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The life cycle of non-polio enteroviruses and how to target it

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Abstract | The genus *Enterovirus* (EV) of the family *Picornaviridae* includes poliovirus, coxsackieviruses, echoviruses, numbered enteroviruses and rhinoviruses. These diverse viruses cause a variety of diseases, including non-specific febrile illness, hand-foot-and-mouth disease, neonatal sepsis-like disease, encephalitis, paralysis and respiratory diseases. In recent years, several non-polio enteroviruses (NPEVs) have emerged as serious public health concerns. These include EV-A71, which has caused epidemics of hand-foot-and-mouth disease in Southeast Asia, and EV-D68, which recently caused a large outbreak of severe lower respiratory tract disease in North America. Infections with these viruses are associated with severe neurological complications. For decades, most research has focused on poliovirus, but in recent years, our knowledge of NPEVs has increased considerably. In this Review, we summarize recent insights from enterovirus research with a special emphasis on NPEVs. We discuss virion structures, host–receptor interactions, viral uncoating and the recent discovery of a universal enterovirus host factor that is involved in viral genome release. Moreover, we briefly explain the mechanisms of viral genome replication, virion assembly and virion release, and describe potential targets for antiviral therapy. We reflect on how these recent discoveries may help the development of antiviral therapies and vaccines.

Acute flaccid paralysis
A type of paralysis that is characterized by a sudden weakness or loss of muscle tone.

Hand-foot-and-mouth disease
A viral disease in which characteristic vesicular lesions appear on the hands, feet and inside the mouth.

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Poliovirus is undoubtedly the best-known enterovirus and one of the most studied viruses. Since its identification in 1908, poliovirus has been intensively investigated to understand its life cycle and to control its spread and the debilitating disease it causes — poliomyelitis. The finding that poliovirus can be propagated in cultured, non-neural human cells not only led to the development of polio vaccines but also hugely contributed to the establishment of molecular virology as a new field. In fact, many important breakthroughs and concepts in molecular virology stem from studies on poliovirus. Poliovirus was the first animal RNA virus for which the complete genome sequence was determined[1] and for which a reverse genetics system was established[2]. Furthermore, poliovirus[3] and rhinovirus[4] were the first animal RNA viruses for which the three-dimensional structure was solved by X-ray crystallography.

The genus *Enterovirus* of the *Picornaviridae* family consists of 13 species, of which seven contain human pathogens. Besides three poliovirus serotypes, these seven species contain more than 250 other types (BOX 1). These non-polio enteroviruses (NPEVs) include many important pathogens, such as coxsackieviruses, echoviruses, numbered enteroviruses and rhinoviruses. Enteroviruses spread either through the faecal–oral route or via respiratory transmission. From their primary sites of replication in the gastrointestinal or respiratory tract, they can disseminate and infect other tissues and organs, including the central nervous system. Although most infections go unnoticed, NPEVs can cause a wide range of disorders with varying presentation and severity, most often in infants, young children and immunocompromised individuals. Coxsackieviruses, echoviruses and numbered enteroviruses are the main viral cause of aseptic meningitis. Furthermore, they can cause neonatal sepsis-like disease, encephalitis, acute flaccid paralysis (AFP), non-specific febrile illness, hand-foot-and-mouth disease (HFMD), herpangina, pleurodynia, pericarditis and myocarditis[5]. Coxsackieviruses B and echoviruses are also implicated as environmental factors in the aetiology of type 1 diabetes by persistently infecting and causing inflammation of pancreatic β-cells[6]. Rhinoviruses cause the common cold but can also trigger severe respiratory tract disease and exacerbations of asthma and chronic obstructive pulmonary disease (COPD)[7]. Despite their name and transmission route, enteroviruses are not associated with gastrointestinal illnesses. Apart from two inactivated enterovirus (EV)-A71 vaccines that were recently

Herpangina
A viral infection of the mouth that is characterized by blisters in the throat.

Pleurodynia
A viral disease that is characterized by a sudden pain in the muscles between the ribs.

Pericarditis
An inflammation of the pericardium, the membrane that surrounds the heart and the beginning of the great vessels.

Myocarditis
An inflammation of the heart muscle.

Bronchiolitis
An inflammation of the bronchioles that is caused by a viral infection.

marketed in China, there are currently no effective measures to prevent or treat NPEV infections.

Some specific NPEVs have emerged as serious public health threats, particularly EV-A71 and EV-D68 (BOX 2). EV-A71 is a major cause of HFMD, a disease that is usually mild and self-limiting, but serious and life-threatening neurological complications, such as brainstem encephalitis, meningitis and poliomyelitis-like paralysis, may occur. In the past 2 decades, large outbreaks of EV-A71 have occurred in Southeast Asia[8,9]. EV-D68 is an atypical enterovirus that replicates in the respiratory tract. EV-D68 has long been considered a rare pathogen, but it is now being increasingly detected during respiratory disease outbreaks worldwide. EV-D68 usually causes mild respiratory disease but can also cause severe bronchiolitis or pneumonia. In 2014, a nationwide outbreak of EV-D68 in the United States was associated with severe respiratory disease and a cluster of AFP and cranial nerve dysfunction in children[10,11]. Another well-known NPEV is coxsackievirus A24 variant, the major cause of large outbreaks and pandemics of acute haemorrhagic conjunctivitis, which affects millions of people[5].

In this Review, we discuss our current understanding of enterovirus structure, host–receptor interactions, mechanisms of uncoating and the recent discovery of a universal enterovirus host factor that is involved in viral genome release. Moreover, we briefly explain the mechanism of viral genome replication and assembly and describe potential targets for antiviral therapy. We reflect on how these recent discoveries may help the development of antiviral therapies and vaccines.

The enterovirus life cycle

Enteroviruses are non-enveloped viruses of ~30 nm in size with a single-stranded RNA genome of positive polarity (+) RNA. The life cycle of all enteroviruses (FIG. 1) starts with binding to one or multiple cell surface receptors, resulting in receptor-mediated endocytosis. Enteroviruses can use different endocytic routes, depending on the serotype and cell type. Receptor binding and/or pH changes in the endosomal system induce virus uncoating, which is the release of the viral genome from the capsid into the cytoplasm via a pore in the endosomal membrane. Although different enteroviruses may use different receptors and entry pathways, many post-entry steps are highly conserved. After delivery to the cytosol, the viral RNA is translated into a single large polyprotein, which is proteolytically processed by viral proteinases $2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$ into ten proteins (capsid proteins VP0, VP1 and VP3 and replication proteins 2A–2C and 3A–3D) and some stable and functional cleavage intermediates. Enterovirus genome replication takes place on virus-induced membrane structures termed replication organelles (ROs)[12]. Genome replication by the RNA-dependent RNA polymerase $3D^{pol}$ starts with the synthesis of a negative-strand copy of the incoming viral genome to generate a double-stranded RNA

Box 1 | Classification of enteroviruses

The genus *Enterovirus* is one of the 35 genera of the *Picornaviridae* family (see the figure, which provides a schematic overview of the *Picornaviridae* family, showing the genera that contain the most relevant pathogens). Other genera containing known human and/or animal pathogens are *Parechovirus* (for example, human parechovirus, which is associated with encephalitis in young children, and Ljungan virus), *Hepatovirus* (for example, hepatitis A virus), *Kobuvirus* (for example, Aichi virus, which causes acute gastroenteritis in humans), *Cardiovirus* (for example, encephalomyocarditis virus (EMCV), Theiler's murine encephalomyelitis virus and Saffold virus — a human pathogen) and *Aphthovirus* (for example, foot-and-mouth disease virus). Enteroviruses are highly abundant viruses and have high mutation and recombination rates, which can lead to the emergence of new pathogenic strains. In the past, enteroviruses were discriminated using serology based on differences in the VP1 capsid protein, which contains epitopes for neutralizing antibodies. However, this has now been replaced by molecular typing methods, which are also based on VP1 and correlate well with serotypes. Currently, enteroviruses are classified into 13 species. More than 100 enterovirus types that infect humans are known, and these all belong to four species, *Enterovirus A–D*; besides poliovirus (*Enterovirus C* species member), these contain the coxsackieviruses (CVs) A and B, echoviruses and several more recently identified viruses that are simply named 'enterovirus' (EV) and then named according to the species to which they belong and sequentially numbered (starting with EV-D68). In 2012, rhinoviruses were reclassified as enteroviruses. Currently, more than 160 rhinovirus types have been identified, and these are grouped in three species, *Rhinovirus A–C*.

