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Review

# Virus–Host Cell Interplay during Hepatitis E Virus Infection

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**The molecular interplay between cellular host factors and viral proteins is a continuous process throughout the viral life cycle determining virus host range and pathogenesis. The hepatitis E virus (HEV) is a long-neglected RNA virus and the major causative agent of acute viral hepatitis in humans worldwide. However, the mechanisms of liver pathology and clinical disease remain poorly understood for HEV infection. This review summarizes our current understanding of HEV–host cell interactions and highlights experimental strategies and techniques to identify novel host components required for the viral life cycle as well as restriction factors. Understanding these interactions will provide insight into the viral life cycle of HEV and might further help to devise novel therapeutic strategies and antiviral targets.**

## Important to Know

At least 20 million HEV infections occur annually, accounting for approximately 3.3 million cases of acute illness and 44 000–70 000 deaths [1]. HEV infections are usually self-limiting and asymptomatic in immunocompetent individuals but can progress to chronicity and cause fulminant hepatitis in immunocompromised patients and other risk groups such as pregnant women [2,3]. Importantly, HEV has also been associated with several extrahepatic manifestations, including neuronal and renal diseases as well as pancreatitis [4]. Current therapeutic options against HEV are constrained to the off-label use of the broad-spectrum antiviral agent ribavirin (RBV) and pegylated interferon-alpha (pegIFN $\alpha$ ) [5]. However, RBV therapy is frequently limited due to adverse side-effects, and recently virus isolates have been identified that have lower sensitivity to RBV, leading to higher treatment failure rates [6,7]. Therefore, novel strategies to efficiently and safely target HEV are urgently needed.

HEV is classified as a member of the species *Orthohepevirus A* within the family Hepeviridae. So far, eight different HEV genotypes (GTs), including five human-pathogenic GTs (GT1/2/3/4/7), have been identified [8]. GTs 1/2 are obligate human pathogens which are present mainly in developing countries and are transmitted via the fecal–oral route, causing waterborne outbreaks [8,9]. By contrast, GTs 3/4/7 are zoonotic pathogens with a broad host range causing sporadic cases of zoonotic hepatitis E in industrialized nations [10]. The genome of HEV encompasses at least three open reading frames (ORFs) which encode: nonstructural proteins (ORF1), comprising a methyltransferase, a papain-like cysteine protease, a helicase, and an RNA-dependent RNA polymerase (RdRp); the capsid protein (ORF2); a small multifunctional protein with key functions in particle assembly and release (ORF3). There is an additional ORF (ORF4) exclusively expressed by HEV GT1 [11,12] (Figure 1). Importantly, many facets of the HEV life cycle and, in particular, host–virus interactions that determine the outcome of infection, remain enigmatic. Recent reports on rat HEV, a phylogenetically distinct relative in the *Orthohepevirus C* species, causing severe hepatitis in immunocompetent patients, make the picture even more puzzling and underline the urge for therapeutic intervention strategies, given its zoonotic potential [13]. Viruses are dependent on the host nucleic acid, protein, and energy metabolism to ensure replication and

## Highlights

The Hepatitis E virus (HEV) is an emerging zoonotic pathogen with broad host range, and various HEV strains can cross species barriers.

HEV exists in a membrane-associated, quasi-enveloped form and a nonenveloped state; both forms employ distinct mechanisms for cellular entry and potentially tissue tropism.

The interplay between cellular host factors and viral proteins determines virus host range and pathogenesis.

The establishment of novel HEV infection models and recent technologies will enable significant progress in the elucidation of the HEV life cycle and interactions with involved host factors.

Understanding the interactions between viral and host cell factors allows us to perturb the viral life cycle and provide a basis to guide the development of novel antiviral strategies.

