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.

Guillain–Barré syndrome
An acute neurological disease (usually reversible) resulting from autoimmune destruction of the peripheral nervous system that is frequently triggered by infection.

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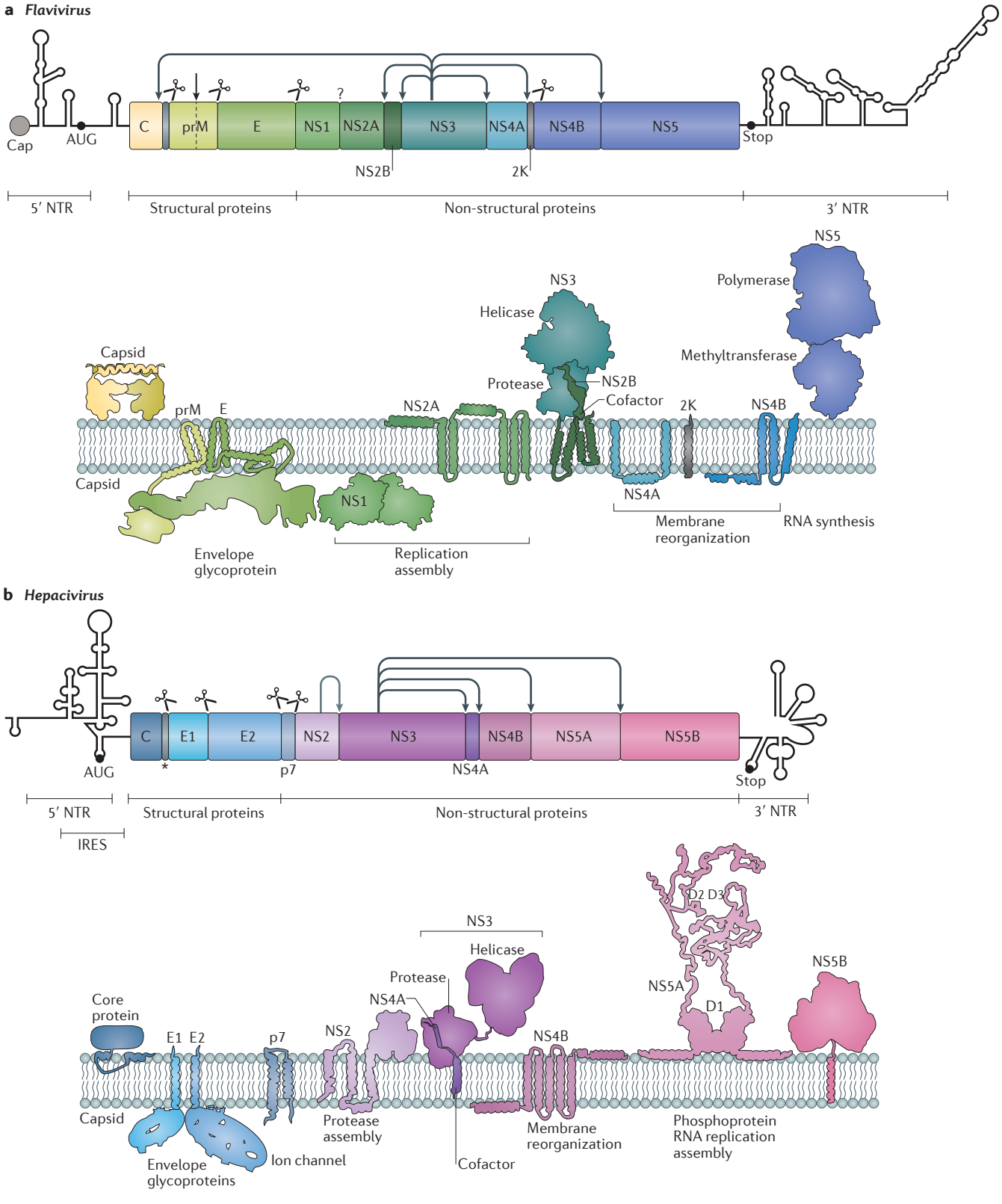
As obligate intracellular parasites, viruses strictly depend on their ability to manipulate the machinery of host cells to propagate. Consequently, viruses have evolved numerous strategies to manipulate infected cells by triggering a series of metabolic and structural changes that facilitate viral replication.

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)[1]. 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[2]. 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 aetiological 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[4]. 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[5].

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 neuroinvasive 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.

differences have been discovered during the past few years, including the dependency on a specific lipid kinase in the case of HCV but not DENV or ZIKV. These differences and similarities reflect the strategies that are used by these viruses to manipulate host cells, offering the opportunity to learn about the roles of host cell factors, pathways and the mechanisms of their manipulation by conducting comparative analyses. In this Review, we summarize the current understanding of how members of the *Flaviviridae* family take control of cellular components or processes in order to create environments that are favourable for viral replication. We will compare and contrast DENV and ZIKV with HCV as representatives of the *Flavivirus* and *Hepacivirus* genera, respectively. Understanding the details of how viruses exploit their host cells opens new avenues for the development of antiviral strategies, and comparing replication strategies within the *Flaviviridae* may help us to identify shared essential host-dependency factors that are suitable for the development of broad-spectrum antivirals with pan-flaviviral activity.

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 peptidases 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; serine 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 peptidases 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.

Architecture of 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), multimembrane 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[8].

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[9–11]. VPs and CMs are interconnected and form a single endomembrane network[9]. 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[9]. 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[12]. 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)[9,13,14]. However, the absence of such structures in either DENV-infected mosquito cells[15] or ZIKV-infected hNPCs[10] 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 zippered ER (zER) membranes, are often observed in ZIKV-infected hepatoma cells[10]. 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.