Genus (number of species)	Selected species	Selected serotypes
Enterovirus (13)	*Enterovirus A*	Several CV-A types (for example, CV-A6, CV-A10 and CV-A16) and several numbered EVs (for example, EV-A71)
	Enterovirus B	CV-B types, echoviruses and one CV-A (CV-A9)
	Enterovirus C	Polioviruses, several CV-A types (for example, CV-A21 and CV-A24v) and several numbered EVs
	Enterovirus D	Several numbered EVs (for example, EV-D68 and EV-D70)
	Rhinovirus A	
	Rhinovirus B	
	Rhinovirus C	
Cardiovirus (3)	*Cardiovirus A*	Encephalomyocarditis virus (2 types)
	Cardiovirus B	Saffold viruses (11 types) and Theiler's murine encephalomyelitis virus
	Cardiovirus C	Boone cardiovirus
Aphthovirus (4)	*Foot-and-mouth disease virus*	Foot-and-mouth disease virus (7 types)
	Bovine rhinitis A virus	
	Bovine rhinitis B virus	
	Equine rhinitis A virus	
Parechovirus (4)	*Parechovirus A*	Human parechoviruses (19 types)
	Parechovirus B	Ljungan virus
Kobuvirus (3)	*Aichivirus A*	Aichi virus 1
Hepatovirus (9)	*Hepatovirus A*	Hepatitis A virus
29 other genera		

Box 2 | **Clinical impact of EV-A71 and EV-D68**

Enterovirus A71 (EV-A71)

Enterovirus (EV)-A71 is a major cause of hand-foot-and-mouth disease (HFMD), a childhood exanthema that is characterized by blisters on hands, feet and buttocks, oral vesicles and fever. This disease is usually mild and self-limiting but may involve serious and life-threatening neurological symptoms — often involving long-term sequelae — and cardiopulmonary complications. In fact, EV-A71 is currently considered the most neurotrophic non-polio enterovirus (NPEV). EV-A71, first isolated in 1965, was found to cause small outbreaks of aseptic meningitis in the United States, Europe and Australia and large outbreaks of polio-like disease in Bulgaria and Hungary that were associated with high morbidity and mortality (20% paralysis and 5% lethality) in the 1970s. Following its global spread, it caused several large outbreaks in Asia and became a major public health threat in the late 1990s[5]. The largest outbreak was in Taiwan in 1998, where ~130,000 cases of HFMD were reported, including >400 cases of severe neurological disease, of which ~20% were lethal[124]. Since then, several large outbreaks associated with lethal cases have been reported in Malaysia, Taiwan, Singapore, China, Hong Kong, Japan, Korea, Vietnam and Cambodia. In China, >10 million HFMD cases were reported, of which >80,000 were associated with neurological disease and >3,000 were fatalities[125]. The large majority of severe and fatal cases were due to EV-A71 (and also other NPEVs; for example, coxsackievirus (CV)-A16, CV-A6 and CV-A10 can cause HFMD but they cause less morbidity and mortality)[126]. In light of this, the Chinese government declared the development of an EV-A71 vaccine a national priority. Two inactivated EV-A71 vaccines have recently been developed and marketed in China[9]. EV-A71 infections have also been detected in the United States and Europe, but their incidence is low, and lethal cases are only occasionally reported. Multiple genotypes of EV-A71 have been identified. Their circulation seems to be regionally and temporally unrestricted. Why outbreaks and disease seem mainly restricted to Asia remains unknown.

Enterovirus D68 (EV-D68)

Enterovirus (EV)-D68 was first isolated from children with respiratory infections in California, United States, in 1962. It was long considered a rare pathogen (only 26 cases were reported until 2005), but since 2008, it has been increasingly detected during outbreaks of respiratory disease. This increase is accompanied by an expanded genetic diversity, and currently three EV-D68 clades (A, B and C) co-circulate worldwide. Infections mostly cause mild respiratory disease but can also result in severe bronchiolitis or pneumonia, occasionally leading to death, especially among children. In 2014, the largest outbreak of severe respiratory disease associated with EV-D68 occurred in the United States, with 1,153 confirmed infections and possibly millions of untested milder cases[11]. Many patients were hospitalized and admitted to the intensive care unit. The 2014 US outbreak coincided with an outbreak of a specific form of acute flaccid paralysis (AFP) called acute flaccid myelitis (AFM), which is a sudden onset of weakness in arms or legs due to brainstem and spinal cord grey matter lesions (which is called poliomyelitis when caused by poliovirus). Infection with EV-D68 was confirmed in 5 out of 11 patients in one study[10] and in 12 out of 25 patients in another study[127]. EV-D68 has been detected in the cerebrospinal fluid only sporadically, but this is also true for established neurotrophic enteroviruses (for example, poliovirus and EV-A71). Clinical improvement has been observed in some of the 159 AFM patients, but most have residual weakness a year or more after onset[128]. In 2016, EV-D68 was detected in patients with neurological complications in several European countries. The EV-D68 strains isolated from AFM cases may belong to a recent genetic cluster (clade B1). Careful surveillance and research on virus spread, evolution and pathogenesis are needed to better understand the role of EV-D68 in AFM.

replication intermediate. The negative strand serves as a template for the synthesis of new positive strands. Newly synthesized viral RNAs either serve as a template for further translation and replication or are encapsidated into new virions. Enterovirus particles form by assembly of the structural capsid proteins VP0, VP1 and VP3 into protomers and pentamers. Together with a nascent viral RNA, pentamers form the provirion — a process that is intimately coupled with viral RNA replication. Finally, RNA-induced processing of VP0 into VP2 and VP4 yields mature virions.

Besides cleaving the viral polyprotein, viral proteinases $2A^{pro}$ and $3C^{pro}$ cleave several host proteins to optimize virus translation, replication and virus spread and to suppress antiviral cellular responses. For example, they induce host shut-off (by cleaving cellular factors involved in transcription and cap-dependent translation of host mRNAs), disrupt nucleocytoplasmic transport (by cleaving nucleoporins), interrupt type I interferon and stress pathways (by cleaving RNA sensors and signalling proteins) and disrupt the cellular cytoskeleton (reviewed in REFS[13–15]). Collectively, these changes induce dramatic changes in cellular morphology (that is, a cytopathic effect) and culminate in death and lysis of the cell (reviewed in REF.[16]). Although enteroviruses are described in textbooks as obligate lytic viruses that are released from ruptured cells, evidence is now accumulating that enteroviruses can also egress before cell lysis in membrane-bound structures that can accommodate multiple virions (reviewed in REF.[17]).

Targeting the viral life cycle

There is a great need for broad-range antiviral drugs to treat NPEV infections. Such drugs may also be instrumental in the global poliovirus eradication initiative to treat so-called chronic shedders[18]. Several enterovirus inhibitors have been identified and some have been tested in clinical trials, mainly to treat rhinovirus infections, but all have failed owing to lack of efficacy or toxicity issues[19] (TABLE 1). Generally, antiviral compounds can target either viral proteins or essential host factors. Drugs targeting viral proteins usually have limited side effects, whereas the advantage of drugs that target host factors is that viruses are less likely to develop resistance. For many years, drug discovery has relied heavily on screens for antiviral activity in cell-based infectivity assays using large compound libraries, followed by stepwise chemical optimization. An advanced understanding of the structure and function of enterovirus proteins

Replication organelles
Virus-induced membranous structures that are generated from remodelled host membranes that accumulate in the cytoplasmic area of the cell and that are thought to support replication by scaffolding the replication machinery and occluding the viral RNA from antiviral defence systems.

Protomers
Assembly intermediates of an enterovirus capsid that is composed of one copy of each of the structural proteins VP0, VP1 and VP3.

Chronic shedders
Immunodeficient people who excrete vaccine-derived poliovirus for prolonged times.

