proteins, including prM, envelope protein E, NS1 and NS4B, are glycosylated, and treatment with α-glucosidase inhibitors was shown to reduce DENV replication and morphogenesis in cell culture and in vivo. Unfortunately, a phase Ib clinical trial aimed at assessing the efficacy of the α-glucosidase inhibitor celgosivir in DENV-infected individuals showed that, although safe and well tolerated, celgosivir does not reduce viral load or fever burden.29,30 Further optimization of celgosivir treatment in mouse models suggests that the drug loses efficacy when administered at the peak of viremia. However, in these conditions, the antiviral action can be improved by changing the dosage and regimen of administration.31 Therefore, a new phase Ib–IIa clinical trial with a revised dosing regimen is currently ongoing.32

Rewiring cellular pathways

The generation of a host cell environment that is permissive to viral replication requires the utilization of numerous host cell pathways. Viruses can either use these pathways without manipulating them or they can hijack or change host cell pathways to benefit virus replication. Examples for both of these scenarios have been observed for components of the host cell protein synthesis and processing pathways in infections caused by members of the Flaviviridae virus family.

Protein folding and chaperones

Replication of Flaviviridae family members also relies on host cell chaperones for proper synthesis and folding of the viral proteins. Indeed, numerous chaperones are reported to be required for specific steps of Flaviviridae replication cycles. For instance, heat shock protein 70 (HSP70) functions at many stages of the Flavivirus replication cycle, from virion entry into host cells to assembly and the release of viral particles.33-35 In the case of DENV, HSP70 cofactors DNAJ homologue subfamily B member 11 (DNAJ11) or DNAJB6 promote either viral replication or particle biogenesis, respectively, whereas DNAJC14 has antiviral activity against both DENV and the yellow fever virus (YFV).36,37 Importantly, inhibition of the HSP70–DNAJ network with the inhibitory drug JG40 significantly decreased virus replication for multiple DENV serotypes as well as WNV, YFV and tick-borne encephalitis virus (TBEV).38 Currently, there is little information on the involvement of host cell chaperones in ZIKV infection. However, the conserved
requirement for these proteins by other *Flaviviridae* family members suggests that components of the host cell molecular chaperone network will likely also have a role in the ZIKV replication cycle.

In HCV-infected cells, viral proteins interact with multiple components of the HSP90 and HSP70 chaperone networks, and the function of these chaperones is required at various stages in virus infection. Specifically, NS5A is reported to form a complex with both HSP70 and heat shock cognate 71 kDa protein (HSC70; also known as HSPA8); the former being required for viral protein synthesis and the latter having a role in virion assembly. Inhibition of the different HSP70 network components, either through protein depletion or by addition of specific inhibitors, blocks viral protein synthesis or virus assembly. This conserved requirement for chaperones in the replication cycles of viruses makes them a promising target for the development of antiviral drugs that might be effective against multiple viruses. An example of this are cyclophilins (CTPs); the CYP chaperone activity, especially CYP-A, was shown to be crucial for HCV replication and has been used with clinical success as a drug target for the treatment of chronic HCV infection (BOX 3).

The unfolded protein response. High levels of viral RNA and proteins in infected cells causes increased cellular stress, leading to the activation of cellular pathways that mitigate stress and promote cell survival or trigger apoptosis (FIG. 3A). The unfolded protein response (UPR) is a pathway aimed at compensating for increases in ER stress by increasing the ER protein-folding capacity, attenuating mRNA production and stimulating the ER-associated degradation (ERAD) of misfolded proteins. Initiation of the UPR is facilitated by the interaction of immunoglobulin heavy chain-binding protein (BIP) with one of three ER sensors: serine/threonine-protein kinase/endoribonuclease (IRE1), eukaryotic translation initiation factor 2-α kinase (PERK; also known as EIF2AK3) and cyclic AMP-dependent transcription factor ATF6-α (ATF6). Additionally, UPR activation leads to increased autophagy, oxidative stress and stress granule formation and has been linked to the potentiation of antiviral inflammatory responses. If these mechanisms are not effective at restoring ER homeostasis, active UPR pathways lead to apoptosis.