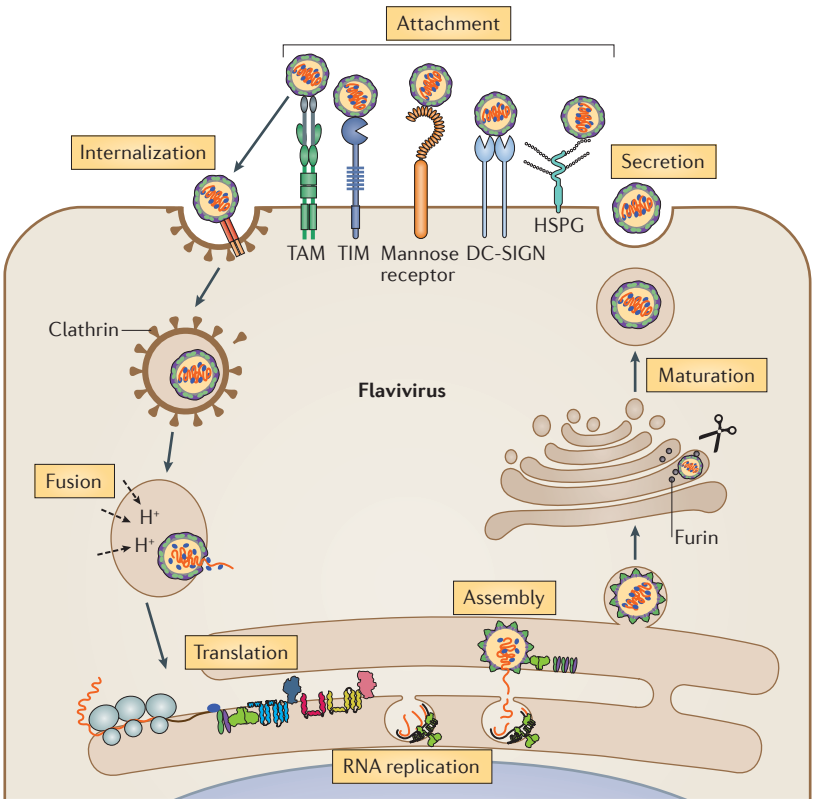
ZIKV, neither CMs nor zERs are formed, and the average diameter of VPs is considerably smaller (~60 nm versus ~90 nm in hepatoma cells), suggesting that cell type-specific factors help determine the architecture of ZIKV ROs.

In contrast to flaviviruses, HCV induces the production of ~150 nm diameter DMVs[16], forming clusters designated as the membranous web (FIG. 2B). Membranous web formation can be induced by the synthesis of the viral replicase 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[159,160]. 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[161]. 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[9]. Maturation of nascent DENV particles containing prM occurs along the secretory pathway by furin-mediated cleavage of prM[162]. *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.

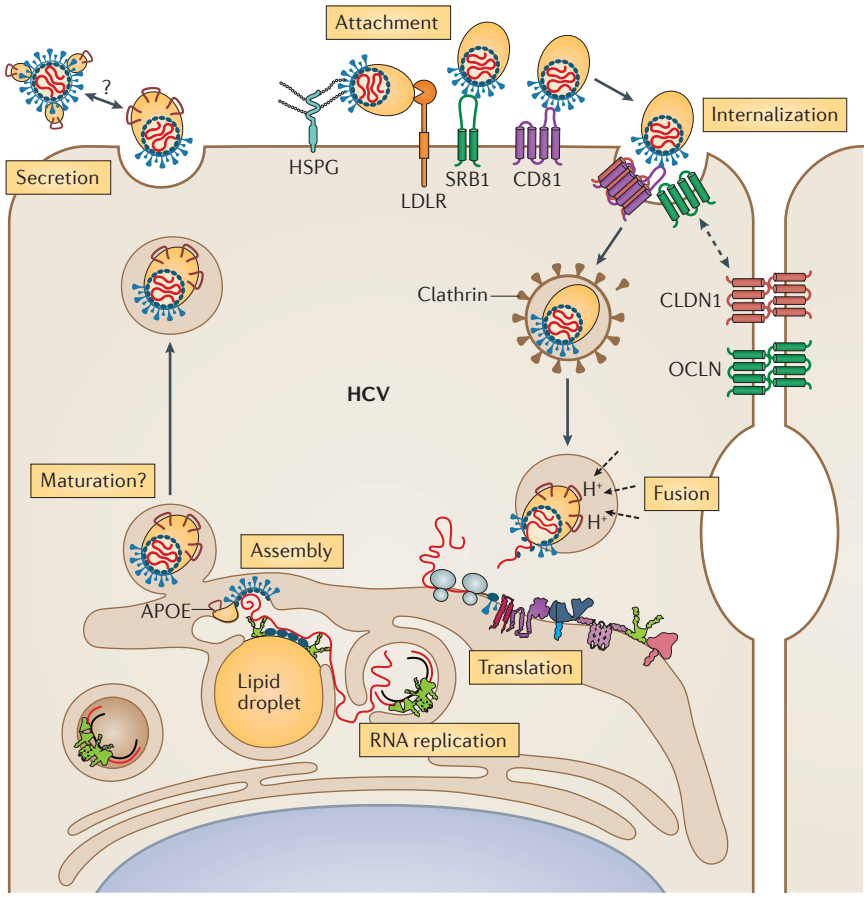


NS5B independent of viral RNA replication. The DMV outer membrane is often linked to the ER, suggesting that DMVs originate as protrusions that extend from the ER towards the cytosol[16] (FIG. 2B, inset). In addition to DMVs, SMVs and MMVs can also be observed within the membranous web (FIG. 2B). Though 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 response[17].

