Abstract
Although the hepatitis E virus represents an uprising threat to the global community by representing the commonest cause of an acute viral hepatitis worldwide, its life cycle is grossly understudied. Albeit HEV is a non-enveloped virus, its progeny is released as quasi-enveloped virions. Thus, the responsible accessory protein pORF3 gained rising attention in the past years. It mediates viral release via the exosomal route by targeting the viral capsid to the endosomal system, more precisely to multivesicular bodies. As this is followed by quasi-envelopment, pORF3 may in terms represent a substitute to a conventional envelope protein. This feature proofs to be rather unique with respect to other enteric viruses, although the protein’s role in the viral life cycle seems to reach far beyond simply maintaining release of progeny viruses. How pORF3 affects viral morphogenesis, how it mediates efficient viral release and how it supports viral spread is summarised in this microreview. With this, we aim to shed light on functions of pORF3 to gain further insights in still enigmatic aspects of the HEV life cycle.

Take Aways
- HEV is released as exosome via multivesicular bodies
- Viral pORF3 mediates release via endosomal complexes required for transport
- pORF3 modulates various cellular processes in infected cells
- Elucidation of pORF3-related processes imply novel clinical strategies

KEYWORDS
exosome, HEV, MVB

1 | THE HEPATITIS E VIRUS – GENOMIC ORGANISATION

The hepatitis E virus (HEV) is the sole member of the genus Orthohepevirus in the Hepeviridae family. It represents a non-enveloped, positive-sense, single-stranded RNA virus with a genome being 7.2–7.4 kb in size (Tam et al., 1991). Three open reading frames (ORFs) are encoded by all genotypes, yet only HEV genotype 1 carries a fourth ORF (Nair et al., 2016). Proteins encoded by ORF1 and ORF4 are initially translated from the genome and mediate genomic replication (Koonin et al., 1992). Whether pORF1 is proteolytically cleaved or acts as single polyprotein remains elusive, yet both the full-length protein and separately expressed subunits display functionality (Parvez, 2017). During this process, an antisense RNA is synthesised (Nanda, Panda, Durgapal, & Jameel, 1994) serving as template for both the viral genome and a 2.2 kb bicistronic subgenomic RNA comprising ORF2 and ORF3 (Graff, Torian, ...
Nguyen, & Emerson, 2006). The positive-sense transcripts are 5’-capped and 3’-polyadenylated (Kabrane-Lazizi, Meng, Purcell, & Emerson, 1999). Following the cap-structure lies a 5’-UTR, whereas a 3’-UTR precedes the genomically encoded polyadenylation. Both UTRs found in the genomic RNA carry regulatory RNA secondary structures or cis-responsive elements (CREs). A third CRE is found within the region separating ORF1 and the overlapping ORF2/3 (Ju et al., 2020). This CRE-mediated separation of the viral genome potentially serves regulatory purposes for initializing and terminating subgenomic RNA synthesis and therefore impacts translation of the proteins encoded by ORF2 (pORF2) and ORF3 (pORF3) (Ding et al., 2018) (Figure 1a). These in turn are the drivers of viral morphogenesis and release.
2 | HEV – SHOULD WE CARE?

The *Hepeviridae* family comprises eight genotypes within the species Orthohepevirus A (Smith et al., 2014). While genotypes 1 and 2 are restricted to humans, genotypes 3, 4 and 7 display zoonotic potential (Doceul, Bagdassarian, Demange, & Pavio, 2016; Sridhar, Teng, Chiu, Lau, & Woo, 2017). Thus, transmission not only occurs via contaminated sewage or blood-products (Colson et al., 2007; Viswanathan, 2013), but also via contaminated meat (Harrison & DiCaprio, 2018). HEV represents the commonest cause of an acute viral hepatitis worldwide. As of 2005, there are over 20 million newly registered cases per year, resulting in 3 million symptomatic cases and 70,000 related fatalities (Rein, Stevens, Theaker, Wittenborn, & Wiersma, 2012). However, these numbers have risen up until today, suggesting an even higher death toll (P. Li et al., 2020). Herein, mortality rates range from 0.1% to 4% in healthy adults to up to ~30% in pregnant women (Jilani et al., 2007). Increased mortality in the latter is due to the fulminant course the disease can take in pregnant women infected with genotype 1 or 2 (Gouilly et al., 2018). Immuno-suppressed patients represent a further risk group for they often develop a chronic hepatitis upon infection with genotype 3 or 4 (Kamar et al., 2008). This further links the course of disease to the geographical spread, as zoonotic genotypes majorly reside in industrialised nations, yet human-only genotypes are endemic predominantly in emergent nations (Aggarwal, 2011). As per disease-prevention, a vaccine is only approved in China (S.-W. Li et al., 2015), and treatment options are limited. Here, PEGylated interferon alpha (Kamar, Abravanel, et al., 2010) and majorly ribavirin (Kamar, Rostaing, et al., 2010) are used, yet both cause interferon alpha (Kamar, Abravan el, et al., 2010) and majorly riba-virin (Kamar, Rostaing, et al., 2010) are used, yet both cause severe adverse effects (Manns, Wedemeyer, & Cornberg, 2006; Yang et al., 2010), and the latter may confer drug resistance due to viral mutation (Debing et al., 2014). In essence, HEV represents a pathogen being on the rise from a global perspective. There is a tremendous lack in disease prevention and management due to a lack of understanding the viral life cycle. Thus, elucidating the release and spread of progeny viruses is of central interest.