Activation of all three UPR pathways has been reported for DENV and HCV infections both in patient samples and in cell culture models, and several UPR proteins have been identified as important factors in *Flaviviridae* infection (reviewed in REF. 60, 69, 70) (FIG. 3A). For DENV infection, a time-dependent induction of each UPR pathway has been reported. At early stages of infection, the PERK pathway is activated, leading to a translational block through the phosphorylation of eukaryotic translation initiation factor 2 subunit 1 (eIF2α), an event that also leads to the production of cytoplasmic stress granules. DENV overcomes this block and antagonizes stress granule formation by reversing eIF2α phosphorylation, presumably through the activation of the negative feedback factor protein phosphatase 1 regulatory subunit 15A (GADD34; also known as PPP1R15A) (REFS 71, 73). Interestingly, a recent report demonstrated that DENV potentiates a host cell translational block by stimulating eIF4E phosphorylation, which in turn limits cap-dependent translation. This translational repression did not decrease viral protein synthesis, supporting a model in which DENV can switch between cap-dependent and cap-independent translation during infection.

PERK activation has also been observed for HCV infection both in patient samples and cell culture systems. However, contrasting reports have suggested that HCV both stimulates and represses eIF2α phosphorylation (FIG. 3A), and it has been proposed that the IRES mediates RNA translation in an eIF2α-dependent or eIF2α-independent manner, depending on the abundance of active eIF2α (reviewed in REF. 75, 76). Additionally, oscillating stress granule formation has been observed in HCV infection, which has been suggested to be a result of eIF2α phosphorylation. This flexibility in translation strategies may confer HCV the ability to overcome some of the host cell antiviral strategies, thus promoting persistence.

Increasing levels of DENV structural proteins cause UPR activation via IRE1 with two major outcomes: induction of the regulated IRE1-dependent decay (RIDD) pathway and stimulation of X-box-binding protein 1 (XBP1) mRNA splicing to make XBP1 (REF. 71). XBP1 is then translocated to the nucleus where it acts as a transcription factor, facilitating the activation of the ERAD pathways and stimulating expansion of the ER membrane, both of which help to alleviate DENV-induced ER stress. DENV has developed mechanisms to block the downstream apoptotic mediators of the IRE1 pathway, thereby taking advantage of the prosurvival properties of this pathway to enhance viral replication (reviewed in REF. 70). Whereas the IRE1 pathway is also activated by HCV infection and might have a proviral function, mainly through the induction of autophagy (see below), in the case of DENV IRE1-mediated XBP1, activation causes membrane expansion that is important for viral replication and is possibly involved in the formation of VPs.

In addition to IRE1, the ATF6 branch of the UPR is activated by both DENV and HCV infection (FIG. 3A), and in both cases, this pathway has proviral activity. In combination with the IRE1 pathway, ATF6 activation also stimulates ERAD, although ATF6 activation is associated with lower levels of ER stress, whereas IRE1 activation occurs under higher levels of stress. Taken together, these studies suggest that *Flaviviridae* members must maintain a balance between activation and suppression of host cell stress responses to maintain cell survival, avoid immune activation and ensure efficient viral protein production. Thus, tipping this balance in one direction could be an important tool for manipulating viral replication.

Protein degradation

The ubiquitin-dependent proteasome system (UPS) and autophagy are the two main cellular degradation pathways that are responsible for protein homeostasis. Both
pathways are involved in the cellular defence against viral infections and, therefore, viruses have evolved mechanisms to manipulate these protein degradation pathways for their benefit.

**Ubiquitin-dependent proteasome system.** Genome-wide knockdown or knockout screens, together with transcriptomic and proteomic analyses conducted in both mosquito and mammalian cells, revealed the involvement of various components of the UPS in Flavivirus infection\(^2\) (Fig. 3B). For example, components of the ERAD pathway were identified in a recent CRISPR–Cas9 knockout screen as essential factors for several flaviviruses\(^4\). For DENV infection, upregulation of UPS-related genes has been observed in both infected cell lines and peripheral blood mononuclear cells derived from infected individuals.\(^5\)
from infected individuals, arguing for the physiological relevance of the UPS in Flavivirus infection. Moreover, the UPS or components thereof are required for efficient virus production in the mosquito midgut, which is consistent with quantitative proteomic analyses of mosquito cells infected with ZIKV that show increased UPS protein levels. Proteasome inhibitors MG132 and bortezomib exert antiviral activity against ZIKV and DENV in vitro and reduce viral load in an in vivo mouse model. Collectively, these data indicate that both early and late events in the Flavivirus replication cycle require components of the UPS. Although a strong dependence on the UPS was not observed for HCV infection, several UPS components are required for efficient viral replication. Interestingly, UPS components of relevance for HCV replication are also required for WNV and DENV replication, suggesting an evolutionarily conserved role in the replication cycles of members of Flaviviridae. UPS-mediated viral protein degradation can also restrict viral replication and enable an adaptive antiviral immune response by increasing the presentation of antigens to cytotoxic T cells. By contrast, HCV exploits UPS-mediated protein degradation to alter the stoichiometry of viral proteins. For instance, it has been reported that the viral NS5B RNA-dependent RNA polymerase is rapidly degraded to prevent possible interference with genome packaging. The proteasome also regulates HCV core stability via ubiquitin-dependent and ubiquitin-independent mechanisms, thus restricting or enhancing HCV particle production, respectively. Finally, all Flaviviridae family members induce proteasomal degradation of specific restriction factors as well as innate signalling components such as signal transducer and activator of transcription 2 (STAT2) in the case of DENV and ZIKV) or signal transducer and activator of transcription 1 and 3 (STAT1 and STAT3, respectively) in the case of HCV.