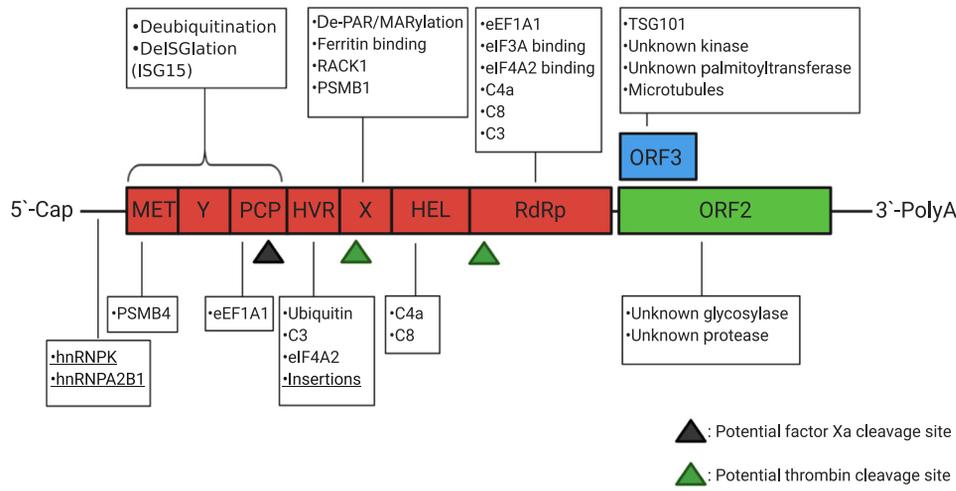
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**Figure 1. Host Interactors with Hepatitis E Virus (HEV).** The genome of HEV encodes three open reading frames (ORFs): the nonstructural polyprotein ORF1 (red), the capsid protein ORF2 (green), and the small multifunctional protein with postulated ion-channel activity and involvement in viral egress (ORF3, blue). The ORF1 polyprotein consists of seven functional domains, namely methyltransferase (MET), Y-domain, papain-like-protease (PCP), the hypervariable region (HVR), the X-domain (macro domain), helicase (HEL), and the RNA-dependent RNA polymerase (RdRp). Host factors directly interacting with viral proteins or domains or the viral RNA (underlined) are indicated in boxes and are listed in Table 1 with further information. The HVR tolerates insertions of host RNA sequences, indicating an interaction of host RNAs with the viral genome. Potential host protease cleavage sites are indicated by a black triangle (factor Xa) or green triangles (thrombin). Created with BioRender.com

persistence. Viral proteins frequently interfere with cellular signaling pathways or processes as well as the antiviral defense mechanisms of the infected host cell. This interplay between viral and host factors shapes the course and outcome of infection. Viral host tropism is therefore determined by a combination of susceptibility and permissiveness: a host cell must be both permissive (support viral replication) and susceptible (possess the receptor complement required for viral entry) for a virus to establish an infection. Hence, deciphering the many interactions that occur between HEV and its host cell over the course of infection is essential to understand the mechanisms of pathogenesis and to develop novel antiviral therapies. In particular, alternative therapeutic strategies targeting host factors required during the life cycle of HEV may greatly reduce the emergence of drug-resistant or insensitive variants [6]. Although the broad host range of HEV has led to several new experimental animal models, such as pig models mimicking chronic HEV infection, which offer promising opportunities for future HEV research [14], specific host susceptibility factors have not been identified in animals yet. In the following sections we therefore focus on the current knowledge and recent advances relating to the interactions between HEV and human host cellular factors with respect to different stages of the virus life cycle.