3 | THE VIRAL CAPSID

The viral capsid protein is represented by pORF2 and is 660 amino acids (aa) in length. It can be separated into three domains: (a) an aminoterminal (N-terminal) shell domain (S), (b) a middle domain (M) and a carboxyterminal (C-terminal) protrusion domain (P) (Yamashita et al., 2009). Further, the full-length protein carries a signal peptide (Figure 1b), which thereby renders localization to the endoplasmic reticulum (ER). Therein, pORF2 is glycosylated at two asparagine residues being part of consensus N-X-S/T motifs (Anekavay et al., 2019). While a study implied that this post-translational modification may fulfil virion-related roles (Graff et al., 2008), a more recent study pointed out that there in fact exist three different forms of the capsid protein. While the glycosylated forms are secreted as dimers only, the unglycosylated form is responsible for forming the viral capsid. Reason for a lack in glycosylation may either be found by translation via a downstream shifted start-codon or by an N-terminal cleavage, which is yet to be clarified in more detail (Montpellier et al., 2018; Yin et al., 2018). Assembly of the capsid structure is mediated by spontaneous homodimerisation within the P-domains near the C-terminal end (Xu, Behloul, Wen, Zhang, & Meng, 2016) and subsequent oligomerisation (T.-C. Li et al., 2005). During this process, 5'-end-dependent RNA-binding and encapsidation is mediated via an N-terminal, positively charged stretch within the S-domain (Surjit, Jameel, & Lal, 2004) (Figure 1c). While the site of RNA encapsidation requires further elucidation, an involvement of the ER and the cis- and trans-Golgi-network was suggested in bringing components into close proximity of each other (Nagashima, Takahashi, et al., 2014; Surjit, Jameel, & Lal, 2007). As a result, an icosahedral capsid structure is formed, which likely reflects a T = 3 symmetry for RNA-loaded particles (Xing et al., 2010). These
virions can be detected in stool samples acquired from infected patients, which display a size of ~27 nm (Balayan et al., 1983).

4 | THE ROLE OF pORF3 IN VIRIONS

Despite HEV virions appearing as non-enveloped in stool samples with a distinctive size, further studies described the presence of larger particles in cell culture supernatants and blood samples. Here, HEV particles were characterised with a size of ~40 nm ranging up to 120 nm (Montpellier et al., 2018; Nagashima, Jirintai, et al., 2014). Along with changes in size went changes in particle density, which range from ~1.07 to 1.15 g/ml in case of blood- and cell culture-derived particles to ~1.21–1.28 g/ml for stool-derived virions. This change is reasoned by these particles carrying a lipid envelope surrounding the capsid structure (Emerson et al., 2010; Qi et al., 2015; Takahashi et al., 2010). This primed investigations regarding the characterisation of the two forms of virions: non-enveloped HEV (nHEV) and quasi-enveloped HEV (eHEV). A central role of the viral protein pORF3 became apparent herein, which seems to be dispensable for the entirety of viral life cycle except for viral egress (Emerson et al., 2010; Emerson, Nguyen, Torian, & Purcell, 2006; Yamada et al., 2009). The protein is ~113 aa in size and resides within the viral quasi-envelope (Takahashi et al., 2008; Takahashi et al., 2016). The exact cause for pORF3 being membrane-associated may either be caused by a predicted transmembrane domain (TMD) (Ding et al., 2017) or via palmitoylation at an N-terminal cysteine-rich region (Gouttenoire et al., 2018), which is yet to be clarified. As further modification, a phosphorylation by host-kinases cyclin-dependent kinase 1 (CDK1) or mitogen-activated protein kinases (MAPKs) at a serine residue on position 70 was described (Zafrullah, Ozdener, Panda, & Jameel, 1997). This directly lies within a region located between aa 57–80, which is described to be crucial for interaction with nonglycosylated pORF2 and thereby rendering a phosphorylation-dependent interaction between pORF3 and nHEV (Tyagi, Korkaya, Zafrullah, Jameel, & Lal, 2002) (Figure 1e). Although following spatio-temporal relationships remain elusive in large parts, a microtubule association of pORF3 mediated via its N-terminal hydrophobic domains (Kannan, Fan, Patel, Bossis, & Zhang, 2009; Zafrullah et al., 1997) may potentially serve purposes of trafficking the capsid to its destination or mediating contact to downstream organelles. In effect, the covering of capsids with pORF3 primes endosomal targeting, which is followed by viral quasi-envelopment.