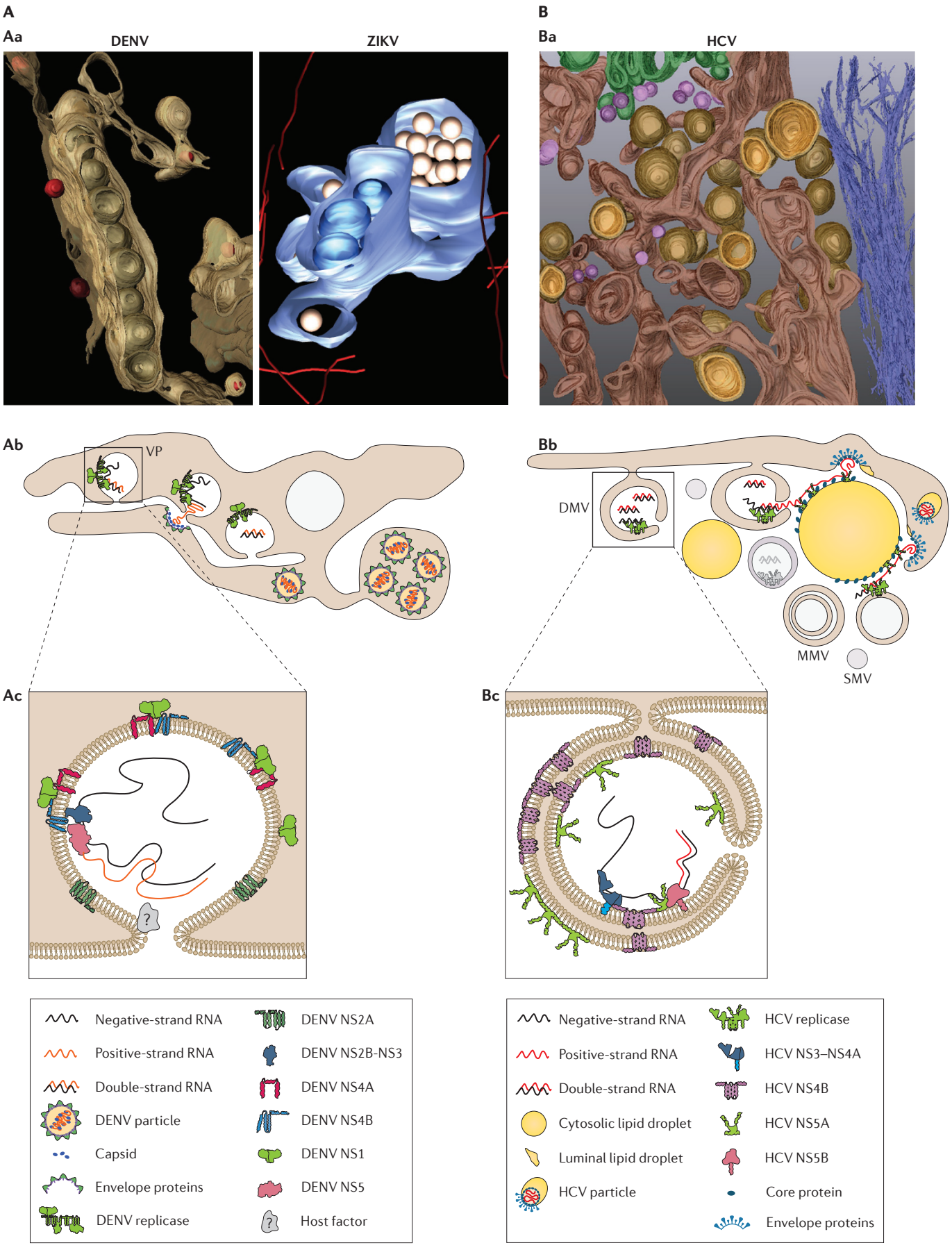
Box 2 | **Hepatitis C virus replication cycle**

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 envelopment of HCV particles and contribute to particle maturation, particle release or acquisition of infectivity[164]. Interestingly, APOE, the secreted glycoprotein E^{RNS} 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 cells[165]. 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 complexes[167,168]. Progeny HCV virions are presumed to exit the cell via secretory pathways.



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.



Cyclophilin
Peptidyl-prolyl isomerases (PPIases) that catalyse the *cis–trans* isomerization of peptide bonds at proline residues and facilitate protein folding.

The correlation between DMV abundance and RNA amplification, as well as the presence of replicase activity in isolated DMVs[18], 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 showed that HCV RNA and replicase activity reside within a nuclease-resistant and protease-resistant environment[18–20], 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[16]. Alternatively, proteinaceous transport complexes, such as nuclear pore complex-like structures, might enable traffic in and out of a closed membrane compartment[21,22].

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[8] (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[23]. 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 remodelling 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[24–26].

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*[27,28]. NS2A is a small hydrophobic protein with five transmembrane α-helices that can alter membrane permeability[29]. In addition, NS2A is enriched in subcellular regions containing viral dsRNA and may interact with replicase proteins[29]. 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[30].

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[16,31]. 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[32]. Moreover, essential residues for DMV formation and RNA replication have been identified within the NS4B carboxy-terminal domain[33]. Regarding NS5A, it is the only HCV protein able to induce DMVs[16], 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[34–36]. 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[34,37].