## Who Wants to Play? – Interplay between Host Cell and Viral Factors during HEV Infection

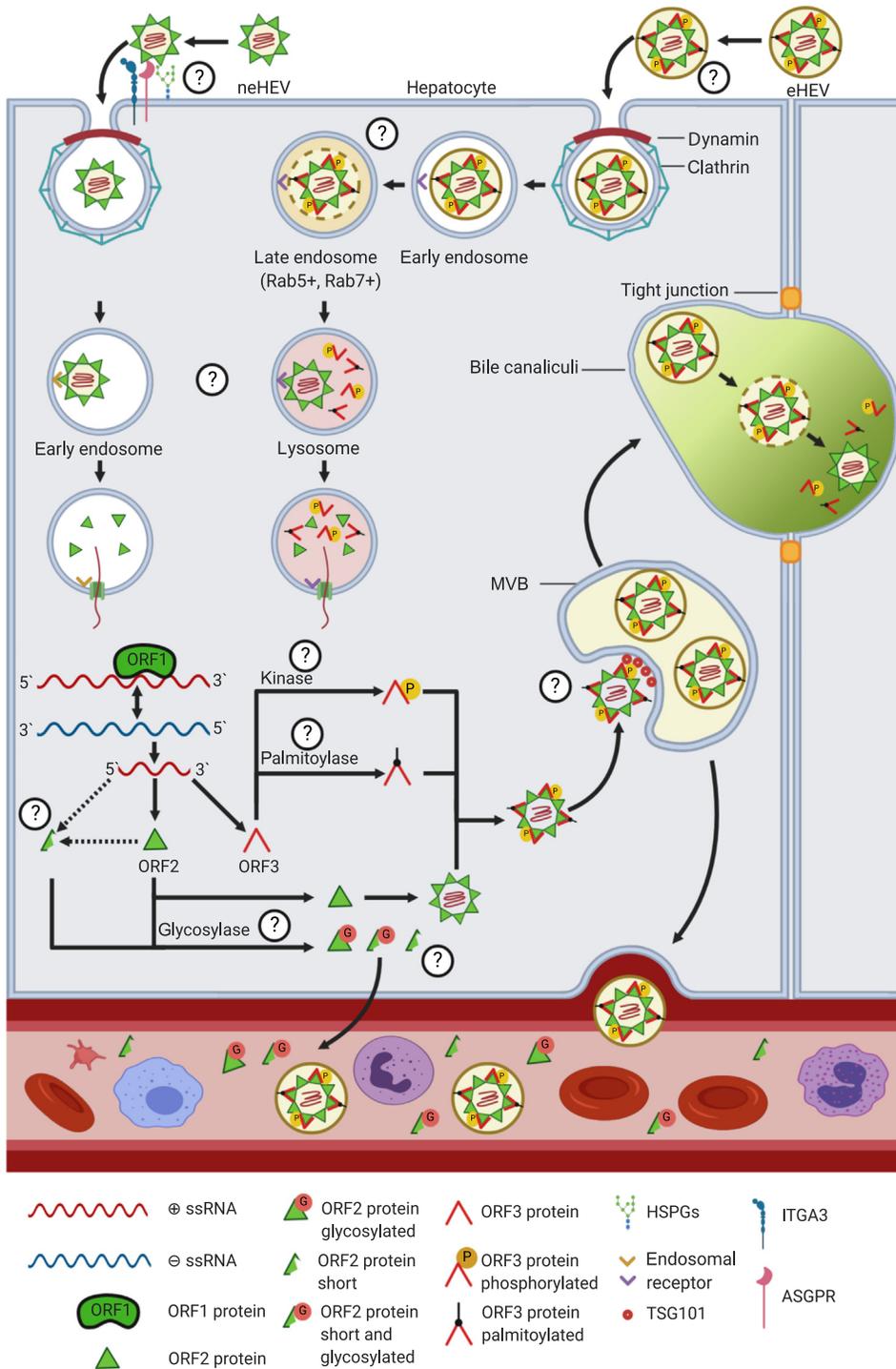
### Attachment and Entry

Surface attachment and entry into a host cell are two initial and essential steps in viral infection cycles and are important determinants regarding viral host ranges, tissue tropisms, and pathogenesis. The expression of specific membrane constituents which allow viral attachment to susceptible host cells frequently determines viral tropism, that is, the ability of a virus to infect a limited set of target cells [15]. HEV is a quasi-enveloped virus circulating in a nonenveloped state (neHEV) in bile and feces whereas, in the blood, HEV is cloaked by a layer of host cell membrane covering