5 | ENVELOPMENT AND VIRAL EGRESS

Central key players within the endosomal system are multivesicular bodies (MVBs). These represent a specified subset of late endosomes, which are characterised by a distinct set of proteins, lipids and accumulated intraluminal vesicles (ILVs). The build-up and sorting into ILVs hereby determines the fate of bound cargo with respect to degradation, subcellular translocation or secretion. ILVs are formed by the MVB membrane-resident endosomal sorting complexes required for transport (ESCRT), which are responsible for cargo recognition and sorting (ESCRT-0 and -I), cargo condensation (ESCRT-II) and budding of vesicles from the MVB surface into the lumen (ESCRT-III) (Rodríguez-Furlan, Minina, & Hicks, 2019). For HEV, MVBs and the ESCRT machinery are essential for particle quasi-envelopment, which is initiated by pORF3-mediating interaction with the host-factor tumour susceptibility gene 101 (TSG101) (Nagashima, Takahashi, Jirintai, Tanaka, Yamada, et al., 2011). The latter is an MVB-resident protein and part of ESCRT-I, thus being involved in cargo- recognition and sorting (Sundquist et al., 2004). An interaction between HEV and this host factor is dependent on a viral late-domain located in pORF3 upstream of the pORF2 interacting domain, where in human pathogenic species of HEV a classical PSAP motif can be found at position 96–99 close to the C-terminus (Emerson et al., 2010) (Figure 1d). Effectively, this leads to a recruitment of capsid structures to the site of ESCRT-mediated formation of ILVs. As a consequence, viral capsids enter MVBs as ILVs, which then leads to endosomal shuttling towards the plasma membrane (PM) in a Ras-related protein 27a (Rab27a)-dependent manner (Nagashima et al., 2017) (Figure 1f). Efficient shuttling and escape from endosomal-lysosomal fusion herein seem to be dependent on cholesterol. Specifically, high cholesterol levels shift the equilibrium of the endosomal flux towards degradative processes, yet withdrawal of cholesterol favours successful release (Glitscher et al., 2021). Ultimately, viral release is achieved by HEV-containing MVBs fusing with the PM. Predominantly, this is suggested to take place at the apical interface of polarised hepatocytes (Capelli et al., 2019), which may be reasoned by pORF3 being found predominantly at the apical interface of polarised cells. This in turn may highlight its role in efficiently mediating release towards the biliary duct to guarantee viral shedding. As virions ultimately are released via MVBs, the viral quasi-envelope comprises host factors such as ALG-2-interacting Protein X (ALIX), vacuolar protein sorting-associated protein 4A (Vps4A), Vps4B, trans-Golgi network integral membrane protein 2 (TGNL2) or tetraspanins, which are characteristic markers for classical exosomes (Nagashima, Takahashi, et al., 2014; Nagashima et al., 2017; Primadharsini et al., 2020) alongside carrying exosome-specific lipids (Chapuy-Regaud et al., 2017) while circulating in the bloodstream (Figure 1g). Just upon entry into the biliary duct, the virions are believed to lose their membranous shell due to the presence of detergents within the bile, which goes along with a tenfold increase in infectivity (Yin, Ambardelar, Lu, & Feng, 2016) and viral shedding into the stool (Figure 1h). This route of egress represents the major mechanism of viral release for HEV, which is highlighted by interference with endosomal/exosomal maturation or pORF3 functionality leading to a loss in productive progeny formation (Anang et al., 2018; Kenney, Wentworth, Heffron, & Meng, 2015; Nagashima, Takahashi, Jirintai, Tanaka, Nishizawa, et al., 2011; Nagashima, Jirintai, et al., 2014; Tangggi et al., 2018). Notably, the importance of pORF3 for this process and its role for subsequent particle production seems to be conserved over different hosts and species of HEV, although alternate late-domain sequences may be in place (Kenney et al., 2012; Primadharsini et al., 2020). Thus, the exosomal release and acquisition of a quasi-
envelope around the otherwise non-enveloped virions of HEV is entirely dependent on pORF3.