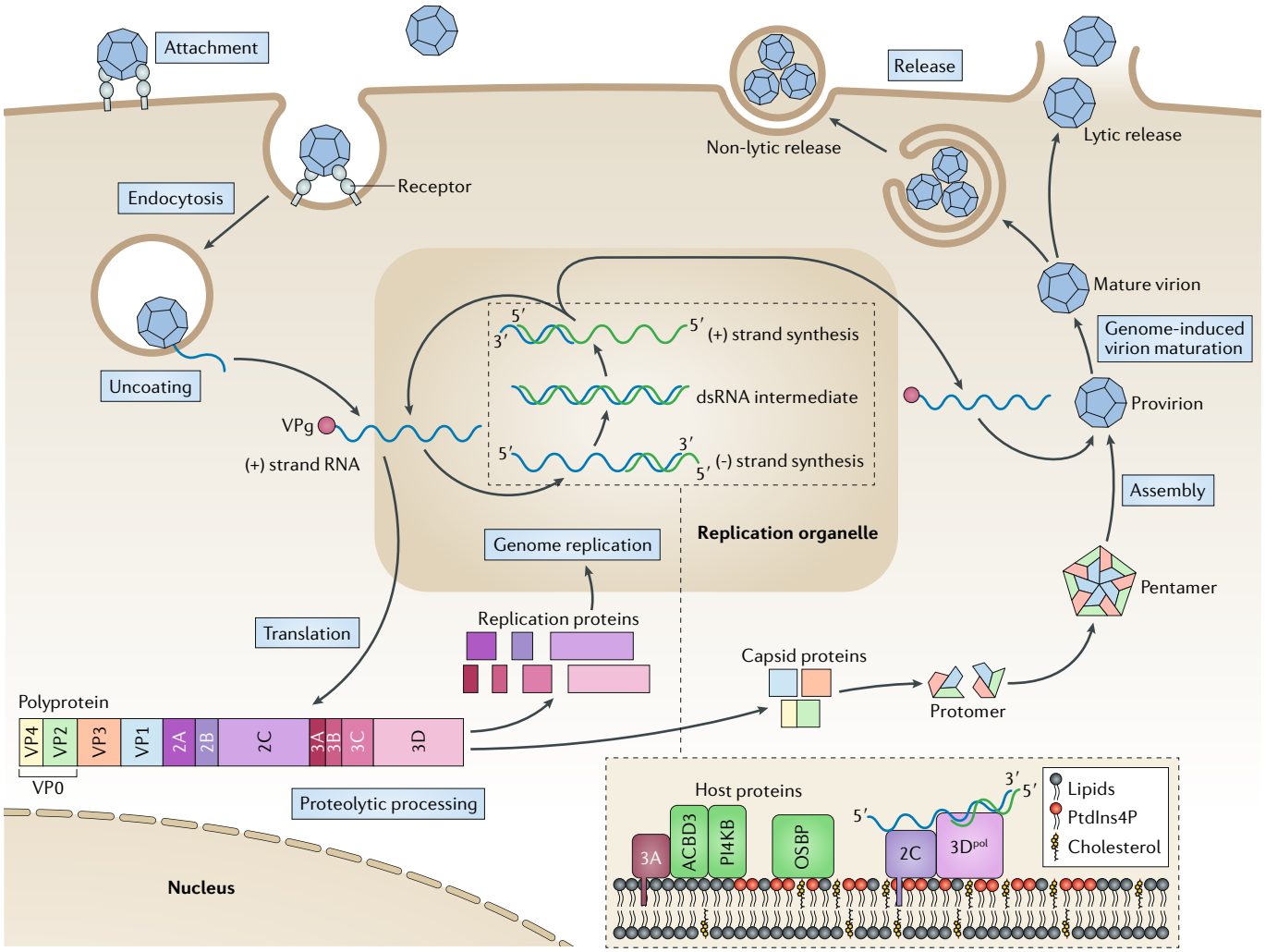

Fig. 1 | **Schematic overview of the enterovirus life cycle.** After binding to its receptor, or receptors, and endocytic uptake in the cell, the virion delivers its positive-strand (+) RNA genome across the endosomal membrane into the cytoplasm. The viral genome is covalently linked to the viral protein VPg (3B), which is required as a primer for replication. Genome translation yields a single polyprotein that is proteolytically cleaved into replication proteins (2A–2C and 3A–3D) and capsid proteins (VP0, VP1 and VP3). Genome replication by the viral RNA-dependent RNA polymerase ($3D^{pol}$) starts with synthesis of a negative-strand (–) RNA that serves as a template for synthesis of new (+) RNA molecules. Replication takes place on membranous replication organelles, in which a favourable lipid environment is created by viral proteins 2BC and 3A, aided by the host proteins acyl-CoA-binding domain-containing protein (ACBD3), phosphatidylinositol 4-kinase-β (PI4KB) (which synthesizes phosphatidylinositol-4-phosphate (PtdIns4P)) and oxysterol-binding protein (OSBP) (which recruits cholesterol). Nascent (+) RNA molecules can either enter a new round of replication or be packaged into progeny virions. Capsid proteins self-organize into protomers and pentamers and, in concert with replication machinery and genomic RNA, assemble into provirions that are converted into infectious, mature virions upon the genome-induced cleavage of VP0 into VP4 and VP2. Mature virions exit the host cell via non-lytic release of extracellular vesicles or via cell lysis in a late stage of infection. dsRNA, double-stranded RNA.

and essential host factors will aid broad-range antiviral drug development by allowing the implementation of dedicated high-throughput in vitro assays and the structure-guided development and optimization of drug candidates. Drug repurposing (that is, the use of compounds that have been developed to treat another condition) is an emerging strategy that can speed up antiviral drug development because safety and pharmacokinetic studies have already been performed. In the following sections, we discuss the various steps of the enterovirus life cycle and highlight examples of inhibitors at each stage.

Enterovirus structure

Rhinovirus 14 and poliovirus were the first animal RNA viruses for which three-dimensional structures were resolved[3,4]. Since then, numerous high-resolution structures have been determined, revealing that enterovirus particles share a similar architecture (FIG. 2a). Enterovirus particles are constructed of 60 repeating protomers, with each protomer consisting of the four structural proteins VP1, VP2, VP3 and VP4. Together they form the icosahedral shell with a pseudo $T = 3$ arrangement that encapsidates the viral genome. The small protein VP4 is myristoylated and located on the inside of the virion,

Table 1 | **Selection of pharmacological inhibitors of the enterovirus life cycle in humans**

Inhibitor	Remarks	Inhibited[a]	Not inhibited[a]	Refs
Capsid				
Pleconaril[b]	• FDA application for common cold (rhinovirus) rejected (safety concerns) • Phase II trial for asthma exacerbations (rhinovirus): no results published • Phase II trial for neonatal sepsis (NPEV): no results published	EV-B, EV-C, EV-D68, RV-A and RV-B	EV-A71, E-24, PV-1, RV-B17, RV-A45 and RV-C	129–136 and reviewed in[19]
Pirodavir[b]	Phase II trial for common cold (rhinovirus): development halted	EV-A, EV-B, EV-C, EV-D, RV-A and RV-B	RV-A8, RV-A25, RV-A45 and RV-C	130–136 and reviewed in[19]
Vapendavir (BTA798)	• Pirodavir analogue • Phase IIb trial for asthma exacerbations (rhinovirus): not active	EV-A71, EV-C, EV-D68[c], RV-A and RV-B	EV-D68[c]	131–133,136,137 and reviewed in[19]
Pocapavir (V-073 or SCH 48973)	Phase II trial for polio eradication: effective	EV-B and EV-C	EV-A71, EV-D68 and RV-B14	133,137–139
NLD	Developed against EV-A71 by structure-based drug design	EV-A71	–	140
3C^{pro}				
Rupintrivir (AG-7088)	• Peptidomimetic inhibitor • Phase II trial for common cold (rhinovirus): development halted	EV-A, EV-B, EV-C, EV-D, RV-A, RV-B and RV-C	–	134,135 and reviewed in[19]
AG7404 (V-7404)	• Rupintrivir analogue • Phase I trial for common cold (rhinovirus): development halted	EV-A, EV-B, EV-C, EV-D, RV-A and RV-B	–	141 and reviewed in[19]
DC07090	Non-peptidomimetic inhibitor	EV-A	–	142
3D^{pol}				
Gemcitabine	Repurposed nucleoside analogue (anticancer) that is effective in animals	EV-A71, CV-B3, RV-A and RV-B	–	85,143
NITD008	Nucleoside analogue that is effective in animals	EV-A	–	144
Ribavirin	Repurposed nucleoside analogue (hepatitis C virus) that is effective in animals	EV-A71, EV-B, EV-C, RV-A and RV-B	–	145,146
Amiloride	Repurposed drug (antikaliuretic diuretic) that acts as a non-nucleoside inhibitor	CV-B3	–	147
2C				
Dibucaine	Repurposed drug (local anaesthetic)	EV-A71, CV-B3 and EV-D68	PV-1, RV-A2 and RV-B14	148
Fluoxetine	Repurposed drug (antidepressant) that was used successfully in a patient	EV-B and EV-D	EV-A, EV-C, RV-A2 and RV-B14	87–89
HBB	Effective in animals	EV-B and EV-C	–	149
Pirlindole	Repurposed drug (antidepressant)	CV-B3 and EV-D68	EV-A71, PV-1, RV-A2 and RV-B14	148
PI4KB				
Enviroxime	Phase II trial for common cold (rhinovirus): development halted	EV-A, EV-B, EV-C, EV-D, RV-A and RV-B	–	130–132 and reviewed in[19]
BF738735	An analogue with increased bioavailability that is effective in animals	EV-A, EV-B, EV-C, EV-D, RV-A and RV-B	–	150
MDL-860	Allosteric inhibitor that is effective in animals	EV-B, EV-C, RV-A and RV-B	–	151,152
OSBP				
Itraconazole	Repurposed drug (antifungal) that is effective in animals	EV-A, EV-B, EV-C, EV-D, RV-A and RV-B	EV-D68[c]	93,94,132
OSW-1	Natural compound	EV-A71, CV-B3, CV-A21 and RV-B14	–	93,153
HSP90				
Geldanamycin	Natural compound of which an analogue is effective in animals	EV-A71, PV-1, CV-B3 and RV-B14	–	96,105