**Autophagy.** Autophagy is a catabolic process involving the formation of DMVs (autophagosomes) that engulf cytoplasmic content, cellular organelles, protein aggregates and pathogens for lysosomal degradation. Increased formation of autophagosomes and modulation of the autophagic flux has been consistently observed in DENV, ZIKV and HCV-infected cells. For flaviviruses, autophagy induction is mediated by NS4A for DENV and NS4A in addition to NS4B for ZIKV. In contrast to DENV, where NS4A-induced autophagy confers protection from cell death, ZIKV proteins dysregulate autophagy through AKT1–mTOR (RAC-α serine/threonine-protein kinase–mechanistic target of rapamycin) inhibition, leading to increased cell death and altered neurogenesis in fetal neural stem cells. For DENV, infectious vesicles containing viral RNA and autophagy markers were detected in supernatants of infected cells and in patient sera, supporting a role for autophagy as an alternative viral transmission route.

For both flaviviruses and HCV, viral proteins and RNA were found to colocalize with autophagosomes, suggesting that viral ROs associate with autophagosomes. Whereas the ER origin of flavivirus ROs refutes this hypothesis, an involvement of autophagic factors in the formation of the HCV-induced membranous web was recently reported. This study showed that the autophagosome membrane elongation complex ATG5–ATG12–ATG16L1 was recruited to the membranous web, potentially through an interaction between viral RNA fragments and infectious full-length viral genomes, consistent with quantitative proteomic analyses of mosquito cells infected with ZIKV that show increased UPS protein levels. Proteasome inhibitors MG132 and bortezomib exert antiviral activity against ZIKV and DENV in vitro and reduce viral load in an in vivo mouse model. Collectively, these data indicate that both early and late events in the Flavivirus replication cycle require components of the UPS. Although a strong dependence on the UPS was not observed for HCV infection, several UPS components are required for efficient viral replication. Interestingly, UPS components of relevance for HCV replication are also required for WNV and DENV replication, suggesting an evolutionarily conserved role in the replication cycles of members of Flaviviridae. UPS-mediated viral protein degradation can also restrict viral replication and enable an adaptive antiviral immune response by increasing the presentation of antigens to cytotoxic T cells. By contrast, HCV exploits UPS-mediated protein degradation to alter the stoichiometry of viral proteins. For instance, it has been reported that the viral NS5B RNA-dependent RNA polymerase is rapidly degraded to prevent possible interference with genome packaging. The proteasome also regulates HCV core stability via ubiquitin-dependent and ubiquitin-independent mechanisms, thus restricting or enhancing HCV particle production, respectively. Finally, all Flaviviridae family members induce proteasomal degradation of specific restriction factors as well as innate signalling components such as signal transducer and activator of transcription 2 (STAT2) in the case of DENV and ZIKV) or signal transducer and activator of transcription 1 and 3 (STAT1 and STAT3, respectively) in the case of HCV.
ER-phagy through viral protease-dependent cleavage of the ER-phagy receptor reticulophagy regulator 1 (FAM134B114–117, also known as RETREG1) (FIG. 3B). By contrast, HCV induces mitophagy to attenuate virus-induced apoptosis and, consistent with these observations, inhibition of mitophagy by knockdown of parkin impairs HCV replication118. To summarize, autophagy has mostly been attributed to a proviral function in HCV, DENV and ZIKV replication cycles; however, further studies are required to better characterize the molecular mechanisms of autophagy–virus interactions.