◀ Figure 2 | **Three-dimensional structure and organization of *Flavivirus* and *Hepacivirus* replication organelles. A** | Architecture of *Flavivirus* replication organelles. **Aa** | Three-dimensional surface rendering of dengue virus (DENV) and Zika virus (ZIKV) replication compartments. The endoplasmic reticulum (ER) is shown in brown and blue for DENV and ZIKV, respectively. Virus-induced vesicles are in light brown for DENV and dark blue for ZIKV. Virus particles are depicted in red (DENV) and gold (ZIKV). **Ab** | Schematic representation of the *Flavivirus* replication and assembly compartments. Genome replication occurs within vesicle packets (VPs) formed upon ER membrane invagination. Pore-like openings connect the interior of vesicles with the cytosol to allow for metabolite exchange and trafficking of the newly synthesized RNA genome. Virions assemble through nucleocapsid budding within ER cisternae close to VPs. Particles arrange in crystalline arrays within swollen ER cisternae connected to VPs. **Ac** | Model of *Flavivirus*-induced vesicle biogenesis. Negative membrane curvatures might be induced by non-structural protein 4A (NS4A) and NS4B amphipathic α-helices that are partially embedded within the ER luminal membrane leaflet. Negative curvature might be stabilized by homo-oligomerization and hetero-oligomerization between NS4A and NS4B along with interaction with NS1 dimers associated with the luminal side of the vesicle. NS2A and other host factors might further contribute to induce membrane alterations and stabilize the pore-like opening. **B** | Architecture of *Hepacivirus* replication organelles. **Ba** | Hepatitis C virus (HCV) membranous web as revealed by electron tomography and three-dimensional reconstruction of infected cells. The ER is shown in dark brown. Double-membrane vesicle (DMV) outer membranes are depicted in light brown and inner membranes in orange. Cytoskeletal filaments, Golgi apparatus cisternae and single-membrane vesicles (SMVs) are shown in blue, green and violet, respectively. **Bb** | Schematic representation of the HCV replication and assembly compartment. DMVs form as ER protrusions and contain the non-structural proteins that are responsible for viral genome replication. Replicase activity might cease upon vesicle closure (grey shaded vesicle). Alternatively, the viral replicase might be associated with the exterior of DMVs. Newly synthetized RNA might be delivered to assembly sites by NS5A and serine protease NS3 in a process assumed to involve core protein-loaded cytosolic lipid droplets. Formation of nucleocapsids is concomitant with particle budding into the ER lumen. **Bc** | Model of HCV-induced DMV biogenesis. The amphipathic α-helices and the oligomerization capabilities of the replicase proteins NS4B and NS5A, perhaps along with components of the autophagy machinery (not shown), might promote positive membrane curvature and DMV formation. MMVs, multimembrane vesicles. Part **Aa** is adapted with permission from REFS 9,10, Elsevier. Part **Bb** is adapted from REF. 16.

Box 3 | **Host cell factors as targets for antivirals**

Antivirals can be classified into two categories depending on their target. Direct-acting antivirals (DAAs) target viral components that are essential for virus multiplication, whereas host-directed therapies (HDTs) target cellular factors that are involved in the virus life cycle. DAAs have the advantage of exerting a potent inhibitory action as exemplified by the successful introduction of DAAs for the treatment of HCV infections; however, an inherent problem is the evolution of drug resistance. Although HDTs are usually more toxic than DAAs, HDTs offer the advantage of imposing a higher barrier to resistance and a broader coverage of different virus species. Numerous HDTs against the dengue virus (DENV) and the hepatitis C virus (HCV) are currently at different stages of preclinical and clinical evaluation (reviewed in REFS 169,170). Two examples of promising HDT candidates are listed below.

Cyclophilin inhibitors to treat HCV infections

Cyclophilin A (CYPA) is a highly abundant cytosolic peptidyl-prolyl isomerase (PPIase) that catalyses 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 (NS5A) protein. In addition to CsA derivatives such as alisporivir (formerly known as Debio 025), NIM811 and SCY635 that retain high-affinity CYPA 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[171,172].

α-Glucosidase inhibitors to treat DENV infections

The endoplasmic reticulum-resident α-glucosidase I and II catalyse 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[173,174]. Further optimization of celgosivir treatment in mouse models suggests that the drug loses efficacy when administered at the peak of viraemia. However, in these conditions, the antiviral action can be improved by changing the dosage and regimen of administration[175]. Therefore, a new phase Ib–IIa clinical trial with a revised dosing regimen is currently ongoing[176].

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 processing and folding

Protein processing and modification. All *Flaviviridae* viruses utilize cellular signal peptidases for proper cleavage of the viral polyprotein, specifically for the liberation of viral structural proteins (FIG. 1). For instance, *Flavivirus* structural proteins are released from the viral polyprotein by host signal peptidase, and HCV structural proteins are cleaved by both signal peptidase and signal peptide peptidase[38,39]. Of interest, efficient viral protease cleavage of the *Flavivirus* capsid (removal of the C-terminal membrane anchor to generate mature capsid protein) is required for efficient cleavage of the capsid–prM precursor by signal peptidase[40]. Moreover, uncoupling the sequential order of the two cleavages impairs nucleocapsid incorporation into virions, arguing that the coordination of virus particle production is regulated by polyprotein processing[41–43]. A recent study demonstrated that individual components of the signal peptidase complex have specificity for specific polyprotein cleavage sites, indicating that individual components of this complex might represent targets for directed pharmacological inhibition of viral replication[44]. Indeed, cavinafungin, a compound that antagonizes signal peptidase activity, has been shown to inhibit DENV and ZIKV propagation[45].

The role of viral protein glycosylation in the replication cycle of *Flaviviridae* family members has been well characterized, as glycoproteins are important for virion structure and stability. Specifically, glycosylation of proteins affects almost every step of the viral life cycle, including evasion from humoral immune responses (reviewed in REFS 46,47), and inhibitors of the glucose-trimming enzymes α-glucosidase I and II are currently being tested in clinical trials for their potential to treat DENV infection (BOX 3). In addition, a recent study demonstrated that specific subunits of the oligosaccharyltransferase (OST) complex, but not its enzymatic activity, are required for mosquito-borne *Flavivirus* infection, whereas no role has been identified in HCV replication[48]. Moreover, this study shows that the OST complex subunits STT3A and STT3B interact with DENV non-structural proteins and are specifically required for viral RNA replication[48]. The importance of the OST complex for *Flavivirus* replication is also supported by a more recent study, which demonstrated that, though STT3A and STT3B enzymatic activity is not required for DENV propagation, the enzymatic activity of the non-canonical OST complex subunit, magnesium transporter protein 1 (MAGT1), is important for DENV replication[49].