Table 1 (cont.) | **Selection of pharmacological inhibitors of the enterovirus life cycle in humans**

Inhibitor	Remarks	Inhibited[a]	Not inhibited[a]	Refs
N-myristoyltransferase 1				
2-Hydroxymyristic acid	Prevents VP4 myristoylation	EV-A71	–	154
Glutathione[d]				
Buthionine sulfoximine	Inhibits glutathione biosynthesis	EV-A, EV-B, EV-C and RV-B14	EV-A71, E-11 and PV-1	99,100,106
TP219	Depletes glutathione			

CV, coxsackievirus; E, echovirus; EV, enterovirus; HSP90, heat shock protein 90; NPEV, non-polio enteroviruses; OSBP, oxysterol-binding protein; PI4KB, phosphatidylinositol 4-kinase-β; PV, poliovirus; RV, rhinovirus. [a]Indicates against which viruses the compound has been tested. Viruses that are not listed have not been tested for sensitivity to the inhibitor. When the name of a species is given, this indicates that multiple (at least two), but not necessarily all, virus types belonging to this species have been tested. [b]In some studies with pleconaril and pirodavir, it was observed that some specific strains within a large panel were not susceptible to drug inhibition. For reasons of clarity, these exceptions are not included in this table. [c]Some studies report an inhibition of EV-D68 by these compounds, whereas others do not find an inhibitory effect. It is currently unclear whether this is due to different strains used or due to technical differences. [d]Susceptibility to inhibition by glutathione depletion is determined by the absence of a surface-exposed methionine, which can be type or strain specific.

whereas the surface of the particle is composed of subunits of VP1, VP2 and VP3, which each adopt the typical eight-stranded, antiparallel β-barrel fold. The surface of the capsid has several highly variable loops, which are readily accessible to the host immune system and account for the high antigenic diversity of enteroviruses. Most enteroviruses have a deep, circular surface depression or canyon encircling each fivefold axis of symmetry that frequently serves as the receptor binding site[20].

Despite these similarities, some enteroviruses display unique structural features. The capsid of rhinovirus C (RV-C) contains protrusions on its surface, contrasting with most enterovirus particle surfaces, which are smoother and spherical[21] (FIG. 2b). As a consequence, RV-C particles have shallow, narrow and non-continuous canyons, much resembling those of EV-D68 (REF.[22]). The biological consequences of such atypical canyons are yet to be determined. The floor of the canyon of all enteroviruses (with the exception of RV-C[21]) harbours a small, hydrophobic 'pocket' that is ordinarily filled with a lipid moiety or pocket factor, which is involved in regulating particle stability. Although not much structural variation exists within the *Enterovirus* genus, a high number of unique and distinctive structural features can be found among other picornavirus genera. For example, the Aichi virus contains poly-L-proline type II helices on its capsid[23], whereas hepatitis A virus (HAV) is more angular, and its surface is devoid of any canyon or depression[24]. Similarly, Ljungan virus lacks a depression on its capsid surface but instead possesses marked protrusions that arise from the fivefold axes[25]. Owing to developments in structural biology (high-resolution cryo-electron microscopy (cryo-EM) and high-speed fixed-target X-ray crystallography at X-ray free-electron lasers)[26], it is likely that the number of high-resolution structures will increase in the near future and enhance our understanding of the structural diversity within the picornavirus family.

One of the earliest explored strategies to inhibit enterovirus infections is to target the viral capsid proteins using so-called capsid binders. These molecules occupy the hydrophobic pocket and displace the pocket factor, thereby increasing particle stability and preventing receptor binding and/or genome release. The first series of capsid binders, known as the WIN compounds[27], were chemically optimized to improve their antiviral and drug-like properties, which eventually resulted in the development of pleconaril. Several clinical trials have been performed with pleconaril and other classes of capsid binders; however, insufficient clinical benefits were observed. Additionally, the rapid emergence of drug-resistant viruses and the unusual structure of RV-C, with a canyon that lacks a pocket that can be targeted by capsid binders[22], pose obstacles for the development of broad-range capsid binders.

Enterovirus entry receptors

One of the key determinants of viral tropism and pathogenesis is the availability of a specific cell surface receptor that can be used for viral attachment and entry. In 1989, poliovirus receptor (also known as CD155) (REF.[28]) and intercellular adhesion molecule 1 (ICAM1) (REF.[29]) were the first enterovirus receptors that were identified for poliovirus and major rhinoviruses, respectively. Since then, many NPEV receptors have been identified, of which the majority belong to the cell surface immunoglobulin-like or integrin receptor family (TABLE 2). The observation that enteroviruses can bind to a large repertoire of different receptors provides a likely explanation for the diverse spectrum of diseases that are caused by these viruses.

In this Review, we categorize receptors as either uncoating or attachment receptors. Uncoating receptors induce conformational changes in the viral capsid that mediate the uncoating process, whereas attachment receptors facilitate cell attachment and may promote virus uptake. Although uncoating receptors are usually proteins, it was recently shown that EV-D68 binds sialylated glycans via its canyon and that these cause the conformational changes that initiate uncoating[30]. This finding represents the first example of an enterovirus that can bind to carbohydrates to trigger uncoating. For influenza viruses, tropism is determined by the distribution of different sialic acids on the respiratory epithelium. Whether a similar correlation exists between the expression pattern of different sialic acids and EV-D68 tropism still needs to be explored. In addition, ICAM5, which is enriched in telencephalic grey matter, was identified as a second uncoating receptor, possibly explaining why EV-D68 is occasionally associated with AFP[31]. The reason why this virus engages two uncoating receptors is currently unknown. Besides uncoating receptors,

Myristoylation
A saturated fatty acid that is linked to the N terminus of structural protein VP4 and has a role in both the entry and assembly process.

Poly-L-proline type II helices
Helical protein structures that consist of repeating proline residues and are frequently involved in protein–protein and protein–nucleic acid interactions.

High-speed fixed-target X-ray crystallography at X-ray free-electron lasers
A novel method in which X-ray free-electron lasers that generate high-intensity X-ray pulses and sample fixation on a micro-patterned pore-containing chip are combined to allow very fast structure determination with limited beam time and sample material.

Telencephalic grey matter
An area of the cerebral cortex that contains the nerve bodies.