**Lipids and lipid metabolism**

*Flaviviridae* replication occurs in strict association with cellular membranes, and members within this virus family have developed the ability to modify the lipid composition of membranes at replication and assembly sites (reviewed in REFs 119,120). These lipid alterations presumably change the physical properties of membranes, such as permeability, fluidity and bending capacity. Several studies highlight the importance of lipid synthetic pathways in *Flaviviruses* and *Hepaciviruses* replication (FIG. 3C). For instance, DENV, WNV and HCV replication is highly sensitive to depletion or pharmacological inhibition of acetyl-CoA carboxylase and fatty acid synthase (FASN)120,121, which are key enzymes in the fatty acid biosynthetic pathway. Indeed, DENV NS3 and HCV NS5B proteins were shown to interact with FASN and mediate its recruitment to the vicinity of viral replication sites, where it likely promotes the local synthesis of fatty acids that are required for efficient virus replication or, alternatively, enhances the catalytic activity of the NS5B polymerase in the case of HCV115. In addition to fatty acids, cholesterol biosynthesis also appears to be required for *Flaviviridae* virus replication. Inhibitors of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCGR), a rate-limiting enzyme in the cholesterol biosynthetic mevalonate pathway, decreases DENV replication in primary human monocytes and cultured cell lines120. It was observed that DENV infection increases the activity of HMGCGR through the inactivation of 5’ AMP-activated protein kinase (AMPK), resulting in higher levels of cholesterol in the ER and increased viral replication122 (FIG. 3C). Consistent with this, treatment with AMPK inhibitors was shown to exert a proviral effect. However, a recent report suggests that DENV infection transiently stimulates AMPK phosphorylation, leading to the inactivation of mTOR complex 1 (mTORC1) and induction of lipophagy117. In this study, pharmacological inhibition of AMPK was found to be antiviral. The reasons behind these conflicting results are currently unknown but may relate to the experimental conditions that were used or a cell type-dependent effect.

For HCV, virus infection or ectopic expression of core protein or NS4B was able to induce the proteolytic activation of transcription factors belonging to the sterol regulatory element-binding protein (SREBP) pathway and increase the level of FASN, HMGCGR and other lipogenic transcripts119. Additionally, the ATP-dependent RNA helicase DDX3X interacts with the HCV 3’ UTR and activates the innate immunity regulator inhibitor of nuclear factor-κB kinase subunit-α (IKKα), which in turn induces the expression of SREBP through CREB-binding protein–histone acetyltransferase p300 (CBP–p300)-dependent gene induction119,123 (FIG. 3C). Alterations in the lipid profile of infected cells were also uncovered by high-resolution mass spectrometry analysis of DENV, WNV or HCV-infected cells124–126. These studies revealed that changes in the lipid composition were especially pronounced in membrane fractions that were enriched in viral ROs, where the content of phospholipids, glycosphingolipids and sphingolipids (mainly ceramide) was increased124–126. Interestingly, although DENV and HCV promote an overall increase in cellular phosphatidylcholine content, only HCV seems to induce the accumulation of this lipid in the perinuclear ER membrane where NS5A is present124,126,127. In addition, purification of HCV DMVs revealed cholesterol enrichment, which is otherwise present at low levels in the ER of uninfected cells128. It was found that different cholesterol transport proteins, such as the oxysterol-binding protein (OSBP) and the Niemann–Pick C1 protein (NPC1), participate in this cholesterol enrichment and are selectively exploited by HCV but not DENV128,129. Overall, these findings support the concept that hepaciviruses and flaviviruses alter the local lipid composition of the ER to promote their replication.

A remarkable example of the importance of the generation of specialized membrane microenvironments during virus replication is the activation of phosphatidylinositol 4-kinase-α (PI4KA) by HCV NS5A, which results in the local enrichment of phosphatidylinositol-4-phosphate (PtdIns4P)125,130. Reduction of PI4PK activity is required for the formation of HCV replication complexes and the Niemann–Pick C1 protein (NPC1), participate in this cholesterol enrichment and are selectively exploited by HCV but not DENV128,129. Overall, these findings support the concept that hepaciviruses and flaviviruses alter the local lipid composition of the ER to promote their replication.