viral surface proteins [16]. Importantly, available experimental evidence suggests that both forms employ distinct mechanisms for cellular entry [17–19]. A number of host factors have been shown to be involved in cell attachment and/or entry of naked HEV (i.e., neHEV) (Figure 2, Table 1). For example, the ectodomain of ASGPR1/2 (asialoglycoprotein receptor 1/2), a cell surface receptor present on the basolateral membrane, has been shown to directly interact with the viral capsid protein (ORF2) in coimmunoprecipitation (CoIP) and ELISA experiments. Ectopic expression of ASGPR further increased HEV binding in HeLa cells, whereas depletion of ASGPR in PLC/PRF/5 cells lowered HEV binding but not virion release. Furthermore, anti-ASGPR antibodies and purified ASGPR ectodomain competitively inhibited the binding of neHEV to hepatocytes [20], implying that ASGPR facilitates HEV infection through ORF2 binding. A microarray analysis of (non-)permissive PLC/PRF/5 subclones further suggested integrin  $\alpha 3$  (ITGA3) as a potential attachment/entry factor, and a direct interaction with neHEV was observed (Figure 2). Nevertheless, a panel of anti-integrin  $\alpha 3$  antibodies could not inhibit the infection of permissive subclones, necessitating further analysis regarding the role of integrin  $\alpha 3$  in the cellular entry of neHEV [21]. Various studies using recombinantly expressed capsid protein (ORF2) to generate virus-like particles (VLPs) as a model for neHEV virions further indicated roles for heparan sulfate proteoglycans (HSPGs), ATP5B (ATP synthase subunit 5 $\beta$ ), and GRP78 (glucose-regulated protein 78) during virus attachment/entry [11,19,22] (Figure 2 and Table 1). By contrast, the absence of viral proteins on the surface of enveloped particles (eHEVs) implies that these virions use different attachment factors and/or cellular receptors to initiate viral entry. In agreement with this, an overall less efficient surface attachment to host cells, independently of HSPGs or ITGA3, has been observed. Interestingly, the eHEV membrane contains phosphatidylserine (PS) which might bind cell surface receptor TIM-1 (T cell immunoglobulin mucin domain 1) on host cells and thereby serve as a potential attachment factor (Figure 2), a mechanism that has been described for multiple other enveloped viruses with outer envelope leaflets enriched in PS [23,24]. However, whether this dual lifestyle of the HEV virion influences its survival, dissemination, and tissue tropism within the host remains unclear. Nevertheless, given that HEV infection has been associated with various types of extrahepatic manifestation, a less specific cell binding by eHEV compared with neHEV may provide an explanation for the detection of HEV beyond the liver (see Outstanding Questions). Overall, although different host factors have been implicated in cell attachment and/or entry of HEV, little is known about the precise role of the different factors in the context of infection, particularly in primary human hepatocytes and most importantly, the receptor responsible for the entry of neHEV into cells remains unknown [11]. The advent of novel infectious cell culture systems for producing large amounts of infectious HEV particles – in combination with recent advances in conducting genomic screens in various formats and genome coverage (e.g., cDNA, CRISPR, and RNAi libraries) along with deep-sequencing data and -omics – now provide effective tools for identifying the host proteins that serve as viral receptors [25].

#### Internalization and Uncoating of HEV

Although essential for infection, passage through the cellular membrane barrier is only the initial step to establishing a successful viral infection. While the quasi-envelope of eHEV represents an elegant strategy for evading antibody-mediated immune responses [26,27] it also imposes a need for additional steps during cellular entry prior to uncoating of the genome. The internalization of both (n)eHEV particles involves clathrin- and dynamin-dependent pathways; however, different release points of the viral genome have been suggested (Figure 2). Perturbations of intracellular trafficking by Rab5/7 knockdown and/or lysosomotropic agents did not alter neHEV infectivity, while eHEV infectivity was greatly reduced [16,28]. Trafficking of eHEV towards lysosomal membranes is believed to be required for degradation of its lipid envelope. In agreement with this, perturbation of different enzymes – for example, NPC intracellular cholesterol transporter 1 (Niemann–Pick C1 protein or NPC1), lysosomal acid lipase (LAL) – required for lipid membrane



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Figure 2. Schematic Representation of the Hepatitis E Virus (HEV) Replication Cycle and Interaction with Host Factors. HEV exists in nonenveloped (neHEV) and enveloped (eHEV) forms. Nonenveloped virions attach to heparan sulfate proteoglycans (HSPGs), to the asialoglycoprotein receptor (ASGPR), and the proposed entry receptor integrin alpha 3

(Figure legend continued at the bottom of the next page.)

degradation in lysosomes selectively reduced eHEV infectivity. A similar mechanism also accounts for the loss of membrane from quasi-enveloped hepatitis A virus (HAV) [29]. Interestingly, nonspecific extracellular vesicles released from uninfected cells were not observed to traffic towards lysosomes, suggesting the presence (or absence) of a specific targeting signal within the eHAV – and likewise eHEV – membrane which redirects quasi-enveloped virions towards lysosomes [29]. Hence, analysis of the protein composition of eHEV versus exosomes released from uninfected cells using quantitative proteomic approaches could provide more support for this hypothesis. Although, the release of virion contents of (n)eHEV into the interior of the cell is temporally and spatially different, it remains unclear whether this process also occurs in a mechanistically distinct manner and involves binding of the viral capsid to a cellular receptor protein as a trigger. However, if the capsid of eHEV interacts with the same protein (following membrane removal) as neHEV, a potential receptor must be present on both late endosome-lysosomal and early endosomal compartments and would thus be a protein that traffics between these compartments (Figure 2). Antiviral strategies targeting early steps of infection, such as viral uncoating, are appealing, particularly when the probability for successful interference with both forms of HEV due a shared uncoating receptor would be given.