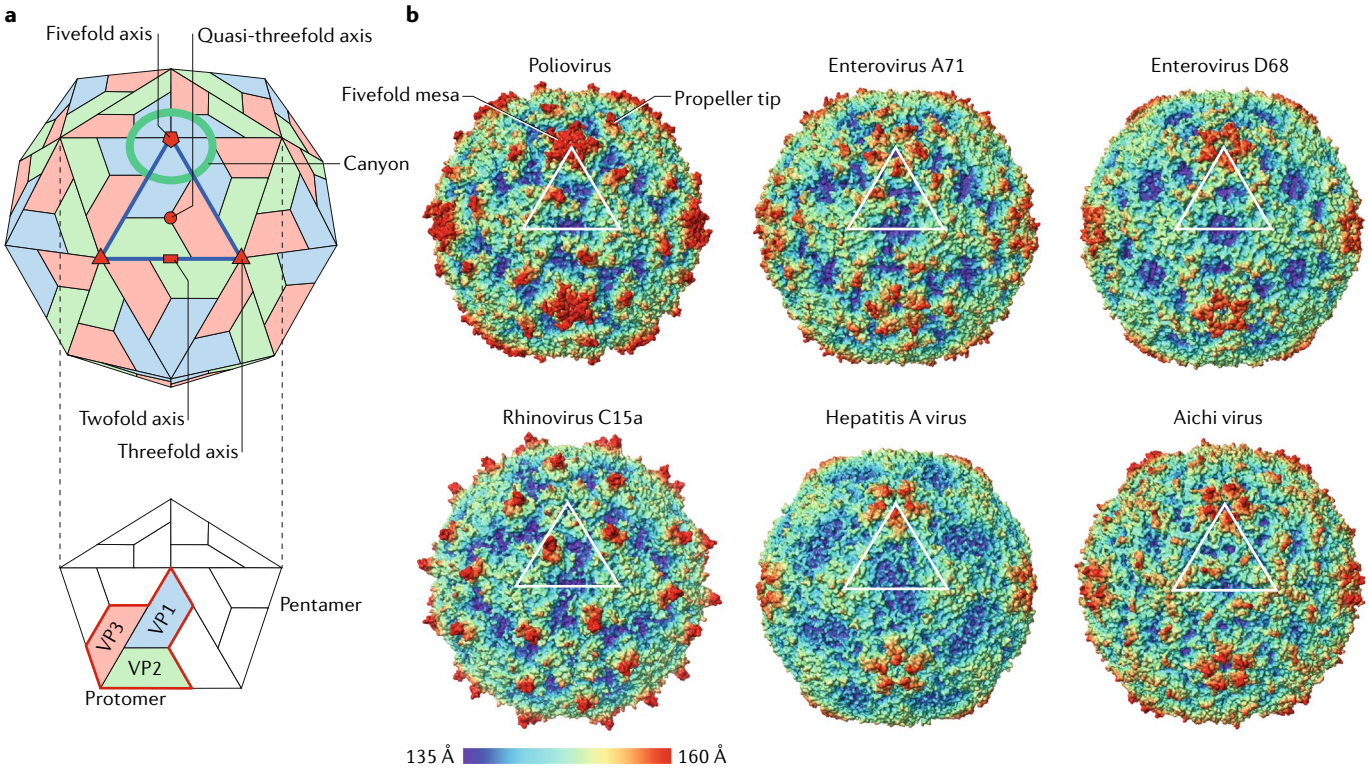

Fig. 2 | **Picornavirus structure. a** | Schematic representation of a picornavirus particle, showing the asymmetric unit (outlined in blue), the different symmetry axes (red) and the location of the canyon (green). Sixty protomers consisting of the surface proteins VP1, VP2 and VP3 and the internal VP4 constitute the capsid shell. **b** | Comparison of capsid surfaces between different picornaviruses belonging to the genus *Enterovirus* (poliovirus, enterovirus A71, enterovirus D68 and rhinovirus C15a); the genus *Hepatovirus* (hepatitis A virus); and the genus *Kobuvirus* (Aichi virus). Surfaces are coloured according to their distance from the centre of the virion, as shown in the colour bar. RCSB Protein Databank entries for the virion structures in part **b**: poliovirus (PDB ID 2PLV), enterovirus A71 (PDB ID 3VBS), enterovirus D68 (PDB ID 4WM8), rhinovirus C15a (PDB ID 5K0U), hepatitis A virus (PDB ID 5WTE) and Aichi virus (PDB ID 5GKA).

attachment receptors can also be of importance for infection. For instance, binding to the attachment receptor complement decay-accelerating factor (DAF; also known as CD55) by group B coxsackieviruses activates signalling events in polarized epithelial cells that liberate the uncoating receptor (coxsackievirus–adenovirus receptor; CAR) from tight junctions, making CAR accessible to the virus[32]. Moreover, attachment factors can be determinants of viral tropism, which was highlighted by a recent study on coxsackievirus A24 variant, in which it was shown that adaptation to the attachment receptor sialic acid was associated with the emergence of acute haemorrhagic conjunctivitis pandemics[33].

For EV-A71, two different receptors have been identified: scavenger receptor class B member 2 (SCARB2)[34] and P-selectin glycoprotein ligand 1 (PSGL1)[35]. SCARB2 was shown to be the receptor of all EV-A71 strains studied thus far[34,36], whereas PSGL1 was found to be the receptor for only some strains (EV-A71-PSGL1-binder (EV-A71-PB))[35]. In contrast to SCARB2, which was shown to induce uncoating[36], it is still unclear how PSGL1 supports EV-A71-PB infection. Autopsies of fatal EV-A71 infection cases revealed viral antigens in the brainstem and spinal cord, but no specific cell type that is targeted by the virus has been identified thus far[37]. SCARB2 is expressed on neurons and glial cells[38], and transgenic mice expressing human SCARB2 develop similar pathological features as humans[39], suggesting that SCARB2 is the main determinant for the development of neurological disease. PSGL1 is primarily expressed on leukocytes, although expression in human neuronal tissue was also reported[38]. Despite this information, the role of PSGL1 in EV-A71-induced disease remains unclear.

Receptor identification is not only useful to better understand viral pathogenicity but also to develop suitable cell and/or animal model systems to study the biology of a virus and to identify antiviral drugs. This is exemplified by RV-C, a major cause of severe respiratory tract infections (BOX 2). Despite its discovery more than 10 years ago[40], our knowledge of this virus was greatly hampered owing to its inability to grow in standard cell culture systems[41]. The recent discovery of the respiratory epithelium-expressed cadherin-related family member 3 (CDHR3) as the protein receptor and the generation of a CDHR3-overexpressing cell line that supports RV-C infection can thus be considered important milestones in RV-C research[42]. In differentiated human bronchial epithelial cell cultures, RV-C was found to preferentially infect CDHR3-expressing ciliated cells, providing the first insight into RV-C tropism[43]. Intriguingly, a coding SNP in *CDHR3* that was

Table 2 | **Enterovirus receptors**

Receptor	Virus	Role	Refs
PVR (CD155)	Poliovirus	Uncoating	28
SCARB2	EV-A71, CV-A7, CV-A14 and CV-A16	Uncoating	34,155
PSGL1	EV-A71-PB, CV-A2, CV-A7, CV-A10, CV-A14 and CV-A16	Attachment	35,156
Annexin II, DC-SIGN, nucleolin and vimentin	EV-A71	Attachment	Reviewed in[157]
Heparan sulfate	EV-A71 and E-5	Attachment	Reviewed in[157,158]
Sialic acid	EV-A71, EV-D70 and CV-A24v	Attachment	33,157
	EV-D68	Uncoating	159
ICAM5	EV-D68	Uncoating	31
LDLR, VLDLR and LRP	Rhinovirus (minor)	Attachment	160
ICAM1	Rhinovirus (major), CV-A21 and CV-A24	Uncoating	29,33,158
CDHR3	Rhinovirus C	Unknown	42
DAF	CV-A21, CV-B1, CV-B3, CV-B5, E-3, E-6, E-7, E-11, E-12, E-13, E-19, E-20, E-21, E-25, E-29 and E-30	Attachment	161 and reviewed in[158]
CAR	CV-B1, CV-B2, CV-B3, CV-B4, CV-B5 and CV-B6	Uncoating	162
Integrin $\alpha_v\beta_3$	CV-A9, E-1 and E-9	Attachment	158,163
Integrin $\alpha_2\beta_1$ (VLA2)	E-1 and E-8	Attachment	164

CAR, coxsackievirus–adenovirus receptor; CDHR3, cadherin-related family member 3; CV, coxsackievirus; CV-A24v, coxsackievirus A24 variant; DAF, complement decay-accelerating factor; DC-SIGN, dendritic cell-specific ICAM-grabbing non-integrin; E, echovirus; EV, enterovirus; EV-A71-PB, EV-A71-PSGL1-binder; ICAM, intercellular adhesion molecule; LDLR, low-density lipoprotein receptor; LRP, LDLR-related protein; PSGL, P-selectin glycoprotein ligand 1; PVR, poliovirus receptor; SCARB2, scavenger receptor class B member 2; Sia, sialic acid; VLA2, very late antigen 2; VLDLR, very low-density lipoprotein receptor.

previously associated with an increased risk of severe childhood asthma exacerbation was shown to enhance RV-C infection in cell culture by increasing CDHR3 expression at the cell surface[44].

Although many NPEV receptors have been identified, the interplay between different receptors and the exact mechanism by which these receptors mediate infection are still poorly understood. Further studies are necessary to improve our understanding of virus tropism and pathogenesis and to define new targets for intervention. Several strategies targeting virus–receptor interactions have been reported, including the use of soluble receptor fragments[45] and antibodies that bind to receptors or to receptor-binding epitopes on the viral capsid[46]. However, these strategies will be limited to a narrow range of viruses that share a common receptor.