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[50] (reviewed in REF. 51). Interestingly, the function of HSP70 in different viral processes seems to be determined by one of nine co-chaperones called DNAJ proteins[50]. 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)[50,52,53]. 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)[50]. Currently, there is little information on the involvement of host cell chaperones in ZIKV infection. However, the conserved

Oligosaccharyltransferase (OST). A heteromeric transmembrane protein complex located in the endoplasmic reticulum lumen that catalyses the transfer of a pre-assembled oligosaccharide to selected asparagine residues within the consensus sequence Asn-X-Ser/Thr of nascent polypeptides.

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[54–58]. 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[55–57]. Inhibition of the different HSP70 network components, either through protein depletion or by addition of specific inhibitors, blocks viral protein synthesis or virus assembly[54,56]. 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 (CYPs); the CYP chaperone activity, especially CYPA, 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[59] (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 3 (PERK; also known as EIF2AK3) and cyclic AMP-dependent transcription factor ATF6-α (ATF6)[60]. Additionally, UPR activation leads to increased autophagy, oxidative stress and stress granule formation and has been linked to the potentiation of antiviral inflammatory responses[61–67]. 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[68] (reviewed in REFS 60,69,70) (FIG. 3A). For DENV infection, a time-dependent induction of each UPR pathway has been reported[71]. 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[71,72]. 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[72]. 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[72,74].

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 REFS 75,76). Additionally, oscillating stress granule formation has been observed in HCV infection, which has been suggested to be a result of eIF2α phosphorylation[73]. This flexibility in translation strategies may confer HCV the ability to overcome some of the host cell antiviral strategies, thus promoting persistence[73].

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[77,78]. 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[71,79,80]. 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[81]. 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.

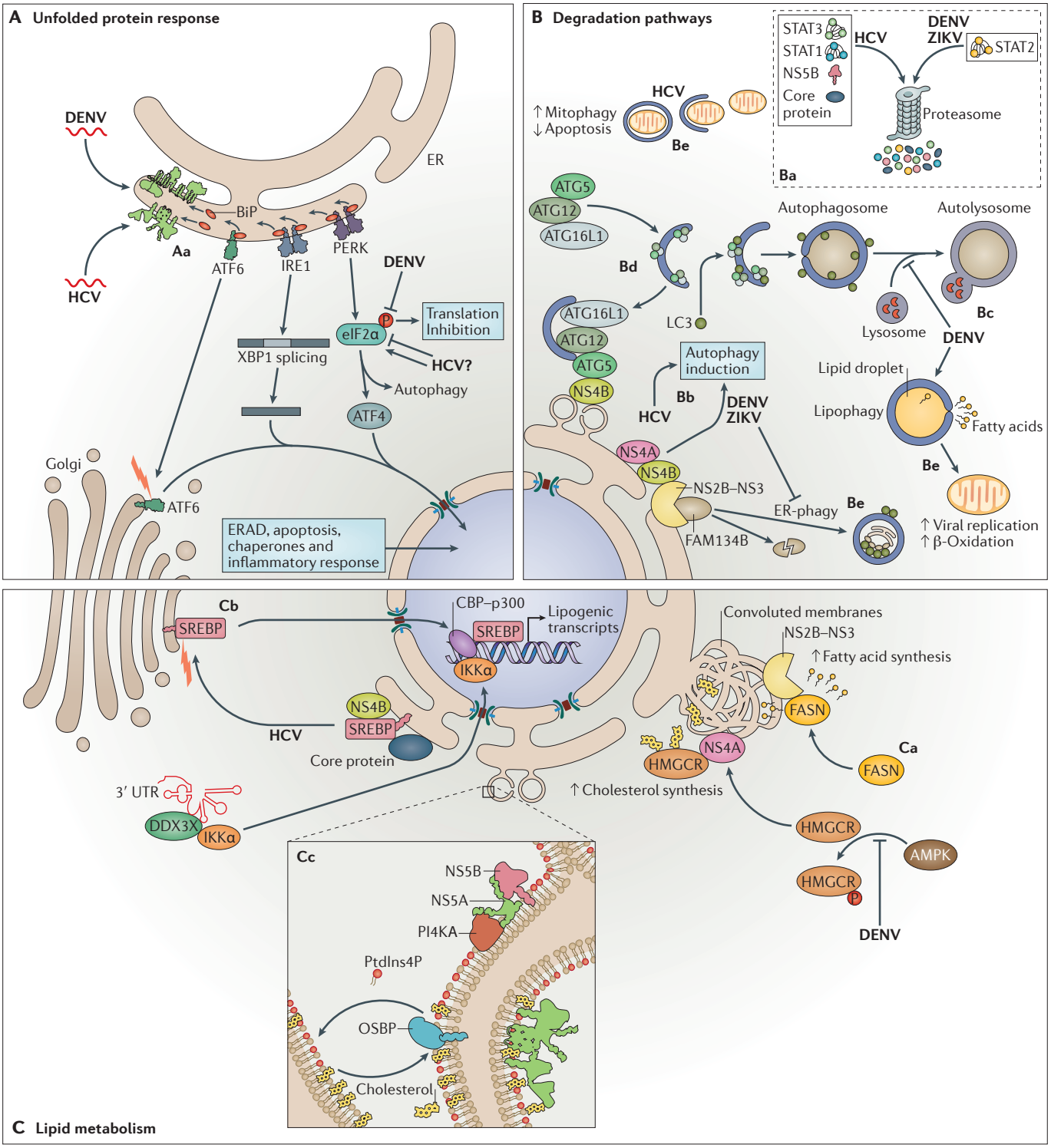
Protein degradation

The ubiquitin-dependent proteasome system (UPS) and autophagy are the two main cellular degradation pathways that are responsible for protein homeostasis. Both

Ubiquitin-dependent proteasome system (UPS). A multicomponent system for regulated protein degradation. Proteins are marked for degradation by conjugation with the ubiquitin polypeptide through the concerted action of modular conjugation machinery. Ubiquitylated substrates are recognized, unfolded and degraded by a large multisubunit protease complex called the proteasome.