### HEV Replication Complex

In the process of host cell invasion, positive-stranded RNA viruses frequently induce the reorganization of intracellular membranes [e.g., endoplasmic reticulum (ER), Golgi, mitochondria, endosomes and/or lysosomes] to establish sites of replication. These replication complexes function as scaffolds to increase the local concentration of viral and cellular cofactors and provide a protected environment which limits the recognition of viral proteins and nucleic acids by the innate immune system [30]. The replicative machinery of HEV is largely encoded by ORF1, which features a methyltransferase, RNA helicase, and an RdRp (Figure 1). Whether the polyprotein is processed into distinct active domains during the HEV life cycle, as observed for various other viruses, remains controversial [31–37]. Nevertheless, conserved across all HEV genotypes, two potential cleavage sites for cellular thrombin and factor Xa were recently identified, and serine protease inhibitors, as well as thrombin and Xa-targeting siRNAs, inhibited viral replication [38] (Figure 1). A yeast two-hybrid screen identified 138 protein–protein interactions between HEV

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(ITGA3), while the enveloped virions are thought to attach to host cells via the interaction of their phosphatidylserine-containing membrane to the T cell immunoglobulin mucin domain 1 (TIM-1) on host cells. Enveloped as well as nonenveloped virions are endocytosed by a clathrin- and dynamin-dependent mechanism. The uncoating is poorly understood, but it is evident that both HEV forms require distinct uncoating mechanisms. The neHEV virions uncoat in early endosomes; the RNA genome is thought to be transferred into the cytoplasm through a pore that forms upon binding of the virion to its receptor. By contrast, eHEV virions need to traffic through late endosomes (Rab5<sup>+</sup>, Rab7<sup>+</sup>) and lysosomes in order to uncoat. Lysosomal acidification, the NPC intracellular cholesterol transporter 1 (Niemann–Pick C1 protein or NPC1, not shown), and lysosomal acid lipase (LAL, not shown) are required for uncoating, indicating that the lipid membrane needs to be degraded for this process. The positive-sense RNA genome is translated into the ORF1 polyprotein. The RNA-dependent RNA polymerase (RdRp) produces a negative-sense full-length RNA as template for genome replication and transcription of the subgenomic RNA, encoding for proteins of ORF2 (ORF2) and ORF3 (ORF3). Several states of the ORF2 protein are postulated; they differ in size and glycosylation status. A smaller form of the ORF2 protein is produced either through proteolytic cleavage by an unknown protease (ORF2c – cleaved) or by alternative translation (ORF2<sup>S</sup> – secreted). Furthermore, both forms can be glycosylated (ORF2g – glycosylated) by a yet to be identified enzyme, leading to three modified forms that are secreted into the bloodstream and are thought to act as immune decoys. Unmodified, infectious ORF2i (infectious) (i.e., ORF<sup>C</sup> – capsid) can self-assemble to form the capsid. ORF3 is phosphorylated by an unknown kinase, which allows for the binding of the protein to unmodified ORF2 and is thought to mark virions for release. Moreover, phosphoORF3 binds to tumor susceptibility gene 101 (TSG101), which is a part of the endosomal sorting complex required for transport (ESCRT). TSG101 is necessary for efficient viral egress and is required for loading of virions into multivesicular bodies (MVBs). Furthermore, ORF3 is palmitoylated by an unknown palmitoyltransferase, which affects the subcellular localization of ORF3 and virion release. The MVBs fuse with the plasma membrane to release the virions either in the bloodstream (basolateral side), where they keep their envelope (eHEV), or in the bile duct (apical side), where the envelope is removed by bile salts, and neHEV virions are produced. Abbreviation: ssRNA, single stranded RNA. Created with [BioRender.com](https://BioRender.com).