Enterovirus uncoating

Uncoating stages. The entry mechanism by which enveloped viruses fuse with the cellular membrane is relatively well understood; however, it is less clear how enteroviruses deliver their genome into the host cell. Although enteroviruses utilize a highly diverse set of receptors and endocytic routes, the uncoating mechanism of poliovirus and NPEVs follows a number of conserved steps, which gradually destabilize the virus particle and lead to genome release at the correct time and location. Early studies on poliovirus identified three distinct particle types that occur during the entry process (FIG. 3a) on the basis of differences in their sedimentation coefficients and antigenic properties: the 160S, 135S and 80S particles[47]. The 160S particle is the native, infectious form of the virion. The 135S particle (altered particle, or A-particle) is a destabilized, infectious particle type that is formed in vitro, but it remains unclear whether this intermediate also exists during the uncoating process within infected cells. The 80S particle is the empty virion, which has released its genome and is a non-infectious end product of the uncoating process. The structure of the native particle is dynamic and undergoes 'breathing', which is the recurrent expansion and shrinkage of the virion, accompanied by transient exposure of VP4 and the N-terminal extension of VP1 (REF.[48]). Cellular uncoating cues (that is, receptor binding or low endosomal pH) lock the virion in its expanded state, forming the A-particle. Unlike the native particle, this uncoating intermediate is hydrophobic owing to the irreversible externalization of the VP1 N terminus, which contains an amphipathic helix that allows the virion to interact with membranes[49] (FIG. 3b). The A-particle is no longer bound to its receptor, lacks the internal protein VP4 (REF.[50]) and has a ~4% increased radius compared with the native virion[47], probably to allow rearrangement of the genomic RNA in preparation of its release.

Uncoating cues. Although all enteroviruses undergo similar structural changes during uncoating, the cues driving these destabilizing events are diverse. For most enteroviruses, the first uncoating cue encountered during cell entry is receptor binding. These uncoating receptors bind in the canyon, where they engage the GH loop of VP1, causing collapse of the hydrophobic pocket and expulsion of the pocket factor[51]. While proteinaceous uncoating receptors directly engage the VP1 GH loop, a recent study describing

Sedimentation coefficients
Quantities that describe the sedimentation rate of a particle when centrifuged in a fluid medium, which depends on particle size, density and shape.

GH loop
A structural loop in structural protein VP1 that forms the border between the hydrophobic pocket and the bottom of the canyon.

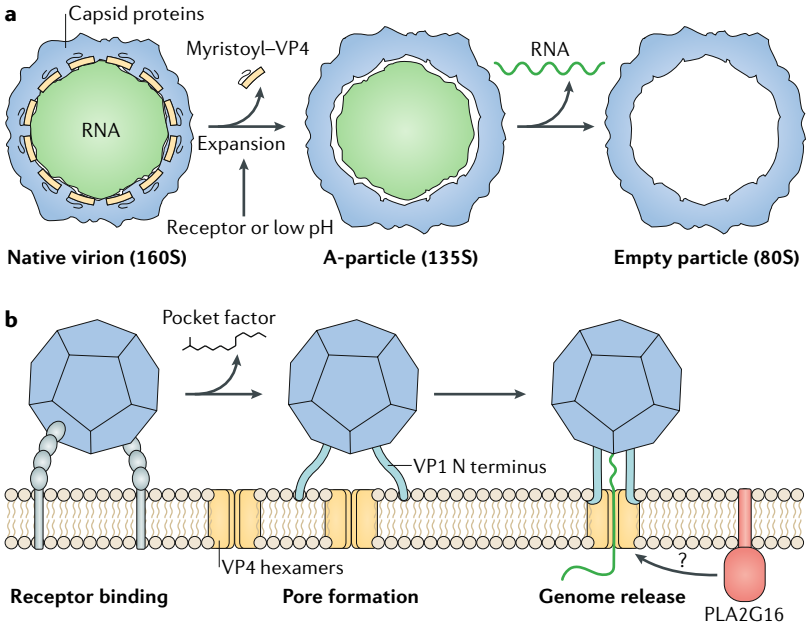

Fig. 3 | **The enterovirus uncoating mechanism. a** | Particle types observed during uncoating of enteroviruses in vitro, with their respective sedimentation coefficients between brackets. Uncoating cues (receptor binding or low pH) induce changes that convert the native virion into the altered particle (A-particle). These changes include capsid expansion, externalization of the capsid protein VP1 N terminus and release of myristoyl–VP4. The subsequent release of the RNA genome yields the empty particle. **b** | During cell entry, the first destabilizing event after receptor binding is the release of the pocket factor (a lipid that stabilizes the virus particle) from the virion. Subsequently, the externalized VP1 N terminus anchors the virion to the endosomal membrane, and myristoyl–VP4 forms hexameric membrane pores. The viral RNA genome is translocated through these pores into the cytoplasm, aided by the host protein phospholipase A2 group XVI (PLA2G16) via an unknown mechanism.

the crystal structure of EV-D68 in complex with its sialic acid receptor[30] revealed that sialic acid indirectly displaces the GH loop via a series of long range conformational changes. Although this unconventional mode of receptor binding triggered pocket factor expulsion, sialic acid did not induce A-particle formation, suggesting that additional uncoating cues are required to complete the uncoating process. In addition to receptor binding, some enteroviruses rely on endosomal acidification as a cue to initiate uncoating. As a low pH is restricted to specific cellular compartments, this chemical cue allows a virus to control the timing and site of genome release. Whereas endosomal acidification is the only known uncoating cue for minor-group rhinoviruses[52], some other enteroviruses, like EV-A71 (REF.[53]), require simultaneous engagement with their receptor and a low pH to fully uncoat.

Pore formation. Numerous structural studies that revealed enterovirus uncoating intermediates have provided extensive information about the molecular events that occur between receptor binding and A-particle formation, but the subsequent process of RNA release is still poorly understood. Because enteroviruses lack an external membrane, the viral genome must be translocated across a membrane to reach the cytoplasm. Early studies on poliovirus genome delivery showed that virions interact with lipid membranes and induce the formation of ion-permeable channels[54], a capacity that was found to be abrogated by mutations in VP4 (REF.[55]). Indeed, recombinant myristoylated VP4 of RV-16 was shown to independently induce membrane permeability[56] by forming multimeric pores[57]. These pores have a channel size consistent with the size that is required for translocation of the single-stranded viral RNA genome[57]. Whether the virion is directly associated with this transmembrane channel during genome release has been unclear for a long time. Yet, an electron tomography study of poliovirus bound to receptor-decorated liposomes revealed approximately 50 Å-long 'umbilical' connectors linking the virion to the membrane[58]. It was proposed that these connectors represent genomic RNA and viral proteins, which might shield the RNA during translocation. Consistent with this observation, a recent study showed that the poliovirus genomic RNA is protected from degradation by co-endocytosed RNase during the entry process[59], supporting a scenario in which a discrete channel connects the virion to the host cell membrane.

Genome release. Other major questions in studies of viral uncoating concern the exit site and orientation of the viral RNA during egress from the virus particle. So far, our understanding of the directionality of RNA release is based solely on a study of RV-A2, which revealed that the 3′ end of the genome exits the virion before the 5′ end[60]. The site of RNA release from the virion was originally proposed to be located at the fivefold axis[61], but cryo-EM structures of uncoating intermediates (A-particles or empty particles) revealed holes in the capsid at the twofold axis and near the quasi-threefold axis[51,62]. The opening at the quasi-threefold axis was found to serve as a channel for externalization of the VP1 N terminus[63]. Indications of the site of RNA release are based on asymmetric cryo-EM reconstructions of heated poliovirus particles that are in the process of genome release[64]. Although the resolution was low (~50 Å), these structures implicated a region spanning the twofold and quasi-threefold axes. A limitation of most cell-free uncoating studies performed to date is that viral uncoating is triggered by numerous cues (for example, heat, acid or soluble receptor molecules) that act on the entire virion (that is, global stimulation), whereas uncoating during a natural infection is probably asymmetrical, because only one side of the virion interacts directly with the membrane. In a recent study, asymmetric coxsackievirus B3 uncoating was induced with receptors embedded in lipid bilayer nanodiscs, generating the first high-resolution structures of an enterovirus uncoating intermediate attached to a membrane[65]. Unlike particles formed upon global stimulation, this structure revealed an opening at the threefold axis. Together, these findings show that more studies employing lipid membranes, as well as high-resolution cryo-EM, are required to determine the exact site of RNA exit from the virion.