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[82] (FIG. 3B). For example, components of the ERAD pathway were identified in a recent CRISPR–Cas9 knockout screen as essential factors for several flaviviruses[48]. For DENV infection, upregulation of UPS-related genes has been observed in both infected cell lines and peripheral blood mononuclear cells derived



CRISPR–Cas9
A site-specific gene-editing system derived from a bacterial adaptive defence system that retains foreign DNA in the CRISPR gene locus. The system is composed of a guide RNA homologous to the target gene and the CRISPR–Cas9 nuclease. Sequence complementarity guides Cas9 to the specific region targeted for cleavage.

Bortezomib
A dipeptide boronic acid derivate that reversibly inhibits the chymotryptic activity of the proteasome. It was the first therapeutic proteasome inhibitor approved by the FDA for clinical use as an anticancer drug.

from infected individuals, arguing for the physiological relevance of the UPS in *Flavivirus* infection[83]. Moreover, the UPS or components thereof are required for efficient virus production in the mosquito midgut[84], which is consistent with quantitative proteomic analyses of mosquito cells infected with ZIKV that show increased UPS protein levels[85]. Proteasome inhibitors MG132 and bortezomib exert antiviral activity against ZIKV and DENV *in vitro* and reduce viral load in an *in vivo* mouse model[85,86]. Collectively, these data indicate that both early and late events in the *Flavivirus* replication cycle require components of the UPS[84,86–88]. Although a strong dependence on the UPS was not observed for HCV infection[48,89], several UPS components are required for efficient viral replication[90]. Interestingly, UPS components of relevance for HCV replication are also required for WNV and DENV replication[90], suggesting an evolutionarily conserved role in the replication cycles of members of *Flaviviridae*.

UPS-mediated viral protein degradation can also restrict viral replication and enhance an adaptive antiviral immune response by increasing the presentation of antigenic peptides 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[91]. 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)[92–99] (FIG. 3B).

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[100,101] (FIG. 3B). For flaviviruses, autophagy induction is mediated by NS4A for DENV and NS4A in addition to NS4B for ZIKV[102–104] (FIG. 3B). 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[105]. For both flaviviruses and HCV, viral proteins and RNA were found to colocalize with autophagosomes, suggesting that viral ROs associate with autophagic membranes[106,107]. 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[9,108]. This study showed that the autophagosome membrane elongation complex ATG5–ATG12–ATG16L1 was recruited to the membranous web, potentially through an interaction between ATG5 and NS4B (REFS 108,109) (FIG. 3B). Knockdown of either ubiquitin-like modifier-activating enzyme ATG7 or ubiquitin-like protein ATG12 expression produced aberrant membranous web structures and reduced viral replication, suggesting that this complex contributes to the formation of HCV ROs. For HCV, autophagy has been suggested to function during the early stages of virus infection, such as translation of incoming HCV RNA or formation of the membranous web[110,111]. Additionally, recent reports indicate roles for autophagy in HCV particle secretion and the release of exosomes, which contain viral RNA fragments and infectious full-length HCV genomes[112,113].

In addition to general autophagy, several selective autophagy pathways are targeted during viral replication. DENV infection alters mTOR signalling to activate lipophagy, whereas both DENV and ZIKV inhibit

◀ Figure 3 | **Cellular pathways co-opted by *Flavivirus* and *Hepacivirus*. A** | Expression of the viral proteins during hepatitis C virus (HCV) and dengue virus (DENV) infection recruits immunoglobulin heavy chain-binding protein (BiP), resulting in depletion of BiP from the endoplasmic reticulum (ER)-stress sensors cyclic AMP-dependent transcription factor ATF6-α (ATF6), serine/threonine-protein kinase/endoribonuclease IRE1 and eukaryotic translation initiation factor 2-α kinase 3 (PERK). **Aa** | These BiP-less sensors become activated to induce the unfolded protein response (UPR) pathway. HCV and DENV have developed strategies to manipulate UPR pathways to promote virus replication (see main text for details). **B** | Manipulation of the cellular degradation pathways. **Ba** | HCV, DENV and Zika virus (ZIKV) infection induce proteasome-mediated degradation of innate signalling proteins to dampen the antiviral immune response. **Bb** | Viral infection triggers autophagy and alters the autophagy flux. **Bc** | For instance, established DENV infection suppresses the autophagic flux, blocking the fusion between autophagosomes and lysosomes. **Bd** | HCV non-structural proten 4B (NS4B) interacts with autophagy protein 5 (ATG5) and recruits the autophagosome membrane elongation complex (ATG5–ATG12–ATG16L1) to the membranous web, contributing to its formation. **Be** | In addition to general autophagy, selective autophagic pathways are hijacked during *Flaviviridae* infection (see main text for details). **C** | Subversion of lipid homeostasis. **Ca** | DENV serine protease NS3 recruits fatty acid synthase (FASN) to replication compartments to stimulate lipid biosynthesis. *De novo* synthesized fatty acids are incorporated into replication organelles (ROs). 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is recruited to DENV ROs, where it colocalizes with NS4A. Inactivation of the protein kinase 5′-AMP-activated protein kinase (AMPK) during DENV infection enhances HMGCR enzymatic activity, which results in higher cholesterol levels within ROs. **Cb** | During HCV infection, both viral proteins and regulatory elements within the 3′ UTR of the viral genome activate the transcription of lipogenic genes through sterol regulatory element-binding protein (SREBP)-mediated pathways (see main text for details). **Cc** | HCV NS5A and NS5B recruit and stimulate phosphatidylinositol 4-kinase-α (PI4KA) activity, thus increasing the local concentration of phosphatidylinositol-4-phosphate (PtdIns4P). Lipid transfer proteins such as oxysterol-binding protein 1 (OSBP) bind to PtdIns4P-rich membranes and release cholesterol in exchange for PtdIns4P. ATF4, cyclic AMP-dependent transcription factor ATF-4; ATG12, ubiquitin-like protein ATG12; ATG16L1, autophagy-related protein 16-like 1; CBP, CREB-binding protein; DDX3X, ATP-dependent RNA helicase DDX3X; eIF2α, eukaryotic translation initiation factor 2 subunit 1; ERAD, ER-associated degradation; FAM134B, reticulophagy regulator 1; IKKα, inhibitor of nuclear factor-κB kinase subunit-α; LC3, microtubule-associated proteins 1A/1B light chain 3B (also known as MAP1LC3B); p300, histone acetyltransferase p300; STAT1, signal transducer and activator of transcription 1; STAT2, signal transducer and activator of transcription 2; STAT3, signal transducer and activator of transcription 3; XBP1, X-box-binding protein 1.