6  |  pORF3 MAKING THE DIFFERENCE

Various different viruses were described to use MVBs and the ESCRT machinery during their viral life cycle or for release of viral by-products. Among these are other hepatotropic viruses such as the hepatitis B virus (HBV) (Hoffmann et al., 2013; B. Jiang, Himmelsbach, Ren, Boller, & Hildt, 2015; Lambert, Döring, & Prange, 2007), the hepatitis C virus (HCV) (Elgner et al., 2016; Masciopinto et al., 2004) or the hepatitis A virus (HAV) (Feng et al., 2013). What makes both HAV and HEV unique in comparison to HBV and HCV is that both normally form non-enveloped virions as they encode no envelope proteins. Conventionally, non-enveloped viruses were believed to be released

![Diagrams](image-url)
strictly via entering a lytic cycle, which in fact was proven untrue and gained increasing interest in the past decade especially for enteric viruses (Owusu, Quaye, Passalacqua, & Wobus, 2021). With respect to this, HAV and HEV have adapted strikingly similar mechanisms corroborating the exosomal route of egress (Feng, Hirai-Yuki, McKnight, & Lemon, 2014). Nonetheless, while HAV enters MVBs directly via its capsid (González-López et al., 2018; W. Jiang et al., 2020), HEV differs insofar as that it dedicated a separate protein for this purpose: pORF3. Due to its membrane localization and interaction with the capsid structure, it features aspects of classical envelope proteins. In this context, the question whether pORF3 represents an integral or a peripheral membrane protein, as suggested via abrogation of palmitoylation (Gouttenoire et al., 2018), needs to be addressed in more detail. Further, the mechanism compromised by pORF3 presents rather reminiscent of the Gag p6-protein of human immunodeficiency virus (HIV), which similarly targets TSG101 via a PxxP-motif (Garrus et al., 2001) and of HBV making use of the host-factor α-taxilin (TXLNA) to initiate TSG101-interaction (Hoffmann et al., 2013). Thus, analysing further host-pathogen interaction potentially being hijacked by HEV may prove useful for additional understanding (Figure 2a,b). Alongside this direct interaction with the exosomal pathway, regulatory roles of pORF3 herein require further attention. Evidently, the viral protein modulates mitochondrial processes (Moin, Panteva, & Jameel, 2007; Tian et al., 2019), possibly via acting as ion channel (Ding et al., 2017). In how far these mechanisms contribute to beneficial viral release, for example, via induction of elevated levels of reactive oxygen species, as described for HCV (Medvedev et al., 2017), presents an issue worth elucidating (Figure 2c). Additionally, pORF3 is described to interfere with cellular host defences such as the inducibility as well as the effectors of interferon signalling (Dong et al., 2012; He et al., 2016; Lei et al., 2018). This in turn further helps the viral infection to be efficiently established. Conclusively, HEV pORF3 may in terms be regarded as an alternate envelope protein of a quasi-enveloped virus, which is partially presented on the surface of eHEV (Qi et al., 2015; Takahashi et al., 2008; Takahashi et al., 2010). In this context, a potential role of pORF3 in viral entry needs to be clarified in more detail, as different routes of viral entry are described for eHEV and nHEV (Yin et al., 2016) (Figure 2d). Knowledge gathered herein will also serve purposes of pinpointing risks of extrahepatic manifestations. From a clinical point of view, the incorporation of pORF3 into eHEV virions also raises questions with respect to diagnostics and disease prevention. First, establishment of ELISA-based assays recognising pORF3 may prove useful to determine actual viral loads in infected patients (Figure 2e). Problems in current assays targeting pORF2 are both shielding of antigens via the quasi-envelope and secretion of pORF2 dimers, which may deteriorate readouts depending on the question being addressed. Further, pORF3 may as well be used as a target for therapeutic vaccinations in addition to the pORF2-based vaccine as experimentally implemented before, for example, in chicken (Syed et al., 2017). As such, it may inactivate eHEV once the virus is in circulation and may in this context be superior to the current pORF2-based vaccine (Figure 2f). In summary, HEV pORF3 represents a small yet central viral protein being of utmost importance for the viral life cycle. As such, it regulates fundamental cellular processes via various different modes of action. Especially its involvement in the unconventional viral egress makes it a target worth being studied, which may help to overcome current gaps in knowledge about HEV and the related lack in options for disease management.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Eberhard Hildt and Mirco Glitscher: Conceptualisation. Mirco Glitscher: Writing – original draft preparation. Eberhard Hildt: Writing – review and editing. Mirco Glitscher: Visualisation. Eberhard Hildt: Supervision. Eberhard Hildt: Project administration. Eberhard Hildt: Funding acquisition.

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