The pan-enterovirus host factor PLA2G16. Although receptor binding is the only known uncoating cue for many enteroviruses, genome release does not take place immediately after receptor binding on the cell surface but after a period of internalization into the cell[66]. For that reason, several studies have suggested the existence of an additional unknown uncoating cue that controls

the moment at which the virion releases its genome[65,67]. A plausible candidate for this role is the host factor phospholipase A2 group XVI (PLA2G16), a lipid-modifying enzyme of which the physiological function remains poorly understood. PLA2G16 was identified as a universal enterovirus host factor that facilitates the displacement of viral genomes from virus-containing endocytic vesicles[68]. It is likely that PLA2G16 is involved in the formation, expansion or maintenance of the membrane pore, either by directly interacting with viral proteins or RNA, or indirectly, by creating a hospitable lipid environment that favours pore formation. For instance, lipids modified by PLA2G16 may affect membrane fluidity or interact with pore components, thereby facilitating their insertion into the endosomal membrane. Interestingly, some (non-*Enterovirus*) picornaviruses possess a non-structural 2A protein with homology to PLA2G16. Although these 2A proteins have never been found in association with virions, the possibility exists that these proteins perform a similar function in virus entry as PLA2G16. Analogously, most parvoviruses have a capsid protein that contains a phospholipase A2 domain, which is thought to modify the membrane of endocytic vesicles to allow viral genome release into the cytoplasm[69]. These findings suggest that various non-enveloped viruses have independently evolved to employ lipid-modifying enzymes for virus entry via mechanisms that are not yet fully understood.

Targeting the uncoating stage of enterovirus infection could be a promising strategy in the development of antiviral drugs against NPEVs. In addition to pocket factor analogues, which stabilize the virion, molecules that prevent genome translocation by targeting viral proteins or host factors that are involved in pore formation might be promising candidates. The pan-enterovirus host factor PLA2G16 could be an excellent drug target, because all enteroviruses tested to date require this factor and because the activity of PLA2G16 is dispensable for the survival of mice[68]. Because no selective PLA2G16 inhibitors have been reported thus far, developing specific in vitro assays to screen for inhibitors of its phospholipase activity could be a promising strategy to develop broadly acting anti-enterovirus drugs.

Translation and genome replication

Following its delivery to the cytosol, the viral genome is directly translated by ribosomes, yielding capsid proteins and viral proteins that mediate genome replication. Translation is mediated by an internal ribosome entry site (IRES) and requires a subset of the canonical translation machinery as well as several IRES *trans*-acting factors (ITAFs) (reviewed in REFS[70,71]). The mechanism of RNA replication is well conserved among all enteroviruses (reviewed in REF.[72]). The core enzyme of the replication machinery is the viral RNA-dependent RNA polymerase $3D^{pol}$. Replication is initiated at higher-order RNA elements in the 5′ and 3′ UTRs and requires a primer, the viral peptide 3B (also known as VPg) coupled to two uridines. VPg uridylylation requires a structural RNA element located in the coding region of the genome. $3D^{pol}$ operates in conjunction with several viral proteins, including $3CD^{pro}$ and 3AB, which together bind to structural RNA elements involved in priming, and 2C, which has ATPase-dependent helicase and ATPase-independent RNA chaperone activities. Besides viral proteins, several host RNA-binding proteins are involved in genome replication, including poly(rC)-binding protein 2 (PCBP2), polyadenylate-binding protein 1 (PABP1) and heterogeneous nuclear ribonucleoprotein C (HNRNPC).

Replication occurs on virus-induced, tubulovesicular ROs, which are thought to originate from endoplasmic reticulum (ER) and/or Golgi apparatus membranes. RO formation requires the viral membrane-associated proteins 2BC and 3A[73] and a specific set of host factors, but the exact composition and mechanism of formation of ROs remains unknown. 3A interacts with acyl-CoA-binding domain-containing protein 3 (ACBD3) to recruit the lipid kinase phosphatidylinositol 4-kinase-β (PI4KB) to membranes, leading to an accumulation of phosphatidylinositol-4-phosphate (PtdIns4P) lipids. PtdIns4P attracts oxysterol-binding protein (OSBP) to form membrane contact sites between the ER and ROs and mediate a PtdIns4P-driven accumulation of cholesterol at ROs (reviewed in REFS[12,74]). Increased levels of PtdIns4P and/or cholesterol are important for RO formation and efficient genome replication, possibly by facilitating proper processing of the viral polyprotein[75–78]. 3A also binds Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1), but its exact role in replication is unclear. The roles of 2BC, 2B and 2C in membrane reorganization are less well understood. It has been suggested that 2BC contributes to PI4KB recruitment[79]. 2B has viroporin activity[80,81], but its exact function in RO formation is unknown. 2C binds reticulon 3, an ER protein that promotes membrane curvature, but the importance of this interaction for RO formation has not been investigated[82]. Some host factors appear to be important for a subgroup of enteroviruses. For example, valosin-containing protein (VCP) was identified as a host factor required for poliovirus replication but not coxsackievirus B3 replication[83]. Of note, several other host proteins have been implicated in enterovirus replication, but their roles in replication are often poorly understood. Additionally, enteroviruses trigger activation of autophagy. Although autophagosomes have been suggested to have a role in genome replication[84], it is currently thought that virus-induced autophagosomes are primarily involved in non-lytic virion release (see below).

The mechanisms of genome translation and replication are highly conserved between enteroviruses and are therefore attractive targets for the development of broadly acting antiviral drugs, which can target viral enzymes, specific host factors or essential cellular processes (TABLE 1). Several inhibitors targeting viral enzymes $3C^{pro}$ and $3D^{pol}$ have been described. $3C^{pro}$ inhibitors include peptidomimetics that target its active site, such as rupintrivir. $3D^{pol}$ inhibitors include nucleoside analogues (for example, the anticancer drug gemcitabine (Gemzar)[85] and non-nucleoside inhibitors (for example, GPC-N114 (REF.[86])), which target a conserved RNA template channel in $3D^{pol}$). Additionally, a number of 2C inhibitors have been reported, but their mode of action is poorly understood. Among them is the antidepressant drug fluoxetine (Prozac)[87,88], which inhibits enterovirus B and enterovirus D members. Fluoxetine was successfully used to

Phospholipase A2 group XVI
(PLA2G16). A lipid-modifying enzyme that catalyses the release of fatty acids from phospholipids in adipose tissue and has been proposed to have acyltransferase activity.

Phospholipase A2 domain
A domain with phospholipase A2 catalytic activity; that is, it cleaves off fatty acids from the second hydroxyl group of the glycerol backbone of a phospholipid.

Internal ribosome entry site
(IRES). A highly structured RNA element in the 5′ UTR that allows cap-independent initiation of translation.

IRES *trans*-acting factors
(ITAFs). Host RNA-binding proteins that bind to the enteroviral IRES and stimulate translation (for example, polypyrimidine tract-binding protein 2, poly(rC)-binding protein 2, polyadenylate-binding protein 1, La and serine/arginine-rich splicing factor 3).

Poly(rC)-binding protein 2
(PCBP2). A host RNA-binding protein that functions as an ITAF to stimulate IRES-mediated translation and that binds to a replication element in the 5′ UTR to stimulate viral RNA replication.

Polyadenylate-binding protein 1
(PABP1). A host protein that binds the viral poly(A) tail and interacts with poly(rC)-binding protein 2 to mediate circularization of the viral genome and initiate negative-strand RNA synthesis.

Heterogeneous nuclear ribonucleoprotein C
(HNRNPC). A host protein that binds the 5′ and 3′ ends of enterovirus genomes and stabilizes interactions between them.

Acyl-CoA-binding domain-containing 3
(ACBD3). A protein that regulates function and structure of the Golgi complex through its interaction with the integral membrane protein giantin.

treat an immunocompromised child with chronic enteroviral encephalitis[89], underscoring the potential of drug repurposing in antiviral therapy. The recently elucidated structure of EV-A71 2C[90] will likely encourage soaking or co-crystallization studies and provide more insight into the mode of action of 2C inhibitors, allowing optimization through structure-guided drug design. In addition, inhibitors of host factors, including compounds targeting PI4KB and OSBP, inhibit a broad range of enteroviruses. Unfortunately, PI4KB inhibition was found to be associated with adverse side effects[91,92]. OSBP inhibitors include the antifungal drug itraconazole (Sporanox)[93], which was shown to have antiviral activity in mice[94].

Virion assembly and release

Assembly. The enterovirus life cycle ends with the formation of new infectious progeny, which is a highly complex, stepwise process that is poorly understood[95]. In a first step, heat shock protein 90 (HSP90) associates with the myristoylated capsid precursor P1, catalysing the proteolytic processing by $3CD^{pro}$ to yield VP0, VP1 and VP3 (REF.[96]). This yields a protomeric particle that subsequently self-oligomerizes to form pentameric particles. These pentamers are stabilized by their myristate moieties[97,98] and, for some enteroviruses, by association with glutathione[99,100]. The current model is that in a next step, pentameric particles condense around replicating viral RNA to form the provirion. Concurrently with encapsidation and as a last quality control step, the viral RNA mediates VP0 cleavage into VP2 and VP4, generating the mature virus particle[101].