**Utilization of organelles and networks**

The utilization or manipulation of cellular organelles and molecular networks of relevance for cellular homeostasis by viruses must tread a fine line between promoting virus propagation and maintaining cell survival. This also applies to *Flaviviridae* members that exploit cytoskeletal, mitochondrial or nucleocyttoplasmic transport networks (FIG. 4).
**Cytoskeleton**

The cytoskeleton has been reported to function at several stages of the replication cycle for members of the *Flaviviridae* family. For DENV infection, entry into host cells depends on microfilament integrity. Additionally, the formation of viral ROs causes substantial remodelling of the intracellular endomembrane system and concomitantly alters the cytoskeletal architecture. DENV and ZIKV rearrange the cytoskeleton to form a cage-like structure that surrounds viral ROs (FIG. 4c). In the case of DENV, the reorganization of the intermediate filament vimentin has been observed, which is thought to arise through interactions with NS4A. Moreover, treatment of infected cells with drugs that disrupt intermediate filaments also inhibits viral replication. In ZIKV-infected cells, nuclei are distorted to accommodate the perinuclear accumulation of viral ROs, which are surrounded by thick bundles of intermediate filaments and microtubules. Treatment with paclitaxel, a microtubule-stabilizing drug, has a strong antiviral effect, suggesting that microtubule flexibility is required for efficient ZIKV infection.
For HCV, microtubules are required for both the entry and post-entry steps of viral infection. Inhibition of actin and microtubule polymerization decreases viral RNA levels in HCV-infected cells, suggesting a role for these structures in translation or viral genome amplification. Recently, a role for septins in HCV replication has been suggested. For instance, septin 9 interacts with microtubules and PtdIns5P to modulate lipid droplet growth and subcellular localization, creating a lipid-enriched environment that is favourable for HCV replication (Fig. 4). Thus, the role of the cytoskeleton may extend beyond viral entry and contribute to the biogenesis and maintenance of viral ROs.

Nucleocyttoplasmic transport machinery

The nuclear pore complex (NPC) mediates macromolecular transport between the cytoplasm and the nucleus and, as such, the NPC and the associated transport machinery have important roles in regulating many cellular pathways. Viruses have evolved ways to utilize or bypass the NPC or nucleocytoplasmic transport machinery in order to manipulate the host cell environment and facilitate viral propagation (reviewed in REF. 139). In the case of Flaviviridae family members, many viral proteins are reported to interact with components of the nuclear transport machinery in order to gain access to the nucleus or to disrupt nucleocytoplasmic transport (Fig. 4). For instance, the DENV capsid and NS5 proteins contain nuclear localization signal (NLS) sequences and localize to the nucleus. Though the majority of ZIKV and DENV NS5 protein is observed in the nucleus, there is no nuclear function currently associated with these proteins, and the disruption of this nuclear localization does not strictly correlate with changes in DENV replication in vitro.

Several HCV proteins, including core protein, NS2, NS3 and NS5, also contain NLS sequences and have been reported to interact with components of the nuclear transport machinery (reviewed in REF. 140) (Fig. 4d). However, unlike DENV capsid and NS5, in HCV-infected cells, substantial levels of viral protein accumulation in the nucleus have not been observed. Two roles for these NLS sequences have been proposed. First, several reports have suggested that nuclear NS5A and core protein directly or indirectly alter host transcriptional activation. Second, recent reports have proposed that the nuclear transport machinery has a cytoplasmic role in protecting HCV RNA from being sensed by PRRs. These reports suggest that HCV recruits components of the NPC and nuclear transport machinery to viral ROs to create a selective barrier between the cytosol and the interior of ROs, thus effectively blocking immune activation while still allowing access to molecules that are required for viral replication.

Selective trafficking of molecules, especially viral genomes and metabolites, between the cytosolic and the membrane compartments of ROs, as well as passive immune evasion through shielding of replication intermediates, has been proposed for other (+)RNA viruses, including DENV and TBEV. These results suggest that cellular transport molecules may be involved in chaperoning or transporting viral genomic material between specific cellular compartments, substantially impacting the spatio-temporal organization of viral ROs. Components of the nucleocytoplasmic transport machinery have also been found in HCV particles, suggesting that these proteins have a structural role in virus assembly. Moreover, recent reports have demonstrated that both HCV and JEV interfere with the nuclear translocation of interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB), thereby attenuating innate immune activation (Fig. 4d). Thus, the NPC and associated transport factors represent a central host network that is a strategic target for Flaviviridae infection.