Table 1. Identified Direct Host Factor Interactions with HEV

Factor	HEV binding partner	Biological function <sup>a</sup>	Refs
ASGPR	neHEV virion	Attachment factor	[20]
C1-inhibitor (SERPING1)	ORF4	Altered complement activation or inhibition <sup>a</sup>	[39]
C3	RdRp, HVR	Altered complement activation or inhibition <sup>a</sup>	[39]
C4a	RdRp, HEL	Altered complement activation or inhibition <sup>a</sup>	[39]
C8	RdRp, ORF4, HEL	Altered complement activation or inhibition <sup>a</sup>	[39]
De-MARylation	X-domain (macro domain)	Immune evasion <sup>a</sup>	[67]
De-PARYlation	X-domain (macro domain)	Immune evasion <sup>a</sup>	[67]
eEF1A1	RdRp, PCP, ORF4	Formation of a translation complex <sup>a</sup> , increased RdRp activity	[12,39]
eIF3A	RdRp, ORF4	Formation of a translation complex <sup>a</sup>	[39]
eIF4A2	RdRp, HVR	Formation of a translation complex <sup>a</sup>	[39]
Factor Xa	PCP	Processing of the ORF1 polyprotein <sup>a</sup>	[38]
Ferritin	X-domain (macro domain)		[87]
hnRNPA2B1	Promoter regions in HEV RNA	Structural (re-)arrangements <sup>a</sup>	[42]
hnRNPK	Promoter regions in HEV RNA	Structural (re-)arrangements <sup>a</sup>	[42]
HSPGs	neHEV virion	Attachment factor	[16,22]
ISG15	MET-PCP	Invading cellular antiviral pathways <sup>a</sup>	[75]
ITGA3	neHEV virion	Entry receptor <sup>a</sup>	[21]
Microtubules	ORF3	Cytoskeleton rearrangement <sup>a</sup>	[59,60]
PSMB1	X-domain (macro domain)	Altered processing of MHC-I complexes <sup>a</sup>	[39]
PSMB4	MET	Altered processing of MHC-I complexes <sup>a</sup>	[39]
RACK1	X-domain (macro domain)	Part of the viral replication/translation complex	[39]
Thrombin	X-domain (macro domain), RdRp	ORF1 polyprotein processing <sup>a</sup>	[38]
TIM-1	Phosphatidylserine on eHEV virion	Attachment factor <sup>a</sup>	[16]
TSG101	ORF3	Loading of virions into MVBs	[61]
Ubiquitin	HVR, MET-PCP	Altered processing of MHC-I complexes <sup>a</sup> , invading cellular antiviral pathways <sup>a</sup>	[39,75]
Unknown glycosylase	ORF2	Production of immune decoys <sup>a</sup>	[47,58]
Unknown kinase	ORF3	Detecting virions ready for release <sup>a</sup>	[57,58]
Unknown palmitoyltransferase	ORF3	Viral egress and subcellular localization	[63]
Unknown protease	ORF2	Production of immune decoys <sup>a</sup>	[47,58]

<sup>a</sup>Speculated.

and host proteins, indicating roles for HEV proteins in modulating host cell processes such as stress and immune responses, the ubiquitin–proteasome system, energy and iron metabolism, and protein translation. Most importantly, a set of host translation factors (eIF4A, eIF3A, and RACK1), required for HEV replication, was identified [39] (Figure 1 and Table 1). As part of the eIF4F complex, eIF4A drives cap-dependent translation initiation in eukaryotes and has been involved in the replication of various RNA viruses. The natural compound silvestrol, a specific inhibitor of eIF4A, was further identified as a highly potent inhibitor of HEV replication *in vitro* and *in vivo* [40,41]. Additional host proteins interacting directly with the HEV genome were identified via affinity chromatography and mass spectrometry. Subsequent *in vitro* analysis revealed binding of two nuclear ribonucleoproteins – the heterogeneous nuclear ribonucleoprotein K (hnRNPK) and the heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNPA2B1) – to promoter regions in the viral RNA [42]. The involvement of these two proteins in packaging of nascent pre-mRNA, as