Although many RNA viruses encode at least one RNA packaging signal to provide specificity and precision during viral assembly, packaging signals have not been identified thus far in the genomes of enteroviruses. For enteroviruses, it is currently believed that encapsidation specificity is governed by direct protein–protein interaction between 2C, as part of the replication complex, and the viral capsid protein VP3 without an apparent involvement of an RNA packaging signal[102]. Intriguingly, parechovirus 1 and foot-and-mouth disease virus (FMDV) were recently shown to contain multiple short regions of ordered RNA structures that bind capsid proteins, dispersed throughout their genomes[103,104]. Whether enterovirus or other picornavirus genomes also contain such structures needs to be investigated.

Enterovirus assembly can be inhibited by geldanamycin[105], an HSP90 inhibitor, but this inhibitor has cytotoxic effects and has mainly been investigated for its antitumour activity. Glutathione levels can be manipulated in several ways, but this is unlikely to be an effective antiviral strategy because only a subset of enteroviruses depends on glutathione for efficient assembly[99,100,106].

Virion release. Enteroviruses are typically considered cytolytic viruses that kill and lyse their host cell for release. However, several studies have revealed that enteroviruses can also be released in a non-lytic manner in vesicles that mediate en bloc transmission of virions[17,107–110]. The current model is that enteroviruses trigger autophagy and upregulate the formation of double-membrane autophagosomes that engulf clusters of virions[84]. Fusion of these autophagosomes with lysosomes is inhibited by the virus to prevent degradation of their contents. Instead, fusion of the outer membrane of virion-containing, double-membrane autophagosomes with the plasma membrane releases single-membrane vesicular structures filled with virions to the extracellular milieu. The membranes of these extracellular vesicles are enriched in the lipid phosphatidylserine, which assists uptake in cells, likely via phosphatidylserine receptors[109]. Nevertheless, the bona fide virus receptor is still needed for infection, implying that at some point, virions are released from the extracellular vesicles[109]. Non-lytic release may be a much more widely adopted transmission strategy of picornaviruses than previously thought and has also been implicated for HAV, which evades neutralizing antibodies by cloaking itself in exosome-like structures[111,112]. Thus, non-lytic release may enhance infection efficiency, but its exact role in enterovirus dissemination and pathogenesis remains to be explored. The seemingly widespread role of autophagy in virion release by enteroviruses and other picornaviruses makes this a potential broad-range antiviral target. However, given the central role of autophagy in cellular and organismal homeostasis, it seems unlikely that autophagy inhibitors are sufficiently safe for clinical application.

Advances in enterovirus vaccine development

Besides antiviral drugs, vaccines are of great importance in combating enterovirus infections. Both inactivated poliovirus vaccines (IPV) and live, attenuated oral poliovirus vaccines (OPV) have been instrumental in preventing poliomyelitis. The major advantages of OPV are that less virus is needed to induce protective immunity and that OPV limits virus spreading by inducing mucosal immunity. However, a major drawback is that OPV can revert and/or recombine with closely related *Enterovirus C* members, leading to vaccine-associated paralytic poliomyelitis and circulating vaccine-derived poliovirus[113]. Currently, safe and effective OPVs are being developed, involving various attenuating traits, including destabilizing mutations in the IRES[114], mutations in $3D^{pol}$ that improve proofreading activity[115,116] and lower RNA recombination capacity[117], and synonymous mutations in the capsid-coding region that alter codon pair bias[118].

Theoretically, inactivated or live, attenuated vaccines can be developed against any NPEV, as exemplified by the EV-A71 vaccine that is currently marketed in China[9]. Development of a polyvalent, broad-spectrum vaccine against many different enterovirus serotypes was considered unfeasible for a long time. However, recently it was shown that combining 50 inactivated rhinovirus types into a single vaccine elicited neutralizing antibodies against 49 types, suggesting that universal, polyvalent, inactivated enterovirus vaccines will be developed[119]. Another promising approach is the use of virus-like particles (VLPs)[120], which assemble upon co-expression of capsid proteins and $3CD^{pro}$, allowing the manufacture of vaccines of viruses that are difficult to culture, for example, *Rhinovirus C* members or viruses that are restricted to high-containment facilities (poliovirus in the post-eradication era). Enterovirus VLPs can differ slightly from native virions in their structure, antigenic properties and stability, owing to the absence of a stabilizing

Phosphatidylinositol 4-kinase-β
(PI4KB). A Golgi-localized lipid kinase that phosphorylates phosphatidylinositol to yield phosphatidylinositol-4-phosphate.

Oxysterol-binding protein
(OSBP). A protein that exchanges phosphatidylinositol-4-phosphate lipids and cholesterol at membrane contact sites.

Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1
(GBF1). A guanine nucleotide exchange factor for ADP-ribosylation factor (ARF) proteins that plays important roles in intracellular membrane transport and homeostasis.

Autophagy
A cellular catabolic process in which intracellular parts of the cell are engulfed by membranes and targeted for breakdown, which yields energy or building blocks or clears aged or damaged parts of a cell.

Soaking
An approach in crystallography in which a protein is crystallized, followed by addition of a ligand to form a co-crystal, allowing determination of the structure of the complex.

Co-crystallization
An approach in crystallography in which a protein and its ligand are crystallized together, forming a co-crystal that is used to determine the structure of the complex.

Glutathione
A tripeptide composed of glutamate, cysteine and glycine that has an important role in cellular redox reactions and as an antioxidant.

genome. However, this problem can be circumvented by introducing specific stabilizing capsid mutations[121]. Recently, stabilized poliovirus VLPs were efficiently produced in plants — which represent a cheap, safe and high-level expression system — and immunization of mice with these VLPs provided protection against a poliovirus challenge[122]. In conclusion, several strategies can be used to develop potent vaccines against NPEVs.

Conclusions and future perspectives

The large group of NPEVs contains many human pathogens that cause a wide spectrum of illnesses, including severe neurological and respiratory diseases. In this Review, we described the current knowledge of the different stages of the life cycle of enteroviruses, most of which is derived from studies on poliovirus. Although these studies have provided valuable insights into the enterovirus life cycle, many important questions need to be addressed. For instance, future studies should elucidate the mechanism of viral genome translocation across the endosomal membrane and the contribution of PLA2G16 to this process; clarify the early post-entry events and identify where the viral RNA is first translated and replicated; and establish the exact origin and function of ROs. In addition, how the viral RNA is encapsidated and which viral and cellular factors are involved in this process, how the viral RNA is organized inside the virion, and how virus-containing extracellular vesicles are formed and what their physiological function is are all important questions that need to be addressed. Recently, we have seen major advances in technologies that may be instrumental in solving these questions. Several new microscopy techniques are becoming increasingly available, for example, three-dimensional scanning electron microscopy approaches that allow the reconstruction of entire cells and the visualization of virus-induced intracellular changes at nanoscale resolutions. Moreover, in situ cryo-electron tomography, an imaging modality that allows structural analysis of macromolecular complexes in their physiological microenvironment, may be applied to gain more insight into viral replication complexes and their association with ROs. CRISPR–Cas9 gene knockout screens can be used to identify essential host factors, and nuclease-inactive Cas9 can be used to bind specific viral RNA sequences, allowing RNA tracking in living cells. Novel advances in mass spectrometry, including phospho-proteomics and crosslink-proteomics, lipidomics and metabolomics, may shed new light on our understanding of virus-induced changes in host cell biology and signalling that are essential for virus replication and/or propagation. Future NPEV research should focus on receptor usage and tropism, pathogenesis, transmission dynamics, epidemiology and evolution. Evolution studies are also needed to better understand how poliovirus evolved from EV-C coxsackievirus[123], which is an essential question in view of the polio eradication campaign.

Intensified efforts should be undertaken to develop preventive and therapeutic options to control NPEV infections. New insights into the viral life cycle, combined with rapid developments in (computational) drug discovery, are expected to spur the development of novel, broad-range enterovirus inhibitors. The successful development of highly effective antiviral drugs against another (+) RNA virus — hepatitis C virus — implies that it should be possible to develop antiviral drugs against enteroviruses, provided that sufficient investments are made. Besides treating NPEV infection, broad-range drugs may also contribute to the eradication of poliovirus. Research on this devastating human pathogen has laid important foundations for the field of molecular virology. Although poliovirus may soon be eradicated, fundamental research on NPEVs is likely to have far-reaching implications that go beyond the field of picornaviruses.

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