Mitochondria

Mitochondria and MAMs are also convergent sites for cellular processes that are important for viral infection, including ATP synthesis, lipogenesis and export, PRR-mediated immune activation and apoptosis initiation. Flaviviridae family members interact with mitochondria and MAMs to either potentiate or mitigate these mitochondria-associated processes. Reports on the impact of flaviviruses, particularly DENV, on mitochondrial structure and function are still somewhat contradictory. Two recent studies demonstrate that DENV or ZIKV infection antagonizes dynamin 1-like protein (DRP1) function, leading to mitochondrial elongation and a reduction in MAMs, which favours viral replication. This virus-induced mitochondrial elongation enhances replication and the sequestration of MAMs in virus-induced CMs appears to contribute to attenuating innate immune activation (Fig. 4). Moreover, the expression of DENV NS4B is sufficient to induce mitochondrial elongation. In contrast to these findings, two other studies have reported DENV-induced mitochondrial fragmentation that is mediated by mitofusin degradation. These studies suggest a mechanism similar to HCV in which virus-induced mitochondrial fission inhibits apoptosis and alleviates immune activation. The differences between these reported observations could arise from differences in experimental setup or host cells that were used for infection. However, in both cases, it is clear that mitochondrial morphodynamics have an important role in Flavivirus infection.

In the case of HCV, viral proteins are enriched on mitochondria and MAMs, and viral infection promotes mitochondrial fragmentation and mitophagy. Additionally, reports have described MAMs as the sites of either viral RNA replication or assembly, further demonstrating the close association between HCV and mitochondria. The mitochondria and MAMs are also important for immune signalling, and both DENV and HCV have been suggested to disrupt the mitochondrial network in order to avoid immune activation.

Conclusions

Virus replication requires sophisticated manipulation of cellular pathways to maintain a balance between host cell survival and efficient virus replication. In the case of the Flaviviridae, virus infection leads to a substantial rearrangement of host cell structures and alterations in...
many pathways, creating an environment that is permissive to virus propagation. Considerable advances have been made in this area that have provided new and important insights into various aspects of the cell biology of virus infection, such as the discovery of a novel pathway to induce the expression of genes that are required for lipid biosynthesis, the identification of new protein folds as illustrated by the three-dimensional structure of DENV NS1 or novel insights into the mechanisms underlying virus-induced neuropathogenesis. Moreover, comparative analyses of flaviviruses and hepacviruses as reviewed here will help us to understand the evolutionary relationship between virus genera. For instance, the biogenesis and three-dimensional structures of the membranous ROs of flaviviruses are more similar to those of alphaviruses and nodaviruses, whereas the ROs of HCV resemble more those of picornaviruses and coronavirus (reviewed in REF 156). This probably reflects the differential use of distinct lipid synthesis and transfer pathways, which offers an interesting yet challenging alternative for the analysis of virus evolution that so far is primarily based on algorithms comparing nucleotide or amino acid sequence similarities. High-content screening projects have led to the identification of several host cell pathways and individual factors as promising targets for broad-spectrum antivirals, such as the signal peptide complex, the OST complex and the host cell chaperone network as well as others.

Although studies in more physiologically relevant systems are required to demonstrate the suitability of these targets for therapeutic approaches, the results presented so far have revealed surprising new insights into the cell biology of these cellular machines. For instance, an individual subunit of the signal peptide complex can be eliminated without overt cytotoxicity in vitro while blocking virus replication, arguing that an approach to target this subunit could have strong antiviral effects with low cytotoxicity. In light of these results, we can expect that additional fundamental discoveries in cell biology will continue to be made while enhancing our understanding of virus replication and pathogenesis. This knowledge may provide insights into how the balance could be tipped in a direction that promotes the activation of pathways that are aimed at limiting virus replication or spread, with the ultimate goal of developing broad-spectrum antivirals.

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This genome-wide CRISPR–Cas9-based screen to identify host dependency factors for dengue virus infection uncovers translation suppression from cellular stress responses.

This genome-wide CRISPR–Cas9-based screen to identify host dependency factors for \textit{Flavivirus} infection uncovers translation suppression from cellular stress responses.

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Author contributions
C.J.N., M.C. and E.G.A. researched data for the article. C.J.N., M.C., E.G.A. and R.B. substantially contributed to discussion of content, wrote the article and reviewed and edited the manuscript before submission.

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