well as alternative transcript splicing and post-translational modifications (PTMs) by the recruitment of regulatory proteins of the eukaryotic RNA metabolism, may point to structural (re-)arrangements during HEV replication [43]. Candidate HEV replication complexes harboring viral RNA and the multifunctional ORF1 protein were found in cytoplasmic dot-like structures which partially overlap ORF2 and ORF3 proteins as well as exosomal markers [44]. However, the structure and composition of the HEV replication complex, as well as its spatial and temporal organization, remain poorly characterized, in particular due to the limited availability of functional tools to directly study the subcellular localization of viral proteins (see Outstanding Questions). Hence, as a first step, unbiased global approaches via morphological profiling approaches, such as the cell painting assay – a microscopy-based assay to evaluate morphological features of single cells, including shape, texture, size, etc. – could be used to define a multiparametric fingerprint to identify even subtle changes in cellular morphology and thus signatures of virus infection [45]. Importantly, small-molecule compounds frequently deliver similar phenotypic profiles. Hence, using reference compounds with known modes of action, such as RBV, this approach further offers the potential to evaluate compound libraries to identify novel molecules targeting HEV replication. Furthermore, novel RNA proximity labeling techniques, such as APEX-seq, can be applied to probe the spatial microenvironment of viral proteins and thus promise new insights into the composition of the HEV replication complex [46].

#### HEV Assembly and Release

The processes of virus assembly usually involve interactions between viral capsid and nonstructural proteins and the coordinated help of host factors. Intriguingly, only a minor fraction of the ORF2 capsid protein is assembled into infectious particles (ORF2<sub>i</sub>). By contrast, large amounts of non-virion-associated ORF2 variants, with potential immunomodulatory function, are secreted through the exosomal pathway (Figure 2). Two heavily secreted glycoproteins were described by Montpellier *et al.* – an ORF2<sub>g</sub> and a smaller, cleaved form, ORF2<sub>c</sub> [47]. While these two forms arise from PTMs, another study found evidence for alternative translational regulation resulting in an ORF2<sup>C</sup>, the regular capsid form, and a secreted form ORF2<sup>S</sup> [48]. To what extent these findings overlap is still unclear, and, as for the ORF2<sup>S</sup>, no glycosylation was verified. Although various studies using VLPs have provided some insights into the mechanical aspects of HEV genome packaging into viral particles [49–53], many fundamental questions regarding the assembly of HEV virions remain open (see Outstanding Questions). Most importantly, the subcellular compartment of virus assembly, the participating host factors, and how these steps are orchestrated in space and time remain unknown. A recently established elegant imaging system allowed visualization of the dynamics and subcellular structures of the Hepatitis C virus (HCV) virus, providing a topological map of how HCV might coordinate the steps of viral replication and virion assembly [54]. Likewise, the combination of confocal and electron microscopy, together with tagged HEV genomes, provides all possible means to study subcellular localization of ORF2 and to identify the potential viral assembly site. Combined with proteomic and genetic approaches, the host factors involved could be further identified; this could not only deepen the understanding of HEV assembly but also indicate attractive drug targets. Following viral particle assembly, progeny virions are released to initiate another round of infection, a process relying on the multifunctional phosphoprotein encoded by ORF3 [55–57]. Phosphorylation of ORF3 through a yet unknown host kinase promotes its interaction with ORF2, which has been suggested as a possible mechanism for ORF3 to recognize the viral particles for release [57,58]. Furthermore, ORF3 was shown to interact with different components of the intracellular transport machinery, including microtubules [59,60] and TSG101, a component of the endosomal sorting complex, required for transport (ESCRT) [61], which loads virions into multivesicular bodies (MVBs) for egress [62]. Interestingly, to fulfill this multifunctional role, ORF3 relies not only on phosphorylation but also

on palmitoylation through an as yet unknown palmitoyltransferase, required for its subcellular localization in order to mediate virion release [63] (Figures 1 and 2). Hence, both host proteins provide attractive strategies to perturb the viral life cycle. Within hepatocytes, polarized trafficking and secretion from MVBs results in the egress of eHEV particles from basolateral membranes to spread within the host, and from the apical membranes into the bile, where high concentrations of human bile acids and salts convert eHEV to neHEV virions. In this context, novel HEV-permissive polarized cell models will allow further study of the mechanisms and determinants of directional HEV secretion [64,65].