ER-phagy through viral protease-dependent cleavage of the ER-phagy receptor reticulophagy regulator 1 (FAM134B[114–117]; 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 replication[118]. 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 biosynthetic pathways in *Flavivirus* and *Hepacivirus* 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 HCV[119]. 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 (HMGCR), a rate-limiting enzyme in the cholesterol biosynthetic mevalonate pathway, decreases DENV replication in primary human monocytes and cultured cell lines[120]. It was observed that DENV infection increases the activity of HMGCR through the inactivation of 5′ AMP-activated protein kinase (AMPK), resulting in higher levels of cholesterol in the ER and increased viral replication[122] (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 inactiation of mTOR complex 1 (mTORC1) and induction of lipophagy[117]. 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, HMGCR and other lipogenic transcripts[119]. 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 induction[119,123] (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 cells[124–126]. 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, glycerophospholipids and sphingolipids (mainly ceramide) was increased[124–126]. 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 present[124,126,127]. In addition, purification of HCV DMVs revealed cholesterol enrichment, which is otherwise present at low levels in the ER of uninfected cells[18]. 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 DENV[128,129]. 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)[35,130]. Reduction of PtdIns4P levels, either through gene PI4KA silencing or pharmacological inhibition, results in the formation of smaller and aggregated DMVs and a consequent reduction in virus replication[35]. PtdIns4P is required to promote the accumulation of cholesterol at viral replication sites through a non-vesicular cholesterol transport mechanism involving OSBP[128] (FIG. 3C, inset). Importantly, PtdIns4P and OSBP activity are required for the formation of the HCV membranous web but are dispensable for DENV replication, highlighting differences between the two virus genera[35,128]. In the case of HCV, it has been shown that PI4Kα is recruited by NS5A and NS5B to DMVs, where the kinase is activated to produce high amounts of PtdIns4P locally. It is assumed that PtdIns4P is used to facilitate the release of cholesterol from OSBP, which is discharged from the protein in exchange for PtdIns4P (reviewed in REF. 8). A similar mechanism might operate for the enrichment of glycosphingolipids in DMV membranes via pleckstrin homology domain-containing family A member 8 (FAPP2)[131] (FIG. 3C).

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 nucleocytoplasmic transport networks (FIG. 4).

Restriction factors
Cellular factors that inhibit pathogen replication.

AKT1–mTOR
An intracellular signalling pathway that regulates several cellular processes, including cell proliferation and survival.

Exosomes
Small extracellular vesicles released directly from cells or upon fusion of multivesicular bodies with the plasma membrane.

Lipophagy
An autophagic pathway that selectively targets lipid droplets and mobilizes lipids to be used for energy production.

ER-phagy
An autophagic pathway that selectively sequesters and degrades portions of the endoplasmic reticulum.

Parkin
An E3 ubiquitin ligase that is involved in autophagic elimination of damaged mitochondria (mitophagy). Mutations in the parkin gene are linked to autosomal recessive juvenile Parkinson disease.

Sterol regulatory element-binding protein
(SREBP). A membrane-bound transcription factor that regulates the transcription of genes involved in fatty acids and cholesterol metabolism.