#### Host Factor Modifications Mediated by HEV Proteins

PTMs of HEV viral proteins (e.g., ORF2/3) realized by host molecules play an essential role in modulating their functions. Furthermore, recent studies also illustrate how enzymes encoded by pathogens modify target host cell proteins to shape an optimized environment for their replication and to facilitate evasion from the immune system [66]. For example, the different domains of the polyprotein encoded by ORF1 have been suggested to alter PTMs of host cell proteins. The X-domain of ORF1, also known as macro domain, functions as a de-MARylation and de-PARylation enzyme [67] (Figure 1, Table 1). PARylation and MARylation regulate key biological and pathological processes, and ADP-ribosylation of viral RNA has been suggested to serve as a signal to initiate a cellular immune response as observed for Venezuelan equine encephalitis virus (VEEV) and severe acute respiratory syndrome coronavirus (SARS-CoV) [68–73]. Interestingly, enzymes encoded by VEEV and SARS-CoV also contain domains with ADP-ribosylhydrolase activity which support viral replication in host cells [74]. Accordingly, the X-domain of HEV might perturb the antiviral response of a given host cell and likewise the Met-PCP domain is able to remove ubiquitin residues and interferon-stimulated gene 15 (ISG15) residues from proteins [75]. ISG15 is one of the most upregulated genes upon viral infection and is a ubiquitin-like modifier that plays a role in resistance to various viruses – for example, influenza viruses A and B (IAV, IBV), herpes simplex virus I (HSV-1), murine gamma herpesvirus 68 (MHV68), and Sindbis virus (SINV) [76]. Therefore, the ability to remove ISG residues (deISGylating) could provide an immune-evasive advantage for HEV. In this context it has been further observed that HEV can block the expression of different ISGs in response to IFN types IFN I–III; however, the mechanism remains unclear [77].

#### Genetic Exchange between Host Cell and HEV

Due to the error-prone nature of the viral RdRp, RNA viruses, such as HEV, diversify into populations with high intrahost variability, providing a potential benefit to the virus population across changing environments (e.g., immune response, antiviral therapy). Moreover, ORF1 of the HEV genome contains a hypervariable region (HVR) (Figure 1) which displays considerable sequence divergence even between isolates of the same virus genotype [78]. Recent reports identified several HEV strains harboring genomic rearrangements in patients at the acute phase of infection and further indicated that enhanced population heterogeneity is associated with HEV persistence and possible RBV insensitivity [7,79–82]. Furthermore, two HEV strains (recovered from the feces of chronically infected patients) containing insertions from human ribosomal subunits (S17 and S19) showed increased replicative capacity in cell culture and an expanded host range [83,84]. Given that sequences which are not required for virus infectivity or replication are normally rapidly lost in small RNA viruses such as HEV, a potential biological role of the HVR during HEV replication and/or pathogenesis can be inferred [85]. In general, two types of recombination-promoting insertion into viral genomes have been described for different viruses: the replicative 'copy-choice' mechanism and the nonreplicative 'breakage–ligation' mechanism [86]. Since insertions into the HVR potentially lead to the emergence of more pathogenic forms of recombinant HEV,

a deeper understanding of the mechanisms governing genetic exchange and plasticity is of great interest – in particular, if insertions are responsible for treatment failures in chronically infected patients or provide a potential determinant for chronicity [6,80].

### Concluding Remarks

The development of novel HEV cell culture systems has provided important advances in the study of HEV infection biology; however, many steps of the viral life cycle remain elusive. For example, although several entry and/or attachment factors have been described, little is known about the precise role of the different adaptors and, most importantly, the receptor responsible for viral entry into the host cell remains unknown. Likewise, endosomal escape mechanisms were shown to differ between eHEV and neHEV, but only a few of the required proteins have been described so far. Moreover, modifications of the ORF3 have been demonstrated to be crucial for viral egress, yet the modifying proteins need to be identified. Finally, recent studies have reported that transcription factors, as well as translation factors, are required for HEV replication.

Antiviral therapies are focused on disturbing virus propagation, mainly by directly targeting the viral genome/proteins or host interaction partners. All the proteins described, and many more host proteins not yet identified, are potential targets for pharmaceutical intervention strategies (Table 1). It is of the utmost importance to shed further light on the life cycle of HEV in order to understand its manipulation and hijacking of host cells and to identify new drug targets.

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### Outstanding Questions

How does eHEV bind to cells, and how is its cell tropism determined? Do eHEV and neHEV differ in their tissue tropism?

Which host factors are essential for HEV RNA replication? In particular, which host factors contribute to the formation of intracellular replication complexes?

Is there a specific signal that directs the endocytosed eHEV virion to lysosomes?

What is the role of the HVR? How do insertions in this region confer cell culture adaptation and potentially therapy resistance?

What is the subcellular site of HEV virion assembly? What is the mechanism to switch from replication to assembly? Which proteins mediate the intracellular trafficking of the assembled virions to promote particle release?

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