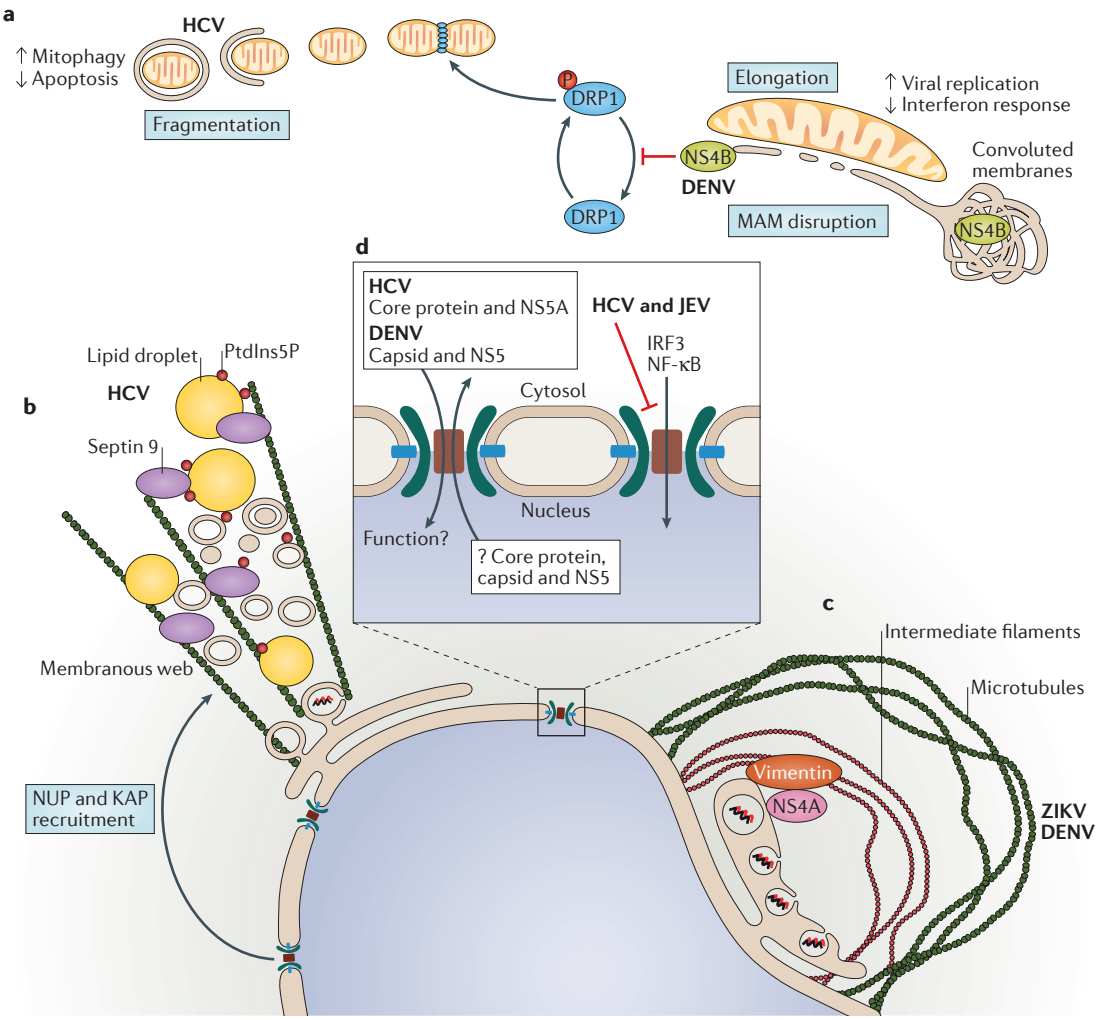


Figure 4 | **Cellular organelles co-opted by *Flavivirus* and *Hepacivirus*. a** | *Flaviviridae* viruses alter mitochondrial morphodynamics, leading to mitochondrial elongation in the case of dengue virus (DENV) or mitochondrial fragmentation in the case of hepatitis C virus (HCV), through the manipulation of specific mitochondrial fission and fusion proteins. **b** | HCV infection increases the intracellular levels of septin 9 and phosphatidylinositol 5-phosphate (PtdIns5P). The septin 9 interaction with PtdIns5P modulates lipid droplet growth and recruitment to the perinuclear area in a microtubule-dependent manner, thus creating an environment that favours viral replication. HCV proteins also recruit components of the nuclear transport machinery to regions of viral replication and assembly. **c** | DENV and ZIKV infection causes the rearrangement of microtubules and intermediate filaments surrounding replication organelles and leads to nuclear distortion in the case of ZIKV infection. **d** | Several *Flaviviridae* virus proteins contain nuclear localization and nuclear export signal sequences and are recruited to the nuclear compartment in infected cells. However, the dynamics of nuclear import and/or export as well as the specific function of these proteins within the nuclear compartment are not clear. HCV and Japanese encephalitis virus (JEV) impair nuclear import of immune transcription factors to limit immune activation in infected cells. DRP1, dynamin 1-like protein; IRF3, interferon regulatory factor 3; KAP, keratin-associated protein; MAM, mitochondrial-associated membrane; NS, non-structural; NF-κB, nuclear factor-κB; NUP, nuclear pore complex protein.

Microfilament
Also known as actin filaments; a component of the cytoskeleton. Microfilaments range from 7–9 nm in diameter and are composed of actin polymers. Microfilaments are involved in several cellular processes, including cytokinesis, cell movement, endocytosis and muscle contraction.

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[132,133]. 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[134]. Moreover, treatment of infected cells with drugs that disrupt intermediate filaments also inhibits viral replication[135]. 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[10].

For HCV, microtubules are required for both the entry and post-entry steps of viral infection[136]. 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[137]. 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[138] (FIG. 4). Thus, the role of the cytoskeleton may extend beyond viral entry and contribute to the biogenesis and maintenance of viral ROs.

Nucleocytoplasmic 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[140]. 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*[141,142].

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. 143) (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[21,22,144].

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[21,22,145]. 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[146]. 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[147,148] (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, lipid biogenesis 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[13,149,150]. 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[14,151]. This virus-induced mitochondrial elongation enhances respiration to increase virus 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[152,153]. These studies suggest a mechanism similar to HCV in which virus-induced mitochondrial fission inhibits apoptosis and alleviates immune activation[118,154]. 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[118,154,155] (FIG. 4). 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

Septins
GTPases that assemble into repeated hetero-oligomers and polymerize into higher-order structures, such as rings or filaments. Septins are the fourth cytoskeletal component and take part in several cellular processes, such as cell division, migration and pathogen interactions.

Interferon regulatory factor 3
(IRF3). A transcription factor that mediates the expression of type 1 interferons and interferon-stimulated genes.

Dynamin 1-like protein
(DRP1). A GTPase of the dynamin superfamily that mediate mitochondrial outer membrane fission events.

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[119,123], the identification of new protein folds as illustrated by the three-dimensional structure of DENV NS1[27] or novel insights into the mechanisms underlying virus-induced neuropathogenesis[7]. Moreover, comparative analyses of flaviviruses and hepaciviruses 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 coronaviruses (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 peptidase complex[44], the OST complex[48] and the host cell chaperone network[50,52] as well as others[68,157,158]. Although studies in more physiologically relevant systems are required to demonstrate the suitability of these targets for therapeutic approaches, the results *per se* have revealed surprising new insights into the cell biology of these cellular machines. For instance, an individual subunit of the signal peptidase complex can be eliminated without overt cytotoxicity *in vitro* while blocking virus replication, arguing that an approach that targets this subunit could have strong antiviral effects with low cytotoxicity[44]. 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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